MOZZARELLA DI BUFALA E BRUCELLOSI: IDENTIFICAZIONE DI GENI DI RESISTENZA ALLA *B. ABORTUS* NEL BUFALO (BUBALUS BUBALIS)

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Liberté, Égalité, Fraternité

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RIASSUNTO

La Mozzarella di Bufala Campana è un formaggio da tavola di pasta filata molle, la cui produzione prevede ancora oggi l'utilizzo di latte crudo. La Denominazione di Origine Protetta per tale prodotto è stata riconosciuta per effetto del reg. Ce 1107/96. Nell'ambito di tale processo di valorizzazione, lo scopo è quello di fornire al consumatore un prodotto con caratteristiche organolettiche e nutrizionali ottime, assicurandone le condizioni di salubrità e non pericolosità. E' fondamentale quindi ridurre il rischio microbiologico ad un livello minimo, in un' area, dove la brucellosi è endemica. *Brucella abortus*, l'agente eziologico della brucellosi, è un patogeno intracellulare facoltativo che infetta i macrofagi dell'ospite. Molte bufale infette liberano brucellae nel latte. In un contesto in cui, i programmi di eradicazione basati sull'abbattimento dei capi infetti si sono rivelati inefficaci, si inserisce l'approccio di migliorare la resistenza degli animali domestici alle infezioni mediante selezione. Nell'uomo alcune varianti alleliche del gene *mbl,* codificante una delle proteine chiave dell'immunità innata*,* rappresentano un fattore di rischio per infezioni virali e batteriche (Garred P. et al, 2003). Durante il triennio di dottorato, l'ipotesi che i polimorfismi al gene *mbl* possano influenzare la suscettibilità all'infezione da *B. abortus* è stata valutata nel bufalo attraverso uno studio caso- controllo. L'interazione tra il gene *mbl* e *B. abortus* è stata investigata in 700 animali, appartenenti a 4 allevamenti casertani, suddivisi in infetti e non infetti mediante diagnosi sierologica e biomolecolare. L'analisi mediante PCR ha identificato quattro alleli all'esone 1 del gene *mbl* (*A, B, C, D*), due alleli (*H, L*) in posizione -550 e due alleli in posizione -221 (*X, Y*) al promotore del gene. Gli animali *AA* sono risultati più frequenti tra i controlli, mentre gli animali *OO* più frequenti tra i casi: (OR: 0.15; CI: 0.09824– 0.2417; $P<10^{-7}$; Tabella 4). Poiché prossimi ai polimorfismi all'esone 1, si è scelto di includere nello studio anche i polimorfismi (*H*, *L*), (*X, Y*). L'analisi mediante PCR ha rivelato 16 coppie di aplotipi al gene *mbl*. I soggetti *HYA/HYA* risultano rappresentati, con solo due eccezioni, solo tra i controlli (OR: 0.001; CI: 0.0006– 0.01847; $P< 10^{-7}$; Tabella 5). D'altra parte, i soggetti *LYD/LYD* sono più rappresentati tra i casi (OR= 9.9; CI: 6.280− 15.857; $P < 10^{-7}$; Tabella 6). L'analisi genetica ha concluso che la coppia di aplotipi *HYA/HYA* è associata alla resistenza all'infezione da *B. abortus,* mentre la coppia di aplotipi *LYD/LYD* è associata alla predisposizione. Molti studi di associazione risultano falsi positivi poiché privi di uno studio che dia un significato biologico all'analisi genetica: in questo studio la funzione della MBL è stata investigata in soggetti *HYA/HYA* e in soggetti LYD/LYD usando diversi approcci. Analogamente a quanto osservato nell'uomo, i sieri provenienti da animali *HYA/HYA* presentano una concentrazione della proteina MBL maggiore di quella osservata in sieri provenienti da soggetti *LYD/LYD* (Figura 4). Il siero proveniente da bufali geneticamente resistenti (*HYA/HYA*) mostra *in vitro* una più elevata attività battericida comparati al siero proveniente da soggetti suscettibili (*LYD/LYD*) (Figura 5). Inoltre, quando i monociti bufalini sono infettati, *in vitro,* con brucellae opsonizzate con sieri provenienti da animali *HYA/HYA*, a differenza di quanto si verifica con sieri provenienti da animali *LYD/LYD*, si osserva un'accelerazione del processo di internalizzazione (Figura 7) e un maggior processamento del patogeno (Figura 8). Questi risultati contribuiscono a definire biologicamente il ruolo protettivo fornito dall'aplotipo *HYA/HYA* e a rendere verosimile la possibilità di controllare l'infezione da *B. abortus* nel bufalo mediante selezione genetica.

