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TITOLO DELLA TESI

IL POLIMORFISMO A625C NEL GENE *MyD88* E' ASSOCIATO ALLA RESISTENZA AL *Mycobacterium bovis* NEL BOVINO

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IN CATTLE HETEROZYGOSITY AT THE A625C POLYMORPHIC SITE OF THE MyD88 GENE IS ASSOCIATED WITH RESISTANCE TO *Mycobacterium bovis*

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INTRODUCTION

Etiology of bovine tuberculosis

Mycobacterium bovis, the agent of bovine tuberculosis – along with *M. tuberculosis*, *M. africanum* e *M. microti* – belongs to the "*Mycobacterium tuberculosis* complex". In addition to cattle and humans, *M. bovis* infects also several domestic and wild species, which provide the pathogen with a reservoir, large and difficult to control. In Ireland and England the reservoir host is the badger, in New Zealand the deer, in Australia the possum **(Lisle et al., 2007)**, in Italy (Sicily) the black pig **(Di Marco et al., 2012)**, in Spain and Portugal the wild boar **(Di Marco et al., 2012)** . England considered eradicating the badger, at least in the areas (the West Country) with high bovine tuberculosis prevalence. However, two independent studies reached contrasting results. One study concluded that badger eradication would markedly reduce the disease prevalence; according to the second study, badger eradication would influence the disease prevalence marginally. In 2008, England decided against badger culling (a case where science acknowledges it cannot give straight answers).

While *M. bovis* is pathogenic for cattle and humans, M. t*uberculosis* instead is pathogenic for humans, but not for cattle: specifically, *M. tuberculosis* infects cattle, which generally controls very well the infection and do not develop clinical signs of the disease **(Ocepek et al., 2005)**. Given that *M. bovis* and *M. tuberculosis* have in common about 99.9% of the DNA sequence **(Garnier et al., 2003)**, the above asymmetry has been attributed to a difference in gene expression between the two bacterial species **(Neil et al., 2005)**. In countries with active bovine tuberculosis programs, the periodical testing of cattle herds for tuberculosis infection, meat inspection and milk pasteurization have reduced to <1% the cases of human tuberculosis

attributable to *M. bovis* and confined them primarily to people infected with HIV or exposed to prolonged contact with animals (veterinarians or abattoir workers) **(Thoen and Lo Bue, 2007)**.

Yet bovine tuberculosis remains relevant as a zoonosis and for the major economic losses it causes to the dairy industry: slaughter of infected and often much valuable animals; quarantine of infected herds; restriction on animal export and milk commercialization. To this, one must add the cost in control measures and compensation. As an estimation of the total cost, the current year the disease will cost the English government £ 1 billion **(Brumfield, 2012)**. Appropriately, the International Office for epizootics (OIE) places bovine tuberculosis within the class B: the class of pathogens which can be transmitted to humans and cause also significant economic losses.

Infection

The contagion occurs via aerosol or ingestion. Animals become infected inhaling the mycobacteria released in the air by animals with the active disease. They can also acquire the pathogen while grazing on contaminated pastures or drinking contaminated water. Once it has infected a new host, the mycobacterium forms the so called "primary complex" - the primary infection site where the bacterium replicates and invades local lymph nodes **(Glickman et al., 2001)**. From the lungs, the mycobacterium can migrate via the lymphatic and blood systems and invade several organs (liver, spleen, bones). *M. bovis* causes in humans a disease which is very similar in symptoms and severity to that caused by *M. tuberculosis*. *M. bovis* is a pathogen very devious and difficult to control since it can remain dormant in the host and can survive outside the host for long time. Cattle, much as people, display great individual variability with respect to the resistance against infection, depending

upon the environment (herd density, co-infections, nutritional status) and the individual genetic background. With reference to human tuberculosis, Schurr E. posed the challenging question as to whether tuberculosis is inherited or acquired, to mean that the presence of the mycobacterium is necessary, but not sufficient to acquire the disease **(Schurr, 2007)**.

In vivo and post mortem diagnosis

The official diagnostic test for bovine tuberculosis in vivo is the purified protein derivative (PPD) test **(Di Marco et al., 2012)**. The tuberculin is a protein isolated from the liquid culture of *M. bovis*. In the infected animal, the tuberculin induces an allergic reaction which causes skin thickening. The ELISA test based on the dosage of interferon –γ (IFN-γ) is also used. This assay measures IFN-γ released in the plasma after stimulation of whole blood with the tuberculin antigen. The two assays both suffer of low sensitivity and specificity **(Alvarez et al., 2012)**. In addition, a negative association exists between exposure to the helminth *Fasciola hepatica* and the tuberculin test **(Claridge et al., 2012)**. The tuberculin test is used as primary test and the INF-γ assay as secondary test. Cattle that show positive reactions in both tests are culled for post mortem examination. When used concurrently, the two assays increase the predictive level of the in vivo diagnosis. However, regardless of the testing scheme used, false-positive and false-negative reactions plague both these assays **(Lim et al., 2012)**. The post mortem diagnosis is the pillar preventing disease transmission from cattle to humans and the reference standard for validation of in vivo diagnostic tests. The post mortem diagnosis is carried out on specimens of diseased tissue and afferent lymph node or on specimens of bronchial, mediastinal and retropharyngeal lymph nodes, when the animal does not show visible lesions. The specimens are analyzed by the culture test, PCR or the histological examination. The culture test, conforming to the Koch's postulate, is still "the

golden standard". However, in the current form, the culture test cannot differentiate between active or latent infection. By definition dormant bacteria cannot grow on standard culture media **(Oliver, 2010)**.

Does the risk to humans justify the high cost of fighting bovine tuberculosis?

Between 1997 and 2000 in England , there were 315 human cases of *M. bovis* tuberculosis. Of the 50 isolates of *M. bovis* recovered, 15 had not been recorded in the cattle population in England and 72% of the human cases of bovine tuberculosis were >50 years old, suggesting reactivation of an infection acquired before routine milk pasteurization was introduced; in addition, many of the remaining cases were people born abroad. These data – indicative of a negligible threat posed by bovine tuberculosis to public health – were used to ask whether the cost required to carry out the programs of badger culling and vaccine development, needed to control bovine tuberculosis, was justified **(Torgerson, 2008)**. The answer came soon. The answer was that without periodical tests and abattoir surveillance, bovine tuberculosis would rapidly advance to infectious stages and lead to an increased risk of transmission to humans **(Smith, 2008)**. Second, the question disregarded the crucial role of disease control in protecting international trade (in 1995 the value of live cattle export in England was $ 115 million **(Gordon, 2008)**. In Smith's words **(Smith, 2008)**, it is not wise getting rid of the cat, just because there are no mice around.

Is *M. bovis* eradication a feasible objective at present?

