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Production of and Applications for a Polyclonal IgY Diagnostic Reagent Specific for *Mycobacterium avium* subsp. *paratuberculosis*

Sung Jae Shin[1], Seung-Sub Lee[1], Elizabeth J. B. Manning[2]*, and Michael T. Collins[2]

[1]*Department of Microbiology and Infection Signaling Network Research Center, Research Institute for Medical Sciences, College of Medicine, Chungnam National University, Daejeon 301-747, Republic of Korea*
[2]*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706-1102, USA*

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Antibodies specific to the cell surface antigens of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) have multiple useful applications, e.g. organism detection, immunoconcentration, and cell visualization. The aim of this study was to produce and compare polyclonal antibodies for such research and diagnostic purposes. Three polyclonal antibodies to MAP were produced using sera from immunized rabbits and chickens plus naturally infected cows. Cross-reactive antibodies in each MAP antibody preparation were removed by absorption with heterologous mycobacterial and non-mycobacterial cells. The specificity of each resulting polyclonal antibody preparation was evaluated by ELISA to multiple bacterial cell wall extract antigens. After absorption, chicken anti-MAP IgY had the highest specificity of the three antibody preparations. FITC-labeled anti-MAP IgY was used to effectively locate MAP in macrophages 12 h post-infection. Also, immunomagnetic beads coated with anti-MAP IgY enhanced recovery of MAP from bacterial suspensions in comparison with non-antibody coated beads. Anti-MAP IgY provides a novel new reagent with broad diagnostic and research applications requiring specific concentration, detection and quantification of MAP.

***Keywords*: *M. paratuberculosis*, polyclonal antibodies for diagnostic purposes, absorption, anti-MAP IgY**

Mycobacterium avium subsp. *paratuberculosis* (MAP) infection causes Johne's disease (paratuberculosis), a chronic inflammatory bowel disease of both domestic and wild ruminants. This pathogen also has been implicated as a possible cause of Crohn's disease in humans (Bull *et al*., 2003; Bentley *et al*., 2008).

A definitive diagnosis of paratuberculosis by detection of the etiologic agent is hampered by the typically low numbers of the organism in clinical or environmental samples (Collins *et al*., 1990). This is true for both PCR and culture methods. Culture of *M. paratuberculosis* is typically laborious, extremely slow (protocols range from 7∼18 weeks) for detectable growth and complete identification (Grant *et al*., 1998). These constraints led us to focus on concentration methods, especially for large volume samples such as milk and water. However, nonspecific concentration techniques, such as centrifugation and filtration, are nonselective and cause high rates of sample contamination.

While multiple MAP detection methods based on culture or PCR are available, an effective immunodiagnostic (antibody-based) method remains elusive due to currently available antibodies' low sensitivity and specificity in binding MAP-specific surface antigens or epitopes. Immunodiagnostic methods employing antibodies specific to target pathogen cell surface antigens have multiple diagnostic and research applications such as immunoconcentration from clinical and environmental samples (Grant *et al*., 1998; Whan *et al*., 2005), plus direct detection of microorganisms or their antigens *in vivo* (Araj *et al*., 1993; Pereira Arias-Bouda *et al*., 2000; Choudhry and Saxena, 2002; Huard *et al*., 2003) or in situ by immunocytochemistry (Humphrey and Weiner, 1987; Coetsier *et al.,* 1998; Baba *et al*., 2008).

Although the efforts have focused on the development of the specific antibodies against *M. paratubercuosis*, antibodies capable of strong biding to the surface of intact *M. paratuberculosis* cells are not readily available. In addition, the specificity of antibodies has been problematic due to the cross-reactivity with other environmental mycobacteria.

The purpose of this study was to develop and evaluate polyclonal anti-MAP antibodies made in rabbit (IgG), chicken (IgY), and in naturally MAP-infected cattle since commercial anti-MAP antibodies do not provide the sensitivity needed for the immunodiagnosis of MAP. We employed pre-absorption method to maximize the specificity of the produced antibodies as a second step. Finally, the valuable applications of the produced antibodies in present study were validated in several clinical trials.

Materials and Methods

Bacterial strains, cultures, and preparation of MAP single cell suspensions.

All bacterial strains used in this study are listed in Table 1. For immunization purposes, *Mycobacterium avium* subsp.

* To whom correspondence should be addressed.
(Tel) 1-608-265-4958; (Fax) 1-608-263-9754
(E-mail) emanning@wisc.edu

Table 1. Bacterial strains

Bacterial species	No. of strain tested	Strain name	Source
Mycobacterial species			
M. avium ss. paratuberculosis	4	ATCC19968, K-10, JTC303, DT114	ATCC[a], JTC[b]
M. avium ss. avium	2	ATCC35712, 104	ATCC
M. avium ss. intracellulare	2	ATCC13950, ATCC25122	ATCC
M. avium ss. silvaticum	1	ATCC49884	ATCC
M. abscessus	1	ATCC19977	ATCC
M. asiaticum	1	ATCC25276	ATCC
M. bovis	1	ATCC19210	ATCC
M. celatum	1	ATCC51130	ATCC
M. flavescens	1	ATCC14474	ATCC
M. fortuitum	1	ATCC49404	ATCC
M. gordonae	1	ATCC14470	ATCC
M. kanasii	1	ATCC12478	ATCC
M. lentiflavum	1	ATCC51985	ATCC
M. malmoense	1	ATCC29571	ATCC
M. nonchromogenicum	1	ATCC19530	ATCC
M. phlei	1	ATCC11758	ATCC
M. scrofulaceum	1	ATCC19981	ATCC
M. smegmatis	1	ATCC14468	ATCC
M. terrae	1	ATCC15755	ATCC
M. tuberculosis	1	ATCC27294	ATCC
Bacteria other than mycobacteria			
Aeromonas hydrophila	1	WSLH	WSLH[c]
Corynebacterium pseudotuberculosis	1	#90	JTC
Enterobacter aerogenes	1	WSLH	WSLH
Escherichia coli	1	ATCC25922	ATCC
Enterococcus faecalis	1	ATCC29212	ATCC
Klebsiella pneumonia	1	ATCC10031	ATCC
Pseudomonas aeruginosa	1	WSLH	WSLH
Proteus vulgaris	1	WSLH	WSLH
Total	33		

[a] American Type Culture Collection, Manassas, VA
[b] Johne's Testing Center, Madison, WI
[c] Wisconsin State Laboratory of Hygiene, Madison, WI

paratuberculosis (MAP) strains ATCC19698, JTC114, JTC303, and K-10 were separately cultivated in 7H9-OADC (oleic acid-dextrose-catalase, Becton Dickinson, USA) broth medium supplemented with 2 μg/ml of Mycobactin J (Allied Monitor, USA) for 1 month at 37°C. A pool of MAP cells from these four strains was used as the immunogen. To remove cross-reacting antibodies to other *Mycobacterium* spp. using intact cells as previously described (Yokomizo *et al*., 1985; Cox *et al*., 1991), *Mycobacterium phlei* ATCC 11758 and *Mycobacterium scrofulaceum* ATCC 19981 were cultivated in modified Watson-Reid (mWR, pH 6.0) broth for 1 month at 37°C. To produce antigens needed to evaluate antibody specificity by ELISA (Table 1), additional mycobacterial species were cultured in 7H9 broth supplemented with 10% (v/v) OADC (Becton Dickinson) for 2 to 4 weeks at 37°C. Non-mycobacterial strains were grown in Luria-Bertani (LB, Becton Dickinson) broth for 1 to 3 days at 37°C.