SUMMARY

Water buffalo Mozzarella is a typical "pasta filata" cheese from Southern Italy, having high moisture (55 to 62%) and high fat in DM (>45%).
The specific characteristics of the final product mainly arise from the raw materials employed, the agro-ecosystem of the area of production and the traditional technology of manufacture. The traditional mozzarella is made from raw water buffalo milk using natural whey cultures as fermentation starters and the microflora occurring in such complex environment is certainly one of the parameters affecting the dairy manufacture. For this reason, on the base of Regulation No. 1107/96 from 12th June 1996 the designation “water buffalo mozzarella cheese” (PDO) has been registered in the List of Protected Designations of Origin and Protected Geographical Designations. The production district of this cheese is defined in its law specifications and encompasses seven provinces across two regions of southern Italy (Campania and Lazio) (Mauriello G. et al, 2003).
In a such enhancement program, the goal is provide to the consumer a product with best organoleptic and nutritional features, ensuring the microbiological safety, in area like Southern Italy, where the brucellosis is endemic.
Brucellosis is a zoonotic infection frequently resulting in abortions and diminished levels of milk production in buffalo and in cattle.
More importantly, brucellosis constitutes a serious public health risk, with the majority of human infections being caused by the consumption of contaminated milk products. The causative agent is *Brucella abortus*, a Gram negative facultative intracellular pathogen that reside predominantly within membrane– bound compartments in two host cell types, macrophages and placental trophoblasts (Roop R.M. et al, 2004). Only a few water buffalo cows that become infected develop clinical signs of the disease (spontaneous abortion) (Borriello G. et al, 2006). However, many infected cows shed *B. abortus* in the milk.
Eradication programs involving the slaughter of infected animals have been carried out for more than 20 to 30 years. However, latent infections, prolonged incubation of the pathogen, incomplete protection provided by vaccines, and difficulties in distinguishing serologically between vaccinated and naturally infected animals have limited the efficacy of eradication programs (Borriello G. et al, 2006).
We are exploring an alternative solution: the control of brucellosis by selective breeding for disease-resistant genotypes. Remarkably, even in water buffalo herds heavily infected with *B. abortus*, about 20% of the subjects remain negative by the serological tests and presumably noninfected all the time. This observation suggests that genetic variation within the host may play a part in the resistance to brucellosis.
In water buffalo, it is known that host genetic factors can influence *B. abortus* infection in water buffalo. It has been shown by Borriello et al. (2006) that subjects with the *BB* genotype at the *Nramp1* locus (*Nramp1BB*) remain seronegative despite prolonged exposure to the pathogen. The monocytes from *Nramp1BB* subjects express a higher level of the *Nramp1* messenger and thus can control more efficiently bacterial replication during the early stages of infection (Capparelli R. et al, 2007).
Indeed, the search for alleles influencing susceptibility to pathogens is not new. In recent years, high-throughput searches for polymorphisms yielded a large number of reports on the association of allelic variants with diseases.