Eradication is the reduction of an infectious disease's prevalence in the global host population to zero **(Dowdle,1998)**. The eradication of *M. bovis* - a pathogen with a vast and diversified

wildlife reservoir and the property of going into dormancy for years - is totally unrealistic. At present, a more practicable objective is reaching the status of bovine tuberculosis officially free (TBOF) country. This status does not indicate that the country has eradicated *M. bovis*, but that the pathogen is under control. The country is declared TBOF when the percentage of bovine herds infected with tuberculosis does not exceed 0.1% of all herds for 6 consecutive years and at least 99.9% of herds are TBOF **(Revierego et al., 2006)**. However, for some countries, this status will be more difficult to reach compared to others, as the case of New Zealand and England demonstrates. At present, both these countries have failed to become officially free from bovine tuberculosis using the test and slaughter control program, that instead was successful in other countries. Wildlife, acting as a *M. bovis* reservoir, is the major factor preventing pathogen eradication in New Zealand and England. New Zealand is expected to reach the "officially free" status by 2013. This target required a very drastic and extensive culling of wildlife, on a scale that in England cannot be publicly accepted. More in general, until an efficient program of wildlife vaccination will be available - we must expect that the control of bovine tuberculosis remains problematic in countries where wildlife acts as a reservoir of the pathogen. Despite of great efforts and resources invested in eradication programs during the last 20 years, only 11 countries of the European Community (Belgium, Czech Republic, Denmark, Germany, France, Luxemburg, Netherlands, Austria, Slovakia, Finland, and Sweden) have been declared TBOF **(Reviriego, 2006)**. The following provinces of Italy are also TBOF: Bergamo, Lecco, Sondrio, and Como (Lombardy), Ascoli Piceno (Marche), Grosseto (Tuscany), Bolzano and Trento (Trentino-Alto Adige) **(Reviriego, 2006)**. These data refer to the year 2006; more recent records were not available. In the United States of America, it took almost 100 years to reduce the prevalence of the disease from about 5% (in 1917) to 0.001% (today) for all herds **(Lim et al., 2012)**. These data explain why *M. bovis* is considered one of the most difficult pathogen to control (or the most successful).

In addition to reservoir host, which factors contribute to the persistence of the disease?

The pathogen ability of causing silent infections, which manifest years later. Limitation in sensitivity and specificity of diagnostic tests. Large herd sizes and animal movements, which facilitate animal contacts. The herd type also influences the risk of the disease. Dairy herds are exposed to a higher risk, due to the high density of animals, high milk production which debilitates the animal and the management (milking practice) which promotes disease transmission **(Lim et al., 2012)**.

The advantage associated with being officially free from bovine tuberculosis

The animals and animal products from single herds or countries declared officially free from tuberculosis have the advantage of free circulation on the national territory and abroad. Herds are officially declared free by the local sanitary agencies (ASL); the countries from the European Community. At present the international commerce of animals and animal products requires that they originate from officially free herds. In the near future the requirement will probably be more stringent, limiting the import from countries (rather than herds) with the officially free status. The European Community in fact tends to consider the whole national territory as a web moving compact towards the status of country exempt from tuberculosis. To reach this objective, it is necessary to convince breeders to observe tougher biosecurity standards (such as buying animals only from herds officially exempt from tuberculosis, avoiding animal overcrowding or the use of contaminated pastureland). All this requires a nationwide cattle database, alerting periodically (on a six months basis) about prevalent (existing) and incident (new) outbreaks.

Cattle vaccination

Several studies show that the BCG – in cattle as in humans – is not 100% efficient, providing total protection to some subjects, only limited protection to others and no protection at all to still other animals **(Buddle et al., 1999; Waddington and Ellwood, 1972)**. Sensitization to environmental mycobacteria prior to BCG vaccination and the strain of used mycobacteria influence protection **(McNair et al., 2007)**. Age at which calves are vaccinated is also important, neonatal calf vaccination induces higher levels of protection than vaccination of calves at 5-6 months of age **(McNair et al., 2007)**. At present, cattle vaccination with the BCG is prohibited in the European Community since it interferes with the diagnostic tests of tuberculosis (both the tuberculin skin test and the conventional IFN-γ blood test). In order to convince the European community to lift the existing prohibition on BCG vaccination of cattle, a test is being developed to differentiate infected from BCG-vaccinated animals **(Vordermeier et al., 2011)**. At present, while BCG is unable to control bovine tuberculosis, a better vaccine remains elusive. A vaccine for cattle, to be effective, should prevent the infection **(McNair et al., 2007)** and thus eliminate disease transmission to humans and should not interfere with diagnostic tests. In contrast, a vaccine for wildlife is asked to prevent transmission of the pathogen to wildlife and domesticated species. In conclusion, vaccination – the tool most useful to reduce prevalence, incidence and spread of infection independently of the infection source (whether represented by wild or domestic animals) - unfortunately is not yet available.

Host-pathogen interaction.

When an animal is infected with *M. bovis*, the bacilli enter its lungs. Once inside the alveoli, they interact with the immune cells, mainly macrophages and dendritic cells. Interaction

initiates the immune response, i. e. the proliferation of antigen-specific lymphocytes, which migrate to the infected site and accumulate around the infected macrophages, forming the so called granuloma. In spite of the immune response, mycobacteria succeed in infecting the macrophages and multiplying within them. Mycobacterial phagocytosis involves several host receptors (complement, Fc, mannose and DC-SIGN receptors), but shortly after infection, DC-SIGN becomes the privileged receptor for the mycobacteria. This is shown by the evidence that pre-incubation of dendritic cells with specific antibodies inhibits *M. tuberculosis* binding to these cells. The mycobacterium can persist and multiply inside host cells because is able to block the maturation of the vacuole in which it resides (the phagosome). When a particle is phagocytosed it becomes encapsulated in the phagosome, which slowly fuses with the lysosome inside the cell. However, in the case of the cell infected by a mycobacterium, the phagosome does not fuse with the lysosome. The lipoarabinomannan (LAM) has been identified as one the several bacterial components inhibiting phagosome-endosome fusion **(Tailleux et al., 2005)**. This evasive tactic works as long as macrophages are resting **(Vandal et al., 2008)**. Once macrophages become activated by INF-γ, the bacterium is exposed to the proton-rich lysosome, where hydrolases, reactive nitrogen, reactive oxygen intermediates operate very efficiently. In activated macrophages, acid resistance is provided by the bacterial membrane bound serine protease encoded by the *Rv3671c* mycobacterial locus, which controls the internal pH of the phagosome by excluding external protons via an as yet undetermined mechanism **(MacMicking, 2008)** (Figure 1).