Single cell suspensions of each MAP strain were prepared as previously described with minor modifications (Sung and Collins, 2003). Briefly, mycobacterial cells grown in either mWR broth or 7H9 were harvested by centrifugation at 10,000×g for 20 min, and washed three times in 10 mM PBS (pH 7.2). Cell pellets were homogenized using the overhead stirrer (Wheaton Instrument, USA) for 1 min on ice to minimize cell clumping. The homogenized mycobacterial cells were passed through an 8 μm-pore size filter (Millipore Corp., USA). The presence of predominantly single cells in the final preparation was confirmed by acid-fast staining. Seedlots of each strain were then kept in small aliquots at -80°C until use.

Polyclonal antibody production

I. Purification of IgG from MAP-infected cattle

Protein G-purified IgG fractions were made from the sera of six naturally infected fecal culture-positive adult Holstein cows with high antibody levels (per ELISA, IDEXX Laboratories, Inc., USA and AGID, Immucell, USA) and sera from two ELISA and fecal culture negative Holstein cows

from a closed and uninfected herd. (Herds managing these animals had been monitored for Johne's disease for multiple years with multiple assays and were located in the Wisconsin, USA). The sample/positive (S/P) ratios of the infected cows by commercial ELISA kit were >1.0 and those of non-infected cows was <0.15.

Bovine IgG purification was performed using ImmunoPure (G) IgG Purification kit (Pierce, USA). The purified bovine IgG was neutralized by 100 μl of Tris-buffered saline (pH 7.8) and dialyzed against 10 mM PBS (pH 7.2) 5 times and finally filter-sterilized using a 0.22 μm pore-size syringe filter (Nalge Nunc International, USA). The concentration of purified bovine IgG was determined using the BCA protein assay kit (Pierce, USA).

II. Production of anti-MAP chicken IgY and anti-MAP rabbit IgG

Antiserum and IgY were produced commercially with rabbits (GeneTel, USA) and chickens (GeneTel), respectively. Two New Zealand White (NZW) rabbits were used for MAP immunization (previously determined to be nonreactive to MAP cellular extract, as assessed by ELISA). Four twenty-four week-old White Leghorn chickens were used for IgY production (collected from their eggs). They chickens had been vaccinated against Marek's disease virus when they were chicks. All animals were raised in biosecure facilities for research purposes (GeneTel).

A pool of heat-killed cells from four MAP strains was used as the immunogen. Before immunization, the immunoreactivity of sera from each individual animal was tested by ELISA to MAP cell wall antigens. Animals having the lowest ELISA optical density (OD mean=0.12) values were selected for production of antisera.

Each rabbit was intradermally injected with 500 μl of 2×10^7 CFU/ml MAP cells in an equal volume of Freund's Incomplete Adjuvant (FIA) (Sigma) four times at two week intervals. At each immunization, the rabbit antibody response to MAP was evaluated by ELISA. After the final immunization, serum from each rabbit was harvested. Rabbit IgG purification was then performed using ImmunoPure (G) IgG Purification Kit (Pierce, USA) as described above.

Laying chickens were first injected subcutaneously with 500 μl of 2×10^7 CFU/ml MAP cells mixed with an equal volume of FIA in the cervical region. Six additional immunizations were given intramuscularly under the wing with the same dose of MAP in FIA at one week intervals for a total of seven injections. Eggs were collected daily after the second immunization, marked, and stored at 4°C until use. Chicken IgY was precipitated from egg yolks by adding 1 volume of 40% polyethylene glycol (PEG) 8000 (Sigma) in PBS to 3 volumes of egg yolk, followed by centrifugation at 13,000×g for 20 min. The purified IgY was dialyzed against 10 mM PBS (pH 7.2) four times. The purity and concentration of IgY was determined using SDS-PAGE and BCA protein assay kit (Pierce), respectively.

Antibody purity evaluation by one-dimensional electrophoresis

The purified polyclonal antibodies were evaluated in commercial precast gels (Jule Biotechnologies, USA) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 20 μg of the purified antibodies were mixed with 20 μl SDS loading buffer (Bio-Rad, USA) and boiled for 10 min prior to separation on an SDS-PAGE gel (10~20% polyacrylamide, 10 wells, 1.5 mm thickness). Gels were electrophoresed for 2 h at 150 V until the blue dye had migrated to the bottom of the gel. The protein bands were assessed via Coomasie Brilliant Blue staining and an image analyzer (Odyssey®, LI-COR Biotechnology, USA).

Cell wall extract antigens

Cell wall extract (CWE) antigens were prepared from non-mycobacterial and mycobacterial strains (Table 1) for use in assessing antibody specificity both before and after Ig absorption using heterologous bacterial cells as previously described (Yokomizo *et al*., 1985; Cox *et al*., 1991). Mycobacterial CWE antigens were prepared from organisms grown in WR or 7H9 broth (Mawuenyega *et al*., 2005; Marques *et al*., 2008). Briefly, bacterial cell pellets were harvested and washed and after addition of a protease inhibitor cocktail (Complete, Mini, EDTA-free®, 0.05% (v/v); Roche Diagnostics GmbH, Germany), 10 ml of the homogenized cells were mixed with 20 g of glass beads (0.10~0.11 mm; Glass-perlen®, B. Braun Biotech International GmbH, Germany). This mixture was vigorously agitated for 30 min using a high speed agitator (model MSK, Braun Instruments, Allentown, PA) while cooling with liquid CO_2. The homogenate was then centrifuged at 3,000×g for 15 min to remove the unbroken cells after which the supernatant was collected. This mycobacterial cell lysate was centrifuged at 30,000×g for 1 h, the pellet was resuspended in 10 mM PBS (pH 7.2) by sonication and then stored at -70°C until use. The concentration of the CWE protein was determined by BCA protein assay kit (Pierce). The cell envelope antigens of non-mycobacterial species were produced and purified as previously described with slight modifications (Schnaitman *et al*., 1970; Eisenbach and Adler *et al*., 1981).

The specificity of each preparation of purified polyclonal bovine, rabbit and chicken anti-MAP antibody was enhanced by mixing each with intact cells of cross-reactive bacteria following methods adapted from commercial paratuberculosis ELISA kits (Yokomizo *et al*., 1985; Cox *et al*., 1991). This protocol reduces or removes cross-reactive antibodies through absorption by *M. phlei* for IgY and bovine IgG and absorbtion by a combination of *M. phlei*, *M. scrofulaceum*, and *E. coli* for rabbit IgG.

For all three polyclonal preparations, cross-reactive antibody absorption was completed using 10^8 CFU/ml of either *M. phlei*, *M. scrofulaceum*, and *E. coli* cells combined or *M. phlei* alone for each 100 μg of anti-MAP antibody. Briefly, 100 μg of anti-MAP antibody was mixed with 10^8 CFU/ml of cell mixture and incubated at 4°C overnight. The mixture was then filtered using 0.2 μm syringe filter (Nalge Nunc International, USA) followed by centrifugation for 3 min at 14,000×g. The concentration of bovine IgG, chicken IgY, and rabbit IgG antibody before and after absorption was determined by the BCA protein assay kit (Pierce). The degree of cross-reactivity for each anti-MAP polyclonal preparation by these methods was calculated by:

% of antibody cross-reactivity = (concentration before ab-

sorption – concentration after absorption) / (concentration before absorption) × (100%)

Anti-MAP antibody specificity estimation via ELISA. The CWE antigens (10 μg/ml) from each mycobacterial and non-mycobacterial strain tested in this study were diluted in coating buffer (10 mM PBS, pH 7.4) (KPL, USA) and 100 μl of each antigen was coated into wells of 96 well microtiter plates (Maxisorp, Nalge Nunc International, USA) at 4°C overnight. After washing, the wells were blocked with 10% (v/v) normal goat serum (Sigma) at room temperature for 2 h. One hundred microliters of pre- or post-absorption antibody preparation (10 μg/ml of bovine IgG, 2 μg/ml of rabbit IgG, and 2 μg /ml of chicken IgY) were added to each well and incubated for 30 min at room temperature. Wells were then washed three times with washing buffer (KPL, USA). Mouse monoclonal anti-bovine IgG antibody conjugated with HRP (Sigma) or rabbit anti-IgY HRP (Gentel, USA) or sheep anti-rabbit IgG HRP (Sigma) at a dilution of 1:10,000 was added to wells and incubated for 30 min at room temperature. Plates were washed 5 times with washing buffer (KPL). One hundred μl of TMB substrate (TMBE-500, Moss Inc., USA) were added and incubated 2 min at room temperature before stopping the reaction by addition of 100 μl stop solution (KPL). The OD of the final product in each well was measured at 450 nm (μQuant, Bio-Tek Instruments Inc., USA).

Applications of resultant polyclonal anti-MAP antibody

I. Visualization of MAP cells in direct staining and in murine bone-marrow derived macrophages.
Post-absorption, anti-MAP chicken IgY was labeled using a fluorescein isothiocyanate (FITC)-protein labeling kit (Pierce) following manufacture's instructions. MAP cells (10^8 CFU/ml) were tagged by incubation with 0.5 μg of FITC-labeled antibodies per ml in 10 mM PBS (pH 7.4) at 37°C for 15 min followed by washing twice with PBS to remove unbound antibody and then suspended in fresh cell culture medium. *M. phlei* was used as the negative control.

Murine bone marrow derived macrophages (BMDM) were harvested from C57BL/6 mice (KOATECH, Korea). Mice were fed a sterile commercial mouse diet and water *ad libitum*. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee of Chungnam National University. The BMDMs were isolated from mice eight weeks old as previously described (Cunnick *et al*., 2006).

Briefly, the BMDMs were differentiated for 5~7 days in macrophage colony stimulating factor (M-CSF)-containing medium as previously described (Cunnick *et al*., 2006; Gersuk *et al*., 2008). The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 10% L929 cell-conditioned medium (as a source of M-CSF), 10% (v/v) heat-inactivated fetal calf serum (FCS, Hyclone, USA), 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μM/ml β-mercaptoethanol (Invitrogen). Adherent differentiated macrophages were collected; these cells were cultured in a 5% CO_2 environment at 37°C in 100mm dia. dishes or 12-well plates.

After the differentiation of BMDMs, the cells (2×10^5 cells/ml) were infected with FITC-labeled anti-MAP antibody tagged MAP at a bacteria-to-cell ratios of 1:1, 5:1, or 10:1. Twelve hours after infection, BMDMs were fixed with 4% p-formaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.1% Triton X-100 for 10min at room temperature and blocked for 1h with PBS containing 3% BSA (w/v). After washing with 10 mM PBS containing 0.05% Tween 20, MAP inside BMDMs were visualized by confocal darkfield microscopy (LSM 510, ver. 2.3; Carl Zeiss, USA).

II. Immunomagnetic separation (IMS) of MAP.
Based on the antigen-detection sensitivity and antibody specificity data in this study, IMS studies were restricted to pre-absorbed chicken IgY. MAP concentration by IMS and detection by IMS-PCR were performed as previously described (Grant *et al*., 1998; Grant *et al*., 2000) with slight modification. Briefly, 10^8 M-280 Dynabeads pre-coated with sheep anti-rabbit IgG (Invitrogen) were coated with rabbit anti-IgY (GeneTel) and then exposed to pre-absorbed chicken anti-MAP IgY for 24 h at 4°C as recommended by the manufacturer. The beads ("IgY IMS beads") were thus covered with three layers of antibody with the anti-MAP IgY being the outermost layer. Uncoated beads were used as a negative control. The IgY IMS beads were added to each 1 ml of test sample and incubated for 30 min at room temperature with gentle agitation on a Dynal sample mixer (Invitrogen). After incubation, the IgY IMS beads were harvested from the mixture using a magnetic particle concentrator (MPC, Invitrogen) for 10 min. The residual liquid was removed by aspiration and three washing steps in 10 mM PBS (pH 7.4), using the MPC for 2 min to harvest IMS beads between each wash. The IMS beads were then resuspended in 100 μl of 10 mM PBS (pH 7.4) and inoculated into tubes of MGIT ParaTB medium™ for MAP quantification as previously described (Shin *et al*., 2007). In addition, IMS-nested PCR was performed as previously described (Naser *et al*., 2004; Romero *et al*., 2005).