Mycobacteria modulate their virulence to cause persistent subclinical infection

Mycobacteria causing tuberculosis are among the most successful pathogens. Their spread among animals and humans is highly efficient. In the case of *M. tuberculosis* it is estimated

that approximately one-third of the human population (about 2 billion people) is latently infected. The majority of these infections (in humans about 90%) cause clinically silent disease, which remain permanently silent, unless the host's immunity is seriously compromised. This behavior results from a complex and strictly coordinated interaction of the bacterium with its host **(Porcelli, 2008)**. The process is controlled by the bacterial secretion system called ESX-1(mycobacteria lack the specialized type I-VI secretion system of Gram-negative bacteria and their virulence is mediated instead by ESX-1) through the secretion of the two proteins ESAT-6 and CFP-10. These proteins are privileged targets of the host's immune system and, at the same time, also virulence factors. ESX-1mutants in fact grow poorly in mouse macrophages. EXS-1 regulates the virulence of the mycobacterium by turning on and off the production of ESAT-6 and CFP-10. The external signals regulating the production level of these proteins are not known at present. ESAT-6 acts also as a membrane-disrupting toxin. Thus the protein helps mycobacteria to escape from phagosome vesicles - in which they are captured for destruction – to the cytoplasm, where they can replicate.

Tuberculosis: unsealing the apoptotic envelope

Manipulation of the cell death process (apoptosis) is a well-known strategy of many viruses and intracellular bacteria, including mycobacteria. Apoptosis of mycobacterium-infected macrophages is a form of altruistic suicide, where the infected cell dies to let the host live. Consistent with their great talent for evading immune-mediated destruction, virulent mycobacteria block the rapid apoptosis of the macrophages they have infected. In contrast, avirulent mycobacterial strains lack this ability **(Porcelli and Jacobs, 2008)**. By blocking the apoptosis of cells early after infection, the mycobacterium delays presentation of its antigens and the T cell response of the host. This helps the pathogen to establish a permanent infection.

The mycobacterium however needs to kill its host to spread to other cells. Thus the bacterium has acquired the ability to deliver a precisely timed "lethal hit". The hit specifies that the infected macrophage must die by necrosis, rather by apoptosis. Macrophages infected with avirulent mycobacteria form a robust and impermeable apoptotic body, that prevents bacteria from escaping. Macrophages infected with virulent mycobacteria instead form a fragile and porous necrotic body, that enables bacteria to escape and spread (Figure 2).

Tuberculosis jumped from humans to cows, not vice versa

Humans have lived closely with their cows for almost 10.000 years, milking, herding them and even sleeping with them for warmth. For long time it was thought that cows gave our ancestors as a gift the mycobacterium causing human tuberculosis. DNA study of 10 species of mycobacteria displayed that humans were infected with strains of *Mycobacterium tuberculosis* long before they began herding cows. Thus, tuberculosis spread from humans to cattle and not vice versa **(Gibbon, 2008)**.

Genetics of the immune response against *M. bovis*

Innate and adaptive immune responses to mycobacteria rely on Toll-like receptors (TLRs), which sense several mycobacterial components. Sensing of the mycobacterial DNA requires TLR-9, while the heat shock protein 65 (HSP65) requires TLR-4 and the lipomannan (LM), lipoarabinomannan (LAM), 19kD lipoprotein (19LP) and soluble tuberculosis factor (STF) require TLR-2 **(Doherty and Arditi, 2004).** TLRs (with the exception of TLR3) all critically depend upon the myeloid differentiation factor 88 (MyD88) to link bacterial recognition by TLRs with NF-kB activation and cytokine production **(Fremond et al., 2004).** Evidence of

the crucial role played by MyD88 as signal transducer is provided by the MyD88$^{-/-}$ mice, which die within 4 weeks from infection with *M. tuberculosis* (Doherty and Arditi, 2004; Fremond et al., 2004). MyD88 $^{-/-}$ mice infected with *M. tuberculosis* display reduced expression of IFN-γ, TNF-α and nitric oxide synthase (NOS). This observation has suggested that MyD88 controls the infection by regulating the production of these mediators **(Scanga et al., 2004).** The above studies and the high genetic similarity (99.95% identity at the nucleotide level) of the *M. tuberculosis* and *M. bovis* genomes **(Garnier et al., 2003)** collectively provided biological plausibility to the hypothesis of a functional role of the MyD88 gene against bovine tuberculosis infection. The present study shows that, in cattle, heterozygosity at the MyD88 A625C polymorphic site is associated with resistance against active - but not the latent - *M. bovis* infection.

MATERIALS AND METHODS

Diagnosis of pulmonary infection

Post mortem sample collection was according to the European Food Safety Authority (EFSA) recommendations **(Anonymous, 2004)**. In the case of animals displaying macroscopic pulmonary lesions, a portion of the diseased tissue and afferent lymph node was collected. In the case of animals without visible lesions, the mediastinal, retropharyngeal and bronchial lymph nodes were collected. Individual lung homogenates consisted of one gram or more of pooled specimens collected from the single animal. To distinguish between subjects with active (ATI) or latent (LTI) pulmonary infection, ten-fold dilutions (10^{-1} to 10^{-8}) of individual lung homogenates in sterile PBS were spotted (10 µl/spot; 5 spots/dilution) on agar-Middlebrook (MB) medium and incubated at 37°C for 4-5 weeks. At the end of the incubation time, the colony forming units (CFUs) were counted. Negative samples were incubated for 10 days in liquid MB medium supplemented (5 µg/ml) with the mycobacterial resuscitation promoting factor B (RpfB) **(Ruggiero et al., 2010)**, spotted on agar-MB, incubated for 4-5 weeks and the CFUs then counted. The optimal concentration of RpfB to use in the assay was found during preliminary experiments. The growth of colonies in the absence of RpfB was indicative of ATI and the growth of colonies only in the presence of RpfB of LTI. Controls were negative to both tests.

Identification of mycobacterial species by PCR

One colony of *M. bovis* was dispersed in 200 µl of distilled H_2O containing lysozyme (Sigma-Aldrich, St. Louis, MO; 20 mg/ml) and incubated at 37°C for 2 h. After incubation,

DNA was isolated using the DNeasy Blood & Tissue Kit from Quiagen (Hilden, Germany). PCR was carried out as described **(Bakshi et al., 2005)**.

Cases and controls

The animals included in the study – both cases and controls - were from three herds declared infected. To exclude sex and age as potential confounders, the animals were all lactating cows of age comprised between 40 and 90 months. This age interval was selected to represent subjects matched for age (as much as it was realistic) and, at the same time, a population sample sufficiently numerous to provide adequate power to the study. The average age of cases and controls were 65.4 ± 5.2 and 69.6 ± 3.9 months, respectively. To curb stratification, cases and controls were both from the same herds and the same breed (Friesian); to keep cases and controls genetically unrelated to each other, when mother and daughter were present, one of the two was excluded.