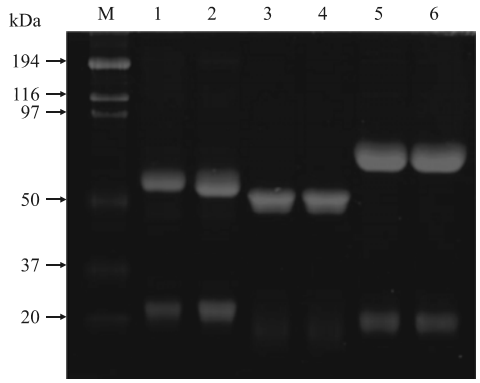

Fig. 1. SDS-PAGE for purity assessment of anti-MAP antibody preparations. Lanes: M, Molecular size marker; 1, purified bovine IgG from a non-MAP-infected cow; 2, purified bovine anti-MAP IgG; 3, purified pre-immunized rabbit IgG; 4, purified rabbit anti-MAP IgG; 5, commercial purified chicken IgY control (GenTel), and 6, purified chicken anti-MAP IgY.

MGIT ParaTB medium™ culture

The number of viable MAP before and after IMS concentration was determined by the MGIT 960 counting method as previously described (Shin *et al.,* 2007). Briefly, ten-fold serial dilutions of MAP JTC303 single cell suspensions or IgY-IMS-MAP were prepared and 100 μl of each dilution then inoculated into MGIT ParaTB medium™ (Becton Dickinson). The time to detection (TTD) in days for each tube was recorded and converted to $\log_{10}$ CFU MAP using a standard curve. All MGIT ParaTB medium™ tubes were

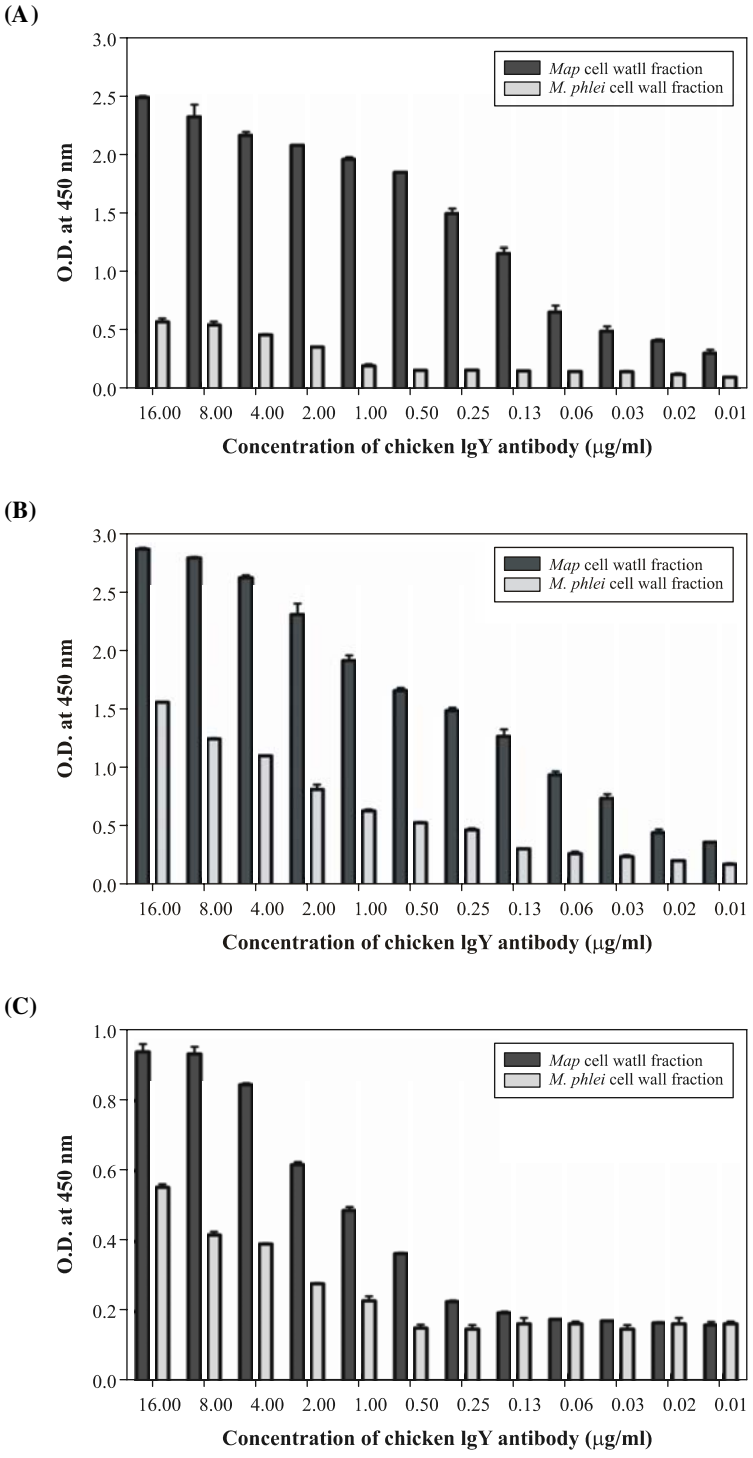

Fig. 2. Comparison of antibody potency and analytical specificity before and after absorption. (A) chicken anti-MAP IgY, (B) rabbit anti-MAP IgG, and (C) bovine anti-MAP IgG.

inoculated in duplicate.

Statistical analysis

Changes in ELISA OD values before and after cross reactive antibody absorption for each of the ELISA solid-phase antigens were evaluated by Paired *t*-test using statistical software (GraphPad Prism version 4.03 for Windows, GraphPad Software, USA). Differences in mean values were considered when $P<0.05$.

Results

Anti-MAP antibody purity

Three polyclonal anti-MAP antibody preparations were made: protein G-purified bovine anti-MAP IgG derived from serum from naturally infected cows (10 mg/ml), protein G-purified rabbit anti-MAP IgG (10 mg/ml) from immunized rabbits and polyethylene glycol (PEG) precipitated chicken anti-MAP IgY (5 mg/ml) from the yolk of immunized hens' eggs. The purity of the antibody preparations and the size of heavy and light chains were confirmed by SDS-PAGE analysis (Fig. 1). Bovine IgG had 56 and 25-kDa heavy and light chains respectively, rabbit IgG had 50 and 16-kDa heavy and light chains, and chicken IgY had 65 and 20 kDa heavy and light chains. No additional bands were observed in the gels.

Anti-MAP antibody potency

The potency of each anti-MAP antibody preparation was evaluated by ELISA using MAP and *M. phlei* CWE as solid-phase antigens (10 μg/ml). The antibody preparation potency was defined as the lowest concentration of anti-MAP antibody showing both a significant ELISA OD above background and a higher OD with MAP CWE than *M. phlei* CWE. The potency of the bovine, rabbit and chicken anti-MAP Ig preparations was 0.25 μg/ml, 0.01 μg/ml, and 0.01 μg/ml, respectively (Fig. 2). Due to the lower potency of bovine anti-MAP IgG it was not further evaluated.

Anti-MAP antibody specificity

Prior to absorption, rabbit anti-MAP IgG and chicken anti-MAP IgY were evaluated for analytical specificity as follows: antibodies were considered as cross-reactive with test bacteria if the S/N was ≥2.0 (signal/noise, i.e. anti-MAP antibody ELISA OD / pre-immune serum ELISA OD). The pre-immunization ELISA ODs for both chicken IgY and rabbit IgG were consistently <0.10 (Table 3). Generally, the rabbit anti-MAP IgG showed more cross-reactivity (i.e. higher

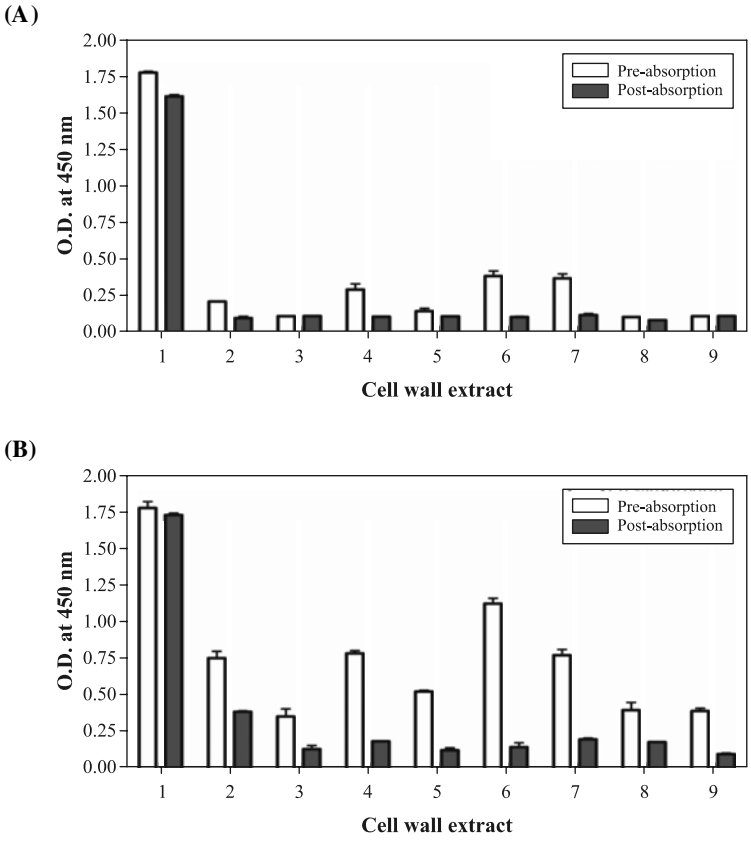

Fig. 3. Enhancement of antibody specificity. Cross reactivity of chicken IgY to other bacteria was reduced by absorption with *M. phlei* cells alone; rabbit IgG cross-reactivity was minimized using a combination of *M. phlei*, *M. scrofulaceum*, and *E. coli* cells. (A) Chicken anti-MAP IgY and (B) Rabbit anti-MAP IgG. 1, *Mycobacterium avium* subsp. *paratuberculosis* ATCC19968; 2, *Mycobacterium agiaticum* ATCC25274; 3, *Mycobacterium bovis* ATCC19210; 4, *Mycobacterium phlei* ATCC11758; 5, *Mycobacterium smegmatis* ATCC11758; 6, *Mycobacterium scrofulaceum* ATCC19981; 7, *Mycobacterium terrae* ATCC15755; 8, *Corynebacterium pseudotuberculosis* clinical isolate, and 9, *Escherichia coli* ATCC25922.

Table 2. Percentage of cross-reactive antibodies after absorption with bacterial cells

Bacteria used for absorption	Expressed as % of cross-reactivity ± SD[a]	
	Chicken IgY	Rabbit IgG
M. phlei	38.9±2.6	35.5±12.8
M. scrofulaceum	32.9±4.8	46.5±9.6
E. coli	22.0±2.9	34.6±8.4
M. avium ss. *avium*	84.8±8.4	87.4±6.9
M. avium ss. *paratuberculosis*	95.2±6.5	96.3±4.9
M. phlei + *E. coli*	38.9±4.3	35.5±3.2
M. scrofulaceum + *M. phlei* + *E. coli*	40.2±5.8	50.6±6.3

[a] % of antibody cross-reactivity = (concentration before absorption – concentration after absorption) / (concentration before absorption) × (100%)

ELISA ODs to non-MAP bacteria) than did chicken anti-MAP IgY (Fig. 3). Chicken anti-MAP IgY showed cross-reactivity primarily with other mycobacteria whereas rabbit anti-MAP IgY showed cross-reactivity to both mycobacteria and non-mycobacteria (Fig. 3).

After absorption of chicken anti-MAP IgY with *M. phlei* ATCC11758, the cross-reactivity with other bacteria mentioned above was significantly reduced (Table 3 and Fig. 3). For rabbit anti-MAP IgG however, significant cross-reactivity to non-MAP mycobacteria remained after *M. phlei* absorption

Table 3. Comparison of antibody reactivity before and after absorption

Bacterial species	Chicken IgY[a]		Rabbit IgG[b]	
	Pre-absorption	Post-absorption	Pre-absorption	Post-absorption
Mycobacterial species				
M. avium ss. paratuberculosis	+++	+++	+++	+++
M. avium ss. avium	+++	+++	+++	+++
M. avium ss. intracellulare	+++	+	+++	+++
M. avium ss. sylvaticum	+++	++	+++	+++
M. abscessus	–	–	++	+
M. asiaticum	+	–	+++	++
M. bovis	–	–	+	–
M. celatum	–	–	+	–
M. fortuitum	–	–	++	+
M. flavescens	–	–	+	–
M. gordonae	–	–	+	–
M. kanasii	–	–	+	–
M. lentiflavum	+	–	++	+
M. malmoense	+	–	+++	++
M. nonchromogenicum	–	–	++	–
M. phlei	+	–	++	–
M. scrofulaceum	+	–	+++	–
M. smegmatis	–	–	+	–
M. terrae	+	–	++	–
M. tuberculosis	–	–	+	–
Bacteria other than mycobacteria				
A. hydrophila	–	–	+	–
C. pseudotuberculosis	–	–	+	–
E. aerogenes	–	–	+	–
E. coli	–	–	+	–
E. faecalis	–	–	+	–
K. pneumonia	–	–	+	–
P. aeruginosa	–	–	+	–
P. vulgaris	–	–	+	–