MyD88 genotyping

The intron/exon boundaries of the bovine *MyD88* gene were established matching the published mRNA sequence of the bovine MyD88 gene (NM_001014382.2) and the DNA sequence of human *MyD88* gene (MC_000003.11). Alignment was carried out using the DNAsis software (Hitachi Solutions America, S. Francisco, USA). DNA was extracted from lung specimen with the QIAamp DNA kit (Qiagen, Hilden, Germany). The primers were: 5'TGAAGGAGTACCC CGCGC3' (forward) and 5'GATGCCTGCCATGTCATT3' (reverse). Conditions of the PCR were: 7 min at 97°C; 45 s at 94°C; 30 s at 60°C; 1.5 min at 72°C (35 cycles) 5 min at 72°C. The 1210 bp fragments from 20 cases and 20 controls were

sequenced using the ABI 3730 DNA analyzer (Applied, Foster City CA, USA) and aligned with the Chromas software (Technelysium, Queensland, Australia). The sequences were used to design primers and Taqman probes targeting specifically on the single nucleotide polymorphism (SNP) located 625 bp downstream of exon1 (A625C). The forward and reverse unlabeled primers were: 5'GGTGGCGTGGTACTTTGC3' and 5'TTTCTCCTCTACGGGCTGTCT3', respectively. The Taqman VIC- and FAM-labeled probes were: 5'TAGCAAGGG**A**GACATT3' and 5'TAGCAAGGG**C**GA CATT3', respectively. PCR conditions were: 30 s at 60°C; 10 min at 95°C; 40 cycles, each lasting 15 s at 95°C and 1 min at 60°C. Genotyping was carried out blindly to the case or control status of the animals being tested.

TaqMan Gene Expression Assay

TNF-α, IFN-γ and NOS2 mRNA levels were measured on lung specimen using the TaqMan Gene Expression Assay and the StepOne instrument (Applied Biosystems, Foster City, CA). Total RNA (2 μg) was reverse transcribed using the High Capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA). The real-time quantitative PCR reactions were carried out following the manufacturer's protocol. The identification numbers of the probes are: Bt03259155_g1 (TNF-α bovine); Bt03212722_g1 (IFN-γ); Bt03249602_g1 (NOS2); Bt03279175_g1 (β-actin). Animals - 5 for each genotype (AA, AC, CC) and class (control, active and latent tuberculosis) - were tested in triplicate. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta Ct}$ method. The endogenous control gene was β-actin. The amplification efficiency of target (TNF-α, IFN-γ and NOS) and reference (β-actin) genes was approximately the same (slope < 0.1).

Sample size calculation

The data relative to 50 cases with active tuberculosis and 50 controls (OR: 0.3; proportion of controls with susceptible genotype: 0.46) showed that a sample of 127 cases and 127 controls would provide 96% power and a two-sided significance level of 0.01. The study enrolled 150 cases of acute tuberculosis, 150 cases of latent tuberculosis and 300 controls.

Statistical analysis

The Fisher's exact test and the ANOVA with Tukey post-hoc test were performed using the GraphPad Prism software version 5 and the binomial logistic regression using the SPSS statistical package version 18; the Hardy-Weinberg equilibrium and the relative risk reduction were calculated as described **(Cavalli-Sforza and Bodmer, 1971; Modiano et al., 2001,** respectively**)**.

RESULTS

Diagnosis of cases and controls

Pulmonary tuberculosis infection can be active (ATI) or latent (LTI); the latter is characterized by the presence of dormant bacteria (viable but not culturable on usual growth media) **(Oliver, 2010)**. The methods commonly used to diagnose latent tuberculosis are the tuberculin skin test or the interferon-γ release assay. However, these methods do not distinguish between hosts still infected and those which successfully controlled infection **(Barry et al., 2009)**. In the present study, grouping together different phenotypes would sensibly reduce the power of the study **(Schurr, 2007)**. *M. tuberculosis* has 5 resuscitation promoting factors (*rpf*) genes coding for as many redundant proteins (RpfA-E), which, in the form of recombinant proteins expressed in *E. coli*, induce resuscitation of *M. tuberculosis* **(Biketov et al., 2007)** and *M. marinum* **(Parikka et al., 2012)**, in *vitro* and ex *vivo*. Based on these findings, an in-house assay was developed aimed at resuscitating dormant mycobacteria with the RpfB protein. From 20 of the animals included in the study, it was possible to have both milk and lung specimens. This material was used to validate the method. The results from milk and lung specimens were fully concordant (Table 1). The test was therefore extended to all the animals, using lung specimens collected post mortem. A PCR assay discriminating between *M. tuberculosis*, *M. bovis* or *M. avium* established that cases (with ATI or LTI) were all infected with *M. bovis*. In conclusion, the cases with ATI were subjects positive to the PCR assay and the bacteriological test in the absence of RpfB; the cases with LTI subjects positive to the PCR assay and the bacteriological test in the presence of RpfB; controls were subjects exposed to *M. bovis* infection (since from the same herds that supplied

also the cases), but free from infection (negative to the PCR assay and the bacteriological tests, in the presence or absence of RpfB) (Figures 3-4).

Study design

The criticism more often leveled at association studies is that they lack reproducibility **(Reviriego Gordejo et al., 2006; Buddle B. et al., 1999)**. To curb this drawback, a two-stages study was designed. The preliminary (hypothesis-generating) stage involved 50 control animals, which were separately confronted with 50 cases with ATI or 50 cases with LTI. This preliminary study displayed a significant association of the MyD88 polymorphic site A625C with ATI (P: 0.01; Table 2), but not with LTI (P: 0.84; Table 2). The A625C polymorphic site is located in the intron 1 of the MyD88 gene (Figure 5). The study yielded also the following valuable data: first, that the association is potentially robust (since detected using a small number of subjects); second, that case stratification according to the form (active or latent) of the infection would definitively provide more power to the study. The SNPs shown in the reference sequence (Figure 5) other than A625C were not present in the sample population studied.