[a] Chicken anti-MAP IgY was absorbed using *M. phlei*.
[b] Rabbit anti-MAP IgG was absorbed using *M. phlei*, *E. coli*, and *M. scrofulaceum*.
+: S/N = 2 to 5
++: S/N = 5 to 10
+++: S/N > 10

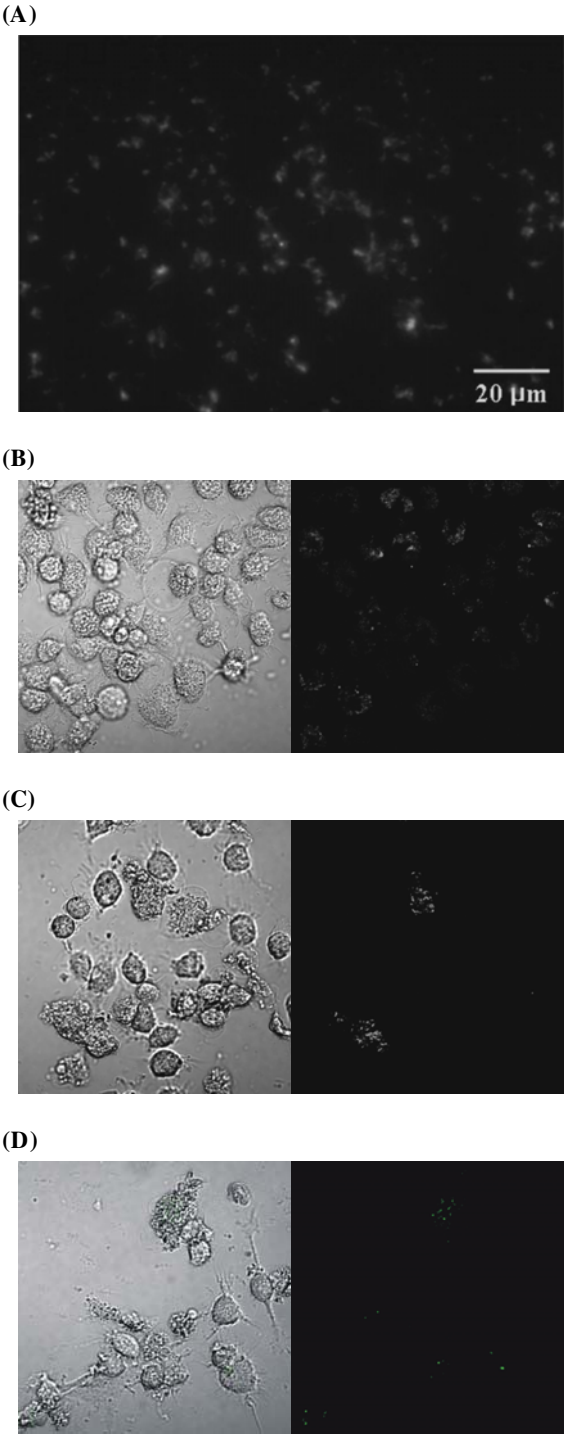

Fig. 4. Staining MAP cells using FITC-labeled anti-MAP IgY. A: Direct visualization of MAP using FITC-labeled chicken anti-MAP, B (MOI 20:1), C (5:1), and D (1:1): Confocal dark field microscopy of MAP in murine BMDM using FITC-labeled chicken anti-MAP IgY tagging.

(data not shown). This cross-reactivity was removed by additional absorption using *E. coli* and *M. scrofulaceum* (Table 3 and Fig. 3B). Absorption of both the chicken and rabbit anti-MAP antibodies with MAP (homologous absorption) virtually eliminated immunoreactivity to MAP. Neither absorption with *M. phlei* alone nor combinations of *M. scrofulaceum* and *E. coli* completely removed cross-reactivity with Mycobacterium Avium Complex (MAC) species other than MAP for either antibody preparations (Table 2 and 3).

Visualization of MAP in murine bone-marrow derived macrophages.

Analytical specificity of the FITC-labeled chicken anti-MAP IgY was influenced by the IgY:FITC ratio and also the concentration of mycobacteria being tested. The optimal ratio for labeling was determined to be 0.5 μg of *M. phlei*-absorbed chicken anti-MAP IgY and 10^7 CFU/ml MAP (data not shown). MAP were readily observed when stained by the FITC-labeled anti-MAP antibody (Fig. 4A) while *M. phlei cells* were not (data not shown). Using this labeling protocol FITC-anti-MAP-tagged organisms were visualized by confocal darkfield microscopy (×800) of *in vitro* infected murine BMDM but not in negative controls (BMDC infected with *M. phlei* at a 20:1 MOI) (Fig. 4B, C, and D).

Concentration of MAP from suspensions using IgY IMS beads

Serial dilutions from $10^{5.8}$ to $10^{0.8}$ CFU/ml MAP cells were used for IMS with MGIT 960 reported time to detection (TTD) in MGIT ParaTB medium™ for MAP quantification. Chicken anti-MAP IgY-coated beads effectively bound and concentrated MAP, thus enhancing MAP recovery by culture in MGIT ParaTB medium™, particularly from suspensions containing low numbers of MAP. The estimated maximum binding capacity of IgY beads was approximately 10^5~10^6 CFU MAP per 10^8 beads (Table 4).

Discussion

In this study, three antibodies specific to MAP were produced. Purified bovine IgG derived from the serum of naturally MAP-infected cattle had low potency and unacceptable cross-reactivity with *M. phlei* and was not further evaluated. Polyclonal rabbit anti-MAP IgG and chicken anti-MAP IgY derived from serum of immunized rabbits and egg yolk of immunized hens were more potent and had higher specificity.

Polyclonal antibodies are relatively inexpensive and easy to produce in the large quantities needed for high volume clinical sample testing focused on efficient identification of MAC species. We had tested several commercially available monoclonal antibodies against *M. avium*, but none of showed greater specificity and sensitivity than polyclonal IgY produced in this study (data not shown).