MyD88 heterozygosity and resistance to ATI

The study was repeated on a larger and independent sample consisting of 300 controls, 150 cases with ATI and 150 with LTI. A separate experiment (with animals not included in the case-control study) showed that crosses between subjects homozygous for the A or C factors (AA x CC) yield only heterozygous (AC) offspring. The experiment proved that A and C are transmitted as codominant alleles (data not shown). Cases with ATI were not in Hardy-

Weinberg equilibrium (χ^2: 4.4; Table 2). When the test was repeated on the cases with LTI, both cases and controls were in equilibrium (χ^2 controls: 0.9; χ^2 cases: 0.3; Table 2). The results suggested an association of the *MyD88* marker with ATI, but not with LTI. The more cogent Fisher's exact test showed that heterozygosity (the AC status) is strongly associated with resistance to ATI (OR: 0.19, P: 6.0 x 10^{-12} Table 2); second, the association remains strong when the homozygous classes are pooled (OR: 0.22; P: 1.8 x 10^{-10}; Table 2); third, the *MyD88* marker does not influence predisposition to LTI (OR: 0.81- 0.83; P: 0.36 - 0.40; Table 2). The binomial logistic regression test supported these conclusions (Table 3). Given the frequency of the AC heterozygotes among controls (135/300 = 0.45; Table 2) and the level of protection afforded (OR: 0.19; Table 2), this genotype prevented 36% [(0.45) x (1- 0.19) = 0.36] of the potential cases of ATI in the population examined **(Modiano et al., 2001).**

MyD88 heterozygosity and inflammation

TNF-α, INF-γ and NOS are known to profoundly influence tuberculosis **(Scanga et al., 2004)**. It is also known that high as well as low levels of inflammation negatively impact into this disease **(French et al., 2001; Narita et al., 1998; Glickman et al., 2001)**. Thus, if the MyD88 heterozygotes displayed intermediate cytokines levels compared to homozygotes, the association between A625C heterozygosity and resistance to *M. bovis* infection would gain strong biological plausibility. To test this hypothesis, the levels of TNF-α, INF-γ and NOS of subjects with different genotype (AA, AC, CC) and status (controls, animals with ATI or with LTI) (6 classes; 5 animals/class) were measured. The expression levels of the subjects with ATI or LTI were then compared with those of control subjects having the same genotype. Heterozygous carriers expressed levels of TNF-α, IFN-γ and NOS significantly lower compared with the AA homozygotes. Instead, carriers expressed levels only slightly higher compared with the CC homozygotes; in this case, the difference did not reach statistical

significance (Figure 6). One possible explanation for this heterogeneity is that the technique used does not discriminate below a threshold level. Taken together, the data support the conclusion that an optimal inflammatory response is associated with the intermediate A625C phenotype.

DISCUSSION

The present study demonstrates that in cattle the animals heterozygous at the *MyD88* A625C polymorphic marker benefit of a five-fold reduced risk for ATI (OR: 0.19; P: 6 x 10^{-12}; Table 2). The reduced risk however does not extend to the animals with LTI (OR: 0.83; P: 0.40; Table 2). Heterozygosity at the A625C SNP is associated with intermediate levels of IFN-γ, TNF-α and NOS (Figure 3). What is the biological advantage of an intermediate level production of these mediators in the case of active tuberculosis? The short answer is that heterozygosity provides the optimal level of inflammation. The deficiency of IFN-γ ,TNF-α or NOS favors tuberculosis **(Doherty and Arditi, 2004; Fremond et al., 2004)**. At the same time, some symptoms of the disease are known to be caused by the immune response of the host, rather than by the mycobacterium **(Glickman and Jacobs, 2001)**. Episodes of disease reactivation and inflammatory syndrome related to pre-existing *M. tuberculosis* **(French et al., 2001)** or *M. avium* **(Narita et al., 1998)** infection have been observed in HIV-co-infected patients after antiretroviral therapy. The study also displays differences in cytokines expression within the same genotype, between animals with acute or latent tuberculosis. This difference is particularly evident in the case of the AA animals (Figure 3). Whether caused by the mycobacterium or the host immune response, these results, though preliminary, point increased expression levels of pro-inflammatory cytokines as potential markers of disease reactivation. The A625C polymorphism – located in the intron 1 of the Myd88 gene - adds evidence to the notion that non-coding regions can influence gene expression. It is not

surprising that this occurs also in the case of inflammation, which needs to be under fine and complex regulation.

In cattle exposure to environmental mycobacteria, which occurs in the majority of the subjects, interferes with the diagnosis of *M. bovis* infection by the tuberculin skin test (TST) or the IFN-γ assay **(Hope et al., 2005**). Variability in the reagents, incubation time and diagnostic cut-off levels also influence specificity and sensitivity of these assays **(Pai et al., 2004).** The post mortem culture test – still the golden standard method **(Thacker et al., 2011)** - was therefore preferred for the diagnosis of infection. Also, the limits of the TST and IFN-γ assays and – on the other side - the high prevalence of *M. bovis* infection among the enrolled animals (150 subjects with acute and as many with latent infection out of approximately 650 randomly tested animals) persuaded the authors that the number of false-positive and false-negative results would be better minimized assuming that controls were all exposed subjects, rather than relying on the TST or the IFN-γ assays for exposure diagnosis. The authors do not claim that the method adopted here is superior to current methods in general; rather they trust that it yields a better defined disease spectrum and more reproducible results under a case-control design.

Tuberculosis is influenced by several genes interacting among themselves **(Chang et al., 2008)** and with the environment **(Schurr, 2007)**. The presence of the mycobacterium is necessary, but not sufficient to acquire the disease, as shown by the control subjects, which, exposed to the pathogen, did not acquire the disease (Table 2); see also Diamond **(Schurr, 1987)**. Environmental factors (climate, herd size, animal purchases, cattle movements) are known to promote bovine tuberculosis **(Neil et al., 2005)**. Even strong genetic effects on the *M. tuberculosis* can be missed when environmental effects are not taken into account **(Schurr, 2007**). We claim that the unusually small OR and P values (OR:0.19; P: 6.0×10^{-12}) reported in the present study reflect how the problems confronting the genetic analysis of this

complex disease were solved. Cases were made homogeneous (active and latent tuberculosis cases being analyzed separately) and the environmental confounders were either excluded (sex and breed) or randomized (age). More importantly, control subjects were from the same source population of cases. Controls were therefore subjects that remained infection-free (negative to the bacteriological and PCR tests) though they had the same opportunity of cases to become infected. Population stratification often has been claimed as responsible for false-positive results in association studies, yet rarely has been demonstrated to be the culprit **(Colhoun et al., 2003; Risch, 2000)**. Human studies have shown that stratification might originate when different ethnicities are mixed **(Healy, 2006)**. In the present study, only one breed was studied. Furthermore, the replication of the association across 2 independent population samples argues against the result being a product of population stratification.

Genetic association studies are characterized by a high rate of false-positive results **(Risch, 2000)**. This condition often is due to the selection of a candidate gene without functional relation to the disease **(Risch, 2000; Lander and Schork, 1994)**. In the present study, *MyD88* was selected on the basis of a large body of experimental data showing that – at least in mice – this gene is critical for signaling downstream the presence of mycobacterial components and induce the production of the innate response mediators (IFN-γ, TNF-α and NO) against mycobacteria (4-5). Further, the two-stages study design allowed to directly prove the reproducibility of the association. Replication of the results at the time they are first described is gaining consensus as an approach for reducing the number of false-positive results **(Colhoun et al., 2003; Tsao and Florez, 2007)**. The two-stages design was also of value to define the precise phenotype (active versus latent *M. bovis* infection) to study (Table 2). In conclusion, high biological relevance of the gene to study, accurate choice of diagnostic criteria, randomization of environmental confounders were all carefully kept in mind during

the present journey in the puzzling field of association studies. However, since the association is being described for the first time, the results of this study are presented as preliminary.