An additional advantage of polyclonal antibodies is their enhanced analytical sensitivity due to their being directed against multiple surface antigens. The polyclonals' analytical specificity (initially lower than monoclonals' specificity) (Coetsier *et al*., 1998) can be markedly enhanced as shown in this study with techniques employed in most commercial paratuberculosis ELISA kits. (Yokomizo *et al*., 1985; Cox *et al*., 1991). After absorption of chicken anti-MAP IgY with *M. phlei* cells and with *M. phlei*, *E. coli*, and *M. scrof-*

Table 4. IMS concentration of MAP as compared to the initial number of MAP CFU/ml as determined by the MGIT counting method

Control	No. MAP recovered on IMS beads	Unbound MAP
Inoculum (Log_{10} CFU/ml)	MAP CFU[a]	MAP CFU[a]
5.8	5.2±0.1	4.8±0.1
4.8	4.2±0.4	2.3±0.3
3.8	3.4±0.3	ND
2.8	2.2±0.1	ND
1.8	1.6±0.0	ND
0.8	0.3±0.1	ND

[a] Calculated by MGIT960 counting method and expressed as log_{10} CFU/ml
* ND, Not determined; TTD>49 days, i.e. below the detection limit.

ulaceum cells for rabbit IgG (Fig. 2), virtually all anti-MAP IgY cross-reactivity with other mycobacteria was eliminated, except for non-MAP species in the *M. avium* complex.

IgY, the egg yolk homologue of IgG, is a useful research and clinical immunodiagnostic reagent. Immunization of hens with purified protein or plasmids with target DNA (Zhang *et al*., 2003) provides polyclonal antibody due to excellent immunogenicity and epitope recognition capacity. In fact, due to the greater phylogenetic distance separating avian and mammalian species, IgY is a more robust and effective immune reagent for many techniques than IgG raised in and then used for mammals (where epitopes highly conserved across many mammal species may not elicit useful antisera) (Schmidt *et al.*, 1989). Used widely with applications ranging from immunofluorescence, immunohistochemistry, immuno-enzyme techniques (ELISA), Western blotting, and immunoelectrophoresis (Tini *et al*., 2002), IgY has permitted both immunodetection and immunocapture of multiple bacterial and parasitic organisms (e.g. *Acanthamoeba, H. pylori*, microsporidia, *E. coli* O157:H7, etc.) (Young *et al*., 2007). The value of IgY is enhanced by the inexpensive, simple, minimally invasive yet swift methods used to produce high titer and abundant (one egg contains as much antibody as an single bleed from a rabbit) antibody (Karlsson *et al*., 2004).

Interestingly, absorption of anti-MAP IgY with *E. coli* did not decrease IgY cross reactivity with *M. phlei* while *M. phlei* absorption of anti-MAP IgY removed all cross reactivity to both *E. coli* and *M. phlei* (S/N ratio=1.0) without decreasing the ELISA OD of the IgY to MAP (S/N ratio =11.31). The rabbit anti-MAP IgG showed cross-reactivity with other mycobacteria such as *M. malmoense* and *M. asiaticum* (Table 3) even after absorption of the antibody preparation with *M. phlei*, *E. coli*, and *M. scrofulaceum* cells.

Antibodies capable of strong binding to the surface of intact MAP cells have not previously been readily available. With the methods described in this study, MAP cells were effectively and specifically labeled with FITC-linked *M. phlei*-absorbed anti-MAP chicken IgY. These tagged MAP could be visualized in infected murine BMDCs. This application avoids the negative effects of FITC on bacterial and eukaryotic cells (Pick *et al*., 1981; Zhang *et al*., 1997; Ward and Cavieres, 1998) as well as interference of bacterial defense during phagocytosis (Weingart *et al*., 1999). This IgY reagent can also be used for detection and tracking of MAP during phago-lysosomal fusion using electron microscopy.

Absorbed chicken anti-MAP IgY was also effectively used as an IMS reagent. Secondary coating of the beads, i.e. first coating the beads with rabbit anti-IgY antibody, was more effective for immunocapture of MAP than direct coating of magnetic beads with absorbed IgY. Roughly one additional log of MAP was recovered using the indirect versus direct bead coating method when tested on concentrations of $>10^5$ CFU MAP/ml. This may be due to the binding capacity of IgY to the beads that were tested or the orientation of IgY capture sites on the bead surface. Recovery efficiency of MAP by IMS using the indirect bead coating method and *M. phlei*-absorbed anti-MAP chicken IgY beads was high. Virtually all MAP were recovered for every dilution from direct MGIT ParaTB medium™. IMS using this antibody and the indirect bead coating method was also effective in a trial using MAP suspended in tap water (data not shown). Anti-MAP IgY beads in conjunction with PCR provide a rapid, sensitive and specific MAP detection method for clinical and environmental samples.

In conclusion, this study demonstrated that IgY antibody as herein prepared is specific, able to capture live organisms and has potential in the development of an immunocapture assay for MAP in a variety of clinical samples.

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References

Araj, G.F., B.H. Fahmawi, T.D. Chugh, and M. bu-Salim. 1993. Improved detection of mycobacterial antigens in clinical specimens by combined enzyme-linked immunosorbent assay. *Diagn. Microbiol. Infect. Dis.* 17, 119-127.

Baba, K., A.M. Dyrhol-Riise, L. Sviland, N. Langeland, A.A. Hoosen, H.G. Wiker, and T. Mustafa. 2008. Rapid and specific diagnosis of tuberculous pleuritis with immunohistochemistry by detecting *Mycobacterium tuberculosis* complex specific antigen MPT64 in patients from a HIV endemic area. *Appl. Immunohistochem. Mol. Morphol.* 16, 554-561.

Bentley, R.W., J.I. Keenan, R.B. Gearry, M.A. Kennedy, M.L. Barclay, and R.L. Roberts. 2008. Incidence of *Mycobacterium avium* subspecies *paratuberculosis* in a population-based cohort of patients with Crohn's disease and control subjects. *Am. J. Gastroenterol.* 103, 1168-1172.

Bull, T.J., E.J. McMinn, K. Sidi-Boumedine, A. Skull, D. Durkin, P. Neild, G. Rhodes, R. Pickup, and J. Hermon-Taylor. 2003. Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J. Clin. Microbiol.* 41, 2915-2923.

Choudhry, V. and R.K. Saxena. 2002. Detection of *Mycobacterium tuberculosis* antigens in urinary proteins of tuberculosis patients. *Eur. J. Clin. Microbiol. Infect. Dis.* 21, 1-5.

Coetsier, C., X. Havaux, F. Mattelard, S. Sadatte, F. Cormont, K. Buergelt, B. Limbourg, D. Latinne, H. Bazin, J.F. Denef, and C. Cocito. 1998. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in infected tissues by new species-specific immunohistological procedures. *Clin. Diagn. Lab. Immunol.* 5, 446-451.