Last, the test used here to differentiate between acute and latent disease potentially could be extended to the periodical testing of cattle for tuberculosis. The count of dormant mycobacteria awakened by RpfB in milk samples would be an easy way to know the prevalence in the population of latent tuberculosis, a parameter greatly influencing the control of the pathogen.

FIGURES AND LEGENDS

FIG 1. Survival tactics of *Mycobacterium tuberculosis* in IFN-γ-activated macrophages.

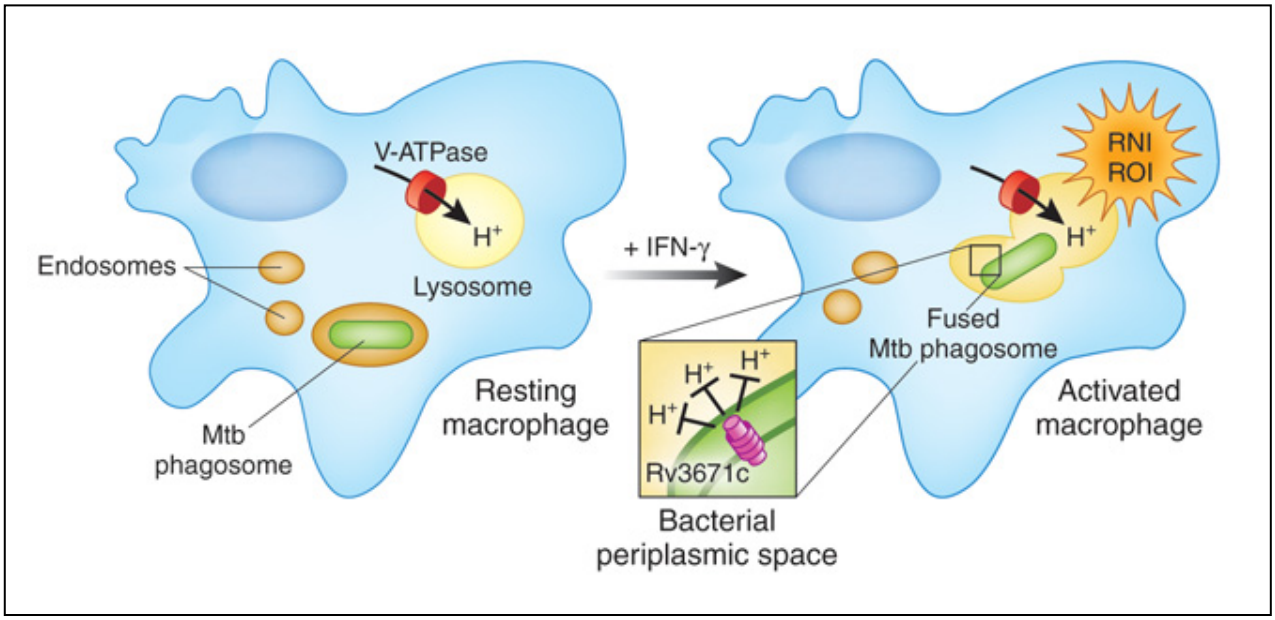

FIG 2. Alternative forms of cell death induced by *Mycobacterium tuberculosis*.

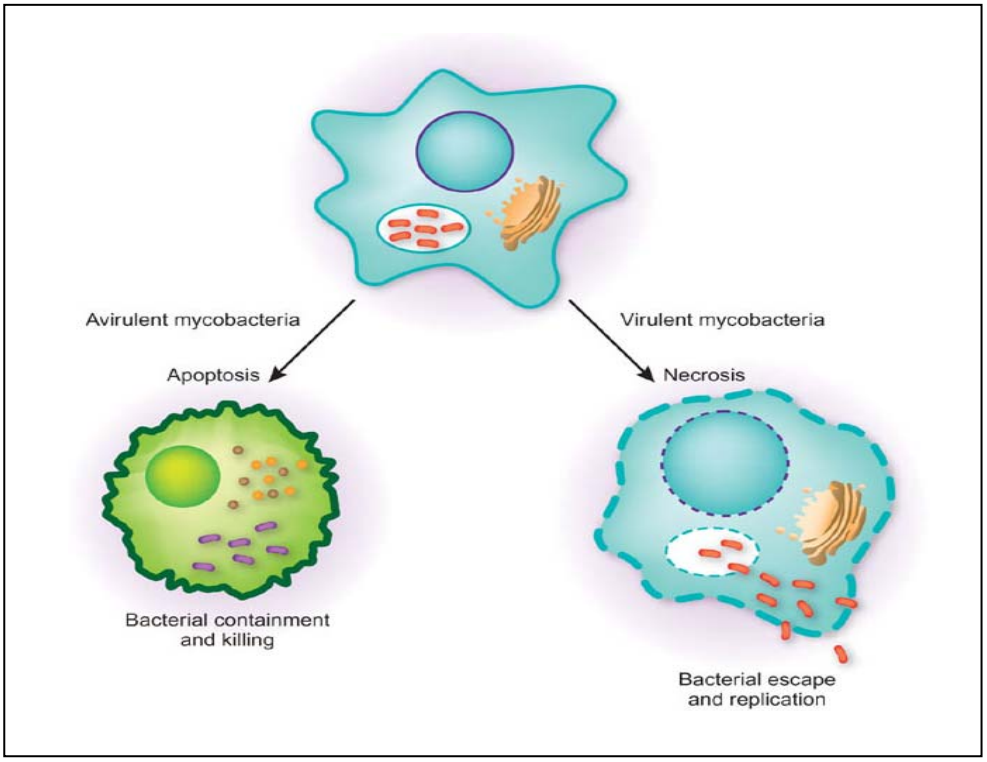

FIG 3. Diagnostic criteria used to classify subjects into controls or subjects with active tuberculosis infection (ATI) or with latent tuberculosis infection (LTI). Controls: subjects negative to the culture - with and without RpfB - and PCR tests. ATI: subjects positive to the culture without RpfB and PCR tests. LTI: subjects positive to the culture with RpfB and PCR tests.

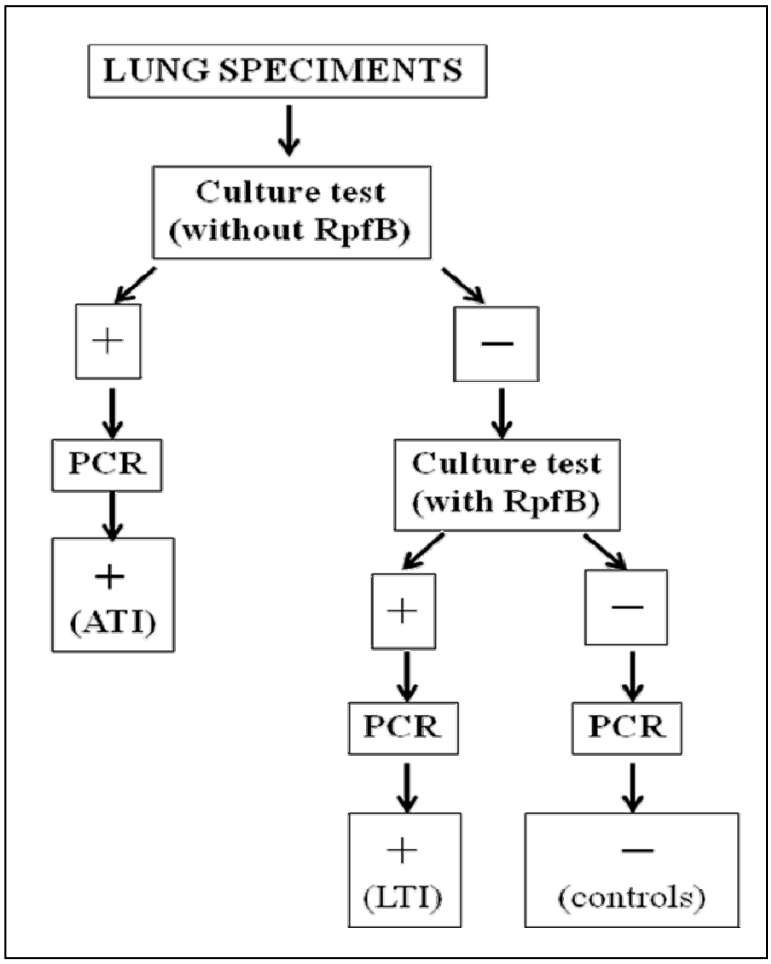

FIG 4. Culture test of a lung specimen from a cow with latent *M. bovis* tuberculosis in the presence and absence of the resuscitation promoting factor B (RpfB).

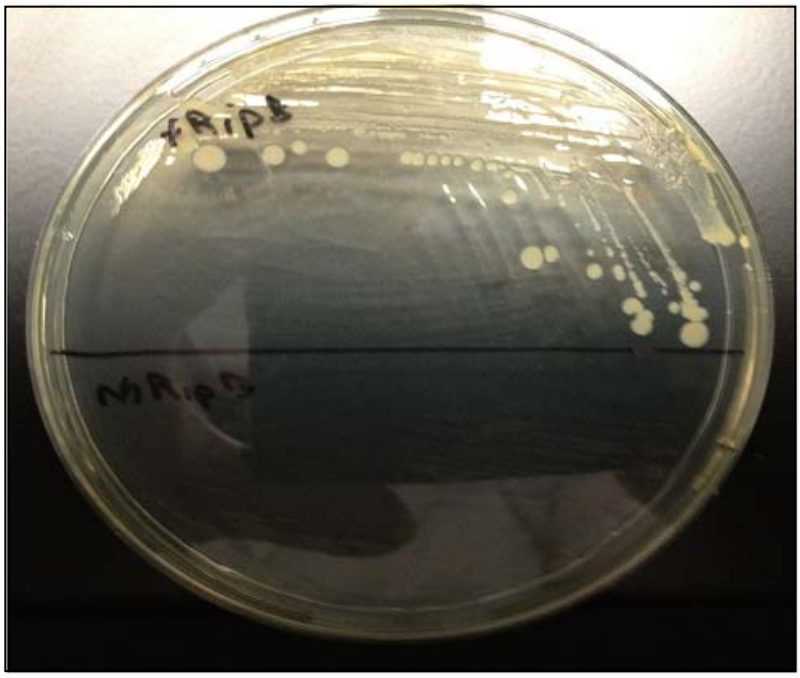

FIG 5. Position of the A625C SNP on the MyD88 gene. The map is oriented 5' to 3'. Source: www.ncbi.nlm.nih.gov/gene/444881.

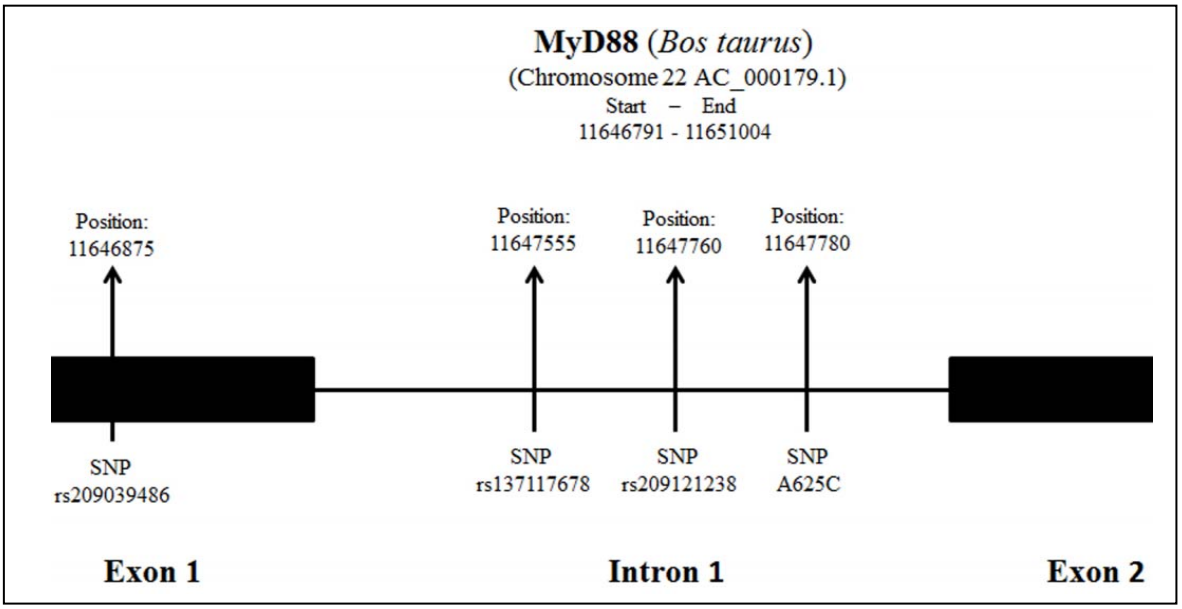

FIG 6. Pro-inflammatory cytokines mRNA levels measured by the TaqMan gene expression assay. Specimens were from controls or animals with active (ATI) or latent (LTI) *M. bovis* infection. Animals were grouped according to the genotype (AA, AC or CC) and class (controls, subjects with ATI or LTI). Each group consisted of 5 subjects. (A-C) mRNA expression levels of TNF-α, IFN-γ and NOS, respectively. Relative sample quantification was carried out by the comparative $2^{-\Delta\Delta Ct}$ method. The endogenous control was the β-actin gene.