Collins, M.T., B.K. Kenefick, D.C. Sockett, R.S. Lambrecht, J. McDonald, and J.B. Jorgensen. 1990. Enhanced radiometric detection of *Mycobacterium paratuberculosis* by using filter-concentrated bovine fecal specimens. *J. Clin. Microbiol*. 28, 2514-2519.

Cox, J.C., D.P. Drane, S.L. Jones, S. Ridge, and A.R. Milner. 1991. Development and evaluation of a rapid absorbed enzyme immunoassay test for the diagnosis of Johne's disease in cattle. *Aust. Vet. J*. 68,157-160.

Cunnick, J., P. Kaur, Y. Cho, J. Groffen, and N. Heisterkamp. 2006. Use of bone marrow-derived macrophages to model murine innate immune responses. *J. Immunol. Methods* 311, 96-105.

Eisenbach, M. and J. Adler. 1981. Bacterial cell envelopes with functional flagella. *J. Biol. Chem.* 256, 8807-8814.

Gersuk, G.M., L.W. Razai, and K.A. Marr. 2008. Methods of in vitro macrophage maturation confer variable inflammatory responses in association with altered expression of cell surface dectin-1. *J. Immunol. Methods* 329, 157-166.

Grant, I.R., H.J. Ball, and M.T. Rowe. 1998. Isolation of *Mycobacterium paratuberculosis* from milk by immunomagnetic separation. *Appl. Environ. Microbiol*. 64, 3153-3158.

Grant, I.R., C.M. Pope, L.M. O'Riordan, H.J. Ball, and M.T. Rowe. 2000. Improved detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by immunomagnetic PCR. *Vet. Microbiol.* 77, 369-378.

Huard, R.C., S. Chitale, M. Leung, L.C. Lazzarini, H. Zhu, E. Shashkina, S. Laal, M.B. Conde, A.L. Kritski, J.T. Belisle, B.N. Kreiswirth, J.R. Lapa e Silva, and J.L. Ho. 2003. The *Mycobacterium tuberculosis* complex-restricted gene cfp32 encodes an expressed protein that is detectable in tuberculosis patients and is positively correlated with pulmonary interleukin-10. *Infect. Immun.* 71, 6871-6883.

Humphrey, D.M. and M.H. Weiner. 1987. Mycobacterial antigen detection by immunohistochemistry in pulmonary tuberculosis. *Hum. Pathol.* 18, 701-708.

Karlsson, M., H. Kollberg, and A. Larsson. 2004. Chicken IgY: utilizing the evolutionary advantage. *World's Poultry Sci. J.* 60, 341-348.

Marques, M.A., A.G. Neves-Ferreira, E.K. da Silveira, R.H. Valente, A. Chapeaurouge, J. Perales, B.R. da Silva, K.M. Dobos, J.S. Spencer, P.J. Brennan, and M.C. Pessolani. 2008. Deciphering the proteomic profile of *Mycobacterium leprae* cell envelope. *Proteomics* 8, 2477-2491.

Mawuenyega, K.G., C.V. Forst, K.M. Dobos, J.T. Belisle, J. Chen, E.M. Bradbury, A.R. Bradbury, and X. Chen. 2005. *Mycobacterium tuberculosis* functional network analysis by global subcellular protein profiling. *Mol. Biol. Cell.* 16, 396-404.

Naser, S.A., G. Ghobrial, C. Romero, and J.F. Valentine. 2004. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 364, 1039-1044.

Pereira Arias-Bouda, L.M., L.N. Nguyen, L.M. Ho, S. Kuijper, H.M. Jansen, and A.H. Kolk. 2000. Development of antigen detection assay for diagnosis of tuberculosis using sputum samples. *J. Clin. Microbiol.* 38, 2278-2283.

Pick, U. 1981. Interaction of fluorescein isothiocyanate with nucleotide-binding sites of the Ca-ATPase from sarcoplasmic reticulum. *Eur. J. Biochem.* 121, 187-195.

Romero, C., A. Hamdi, J.F. Valentine, and S.A. Naser. 2005. Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subspecies *paratuberculosis* DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm. Bowel. Dis.* 11, 116-125.

Schmidt, P., R. Wanke, E. Linckh, V. Wiedemann, R. Kuhlmann, and U. Losch. 1989. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. II. In vitro studies on gastric and enteric digestion of egg yolk antibodies specific against pathogenic *Escherichia coli* strains. *J. Vet. Med.* 36, 619-628.

Schnaitman, C.A. 1970. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. *J. Bacteriol.* 104, 882-889.

Shin, S.J., J.H. Han, E.J. Manning, and M.T. Collins. 2007. Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of the BACTEC MGIT 960 system. *J. Clin. Microbiol.* 45, 1941-1948.

Sung, N. and M.T. Collins. 2003. Variation in resistance of *Mycobacterium paratuberculosis* to acid environments as a function of culture medium. *Appl. Environ. Microbiol.* 69, 6833-6840.

Tini, M., U.R. Jewell, G. Camenisch, D. Chilov, and M. Gassmann. 2002. Generation and application of chicken egg-yolk antibodies. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 131, 569-574.

Ward, D.G. and J.D. Cavieres. 1998. Photoinactivation of fluorescein isothiocyanate-modified Na,K-ATPase by 2'(3')-O-(2,4,6-trinitrophenyl)8-azidoadenosine 5'-diphosphate. Abolition of E1 and E2 partial reactions by sequential block of high and low affinity nucleotide sites. *J. Biol. Chem.* 273, 14277-14284.

Weingart, C.L., G. Broitman-Maduro, G. Dean, S. Newman, M. Peppler, and A.A. Weiss. 1999. Fluorescent labels influence phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect. Immun*. 67, 4264-4267.

Whan, L., H.J. Ball, I.R. Grant, and M.T. Rowe. 2005. Occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in untreated water in Northern Ireland. *Appl. Environ. Microbiol*. 71, 7107-7112.

Yokomizo, Y., H. Yugi, and R.S. Merkal. 1985. A method for avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Nippon Juigaku. Zasshi.* 47, 111-119.

Young, C.A., F. G. Silversides, and R.M. Jones. 2007. Chicken-derived IgY recognizes developing and mature stages of *Loma salmonae* (Microsporidia) in Pacific salmon, *Oncorhynchus* spp. *Aquaculture* 273, 398-404.

Zhang, W.W. 2003. The use of gene-specific IgY antibodies for drug target discovery. *Drug Discov. Today* 8, 364-371.

Zhang, J.L., S. Yokoyama, and T. Ohhashi. 1997. Inhibitory effects of fluorescein isothiocyanate photoactivation on lymphatic pump activity. *Microvasc. Res.* 54, 99-107.