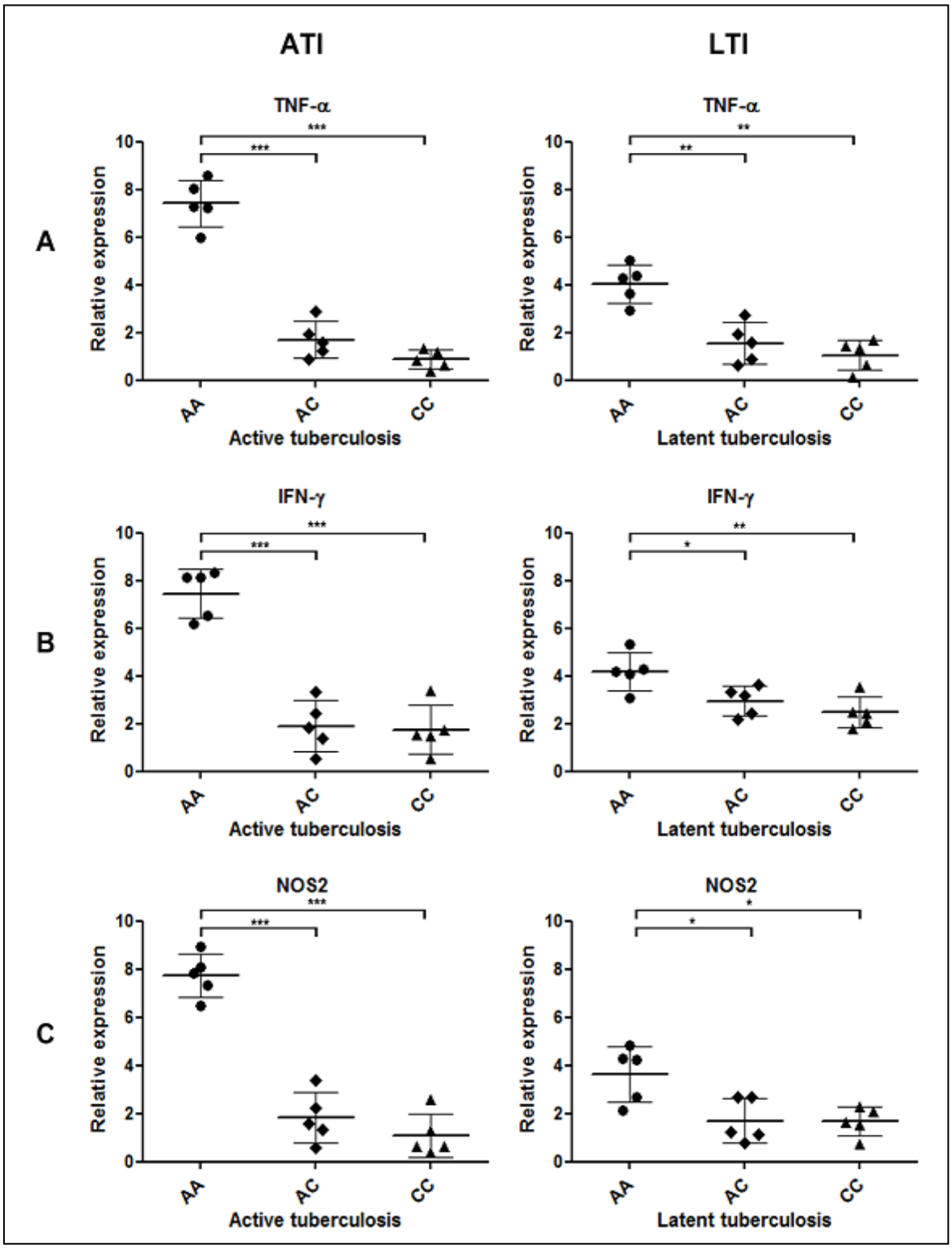

Table 1.

	Specimen	
RpfB	**Milk**	**Lung**
Presence	7/20	7/20
Absence	0/20	0/20

Table 1. Resuscitation of dormant *M. bovis* grown in MB medium supplemented with RpfB.

Table 2.

Study Stage	TB[a]	Status	Genotype			Total	χ^{2b}	AC vs AA		AC vs (AA+CC)	
			AA	AC	CC			OR (C.I.)	P	OR (C.I.)	P
1	Active	Cases	36	10	4	50	5.2	0.30 (0.12-0.74)	0.001	0.29 (0.12-0.71)	0.01
		Controls	25	23	2	50	1.4				
	Latent	Cases	28	21	1	50	1.7	0.81 (0.36-1.81)	0.68	0.85 (0.38-1.87)	0.84
		Controls	25	23	2	50	1.4				
2	Active	Cases	123	23	4	150	4.4	0.19 (0.11-0.32)	$6x10^{-12}$	0.22 (0.12-0.37)	$1.8x10^{-10}$
		Controls	140	135	25	300	0.9				
	Latent	Cases	75	60	15	150	0.3	0.83 (0.53-1.28)	0.40	0.81 (0.53-1.23)	0.36
		Controls	140	135	25	300	0.9				

[a] *M. bovis* pulmonary tuberculosis infection.
[b] $\chi^2_{0.05}$ (1 degree of freedom) = 3.8.

Table2. Heterozygosity at the A625C SNP influences active pulmonary tuberculosis infection.

Table 3.

Ref. Gen.[a]	TB[b]	Binomial logistic regression			
		Wald	P value	e	H-L P[c]
AA	Active	40	1.8×10^{-10}	0.19	1
	Latent	0.78	0.37	0.83	1
CC	Active	0.01	0.91	1	1
	Latent	0.68	0.40	0.74	1

[a] Reference genotype.

[b] *M. bovis* pulmonary tuberculosis infection.

[c] The non-significance of the Hosmer-Lemeshow P value indicates that the model predicted by the logistic regression fits with observed data

Table 3. Heterozygosity at the A625C SNP and resistance to active pulmonary tuberculosis shown by binomial logistic regression

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PUBLICATIONS

Bacteriophage Therapy of Salmonella enterica: A Fresh Appraisal of Bacteriophage Therapy. Rosanna Capparelli, Nunzia Nocerino, Marco Iannaccone, Danilo Ercolini, Marianna Parlato, **Medaglia Chiara** and Domenico Iannelli. **The Journal of Infectious Diseases 2010.**

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In cattle heterozygosity at the A625C polymorphic site of the MyD88 gene confers resistance to Mycobacterium bovis infection.

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