Case-control studies can detect associations between host genes and disease resistance very efficiently. The design of these studies is also conceptually simple: the frequency of the allele conferring resistance in a sample of cases is compared with the frequency in a sample of controls. The expectation is that the allele conferring resistance will display a higher frequency among the controls.
My PhD thesis describes the identification in the water buffalo of a gene conferring resistance to *B. abortus* infection, the mannose-binding lectin gene (*mbl*) coding an important protein of the humoral innate immune system.
MBL is a major pattern-recognition molecule of the innate immune system. It shows selective and calcium (Ca^{2+})- dependent binding to terminal sugars on the surface of microorganisms (mannose and N-acetyl-D- glucosamine for example), enabling it to distinguish self from non-self. It can also bind to phospholipids, nucleic acids and non-glycosylated proteins. MBL has been shown to bind promiscuously to a wide range of bacteria, viruses, fungi and protozoa (Neth O. et al, 2001).
On binding to its ligands, MBL activates the complement in an antibody-independent manner via MBL-associated serine protease (MASP)-1 and MASP-2.
MASP-2 cleaves C4 and C2 (Thiel S. et al, 1997). Additionally, MBL opsonizes bacteria using the C1q receptor on macrophages without the involvement of complement (Takahashi K. et al, 1997) and regulates the inflammatory cytokine release by phagocytic cells (Jack D.L. et al, 2001; Kilpatrick D.C. 2002).
The protein consists of multimers of an identical polypeptide chain of 32 kDa. Each chain comprises four distinct regions encoded by different exons of the MBL-2 gene.
Each chain has a C-terminal, calcium-dependent carbohydrate-recognition domain (CRD); a short, a-helical, hydrophobic neck region (in the so-called coiled-coil configuration); a collagenous region containing 19 Gly- Xaa-Xaa triplets and a cysteine-rich N-terminal region (Garred P. 2008; Garred P. et al, 2008).
Three polypeptide chains form a triple helix within the collagenous region, stabilized by hydrophobic interactions and interchain disulphide bonds within the N-terminal cysteine-rich region. This is the basic building block of all circulating molecular forms of MBL. In serum, MBL consists of oligomers ranging from dimers to hexamers, but the ability of the protein to bind effectively to microorganisms and activate complement appears to depend on the presence of higher order oligomers (tetramers and above) (Eisen D.P. et al, 2003; Dommet R.M. et al, 2006) (Fig.1).
In humans the exon 1 of the *mbl*2 gene (*mbl*1 is a pseudogene) contains four alleles (*A, B, C, D*). Additional polymorphisms have also been described at nucleotide -550 (*H, L*), -221 (*X, Y*), and +4 (*P, Q*) of the promoter (Eisen D.P. et al, 2003).
In humans, certain *mbl* genotypes represent a risk factor for bacterial fungal (Dyment D.A. et al, 2005), and viral (Eisen D.P. et al, 2003; Garred P. et al, 1997) infections.
The hypothesis that the *mbl* gene polymorphism influences *B. abortus* infection was tested in water buffalo.
The interaction between the *mbl* gene and *B. abortus* was investigated in water buffaloes from four herds located in the province of Caserta (southern Italy), an area where brucellosis is endemic, and genes conferring resistance against the disease are expected to be subject to selection.
Accurate diagnosis is fundamental for meaningful results from association studies (Healy D.G. 2006; Zondervan K.T. et al, 2002). Since no single test is fully specific for brucellosis (Godfroid J. et al, 2002), a variety of tests was used. The subjects were tested twice at a 1 month interval for the presence of anti- *B. abortus* antibodies in the serum (by agglutination and flow cytometry) and for the presence of shedding brucellae in the milk (by culture test and by PCR).

Cases included animals consistently positive to all these tests; controls included animals exposed yet negative by the same tests.
Cases and controls were sampled from the same source population (the four herds mentioned above). Thus, controls had the same opportunity as cases of becoming infected and being included in the study.
To exclude confounding effects due to vaccination, sex or age, cases and controls were all unvaccinated lactating cows of 1–10 years age.
MBL genotypes were determined blindly (without knowing the case or control status of the subjects), using an allele-specific PCR (Fig.2).
In particular, promoter genotypes were determined by the presence or absence of a 373 bp band when using appropriate primer combinations (Table 2), and exon 1 genotypes were determined by the presence of a 128 (alleles *D, A*), 135 (alleles *B, A*), or 143 (alleles *C, A*) bp band when using appropriate primer combinations (Table 3).
PCR analysis identified four alleles in the exon 1 of the *mbl* gene (*A, B, C, D*), two alleles (H, L) at position −550 and two more at −221 (*X, Y*) of the *mbl* promoter. Because of the known influence of the exon 1 genotype on pathogen opsonization in humans (Kilpatrick D.C. 2002; Madsen H.O. et al, 1998), the primary analysis investigated whether animals with two copies of the wild allele (*AA*),with one mutant allele (*AB, AC, AD*; collectively referred to as *AO* animals) or with two mutant alleles (*BB, CC, DD, BC, BD, CD*; collectively referred to as *OO* animals) were present at different frequencies in cases and controls. The *AA* animals were more frequent among controls, while the *OO* animals were more frequent among cases than controls (OR: 0.15; CI: 0.09824–0.2417; $P<10^{-7}$; Table 4). The frequency of the allele A among controls (0.67) was significantly higher than among cases (0.42; Fig. 3a). These results provided the first line of evidence that the AA genotype conferred resistance against *B. abortus* infection.
The −550 (*H, L*) and the −221 (*X, Y*) promoter alleles are inherited on the same chromosome (*in cis*) with the structural alleles in the form of haplotypes (Madsen H.O. et al,1998). Haplotypes contain the information relative to the whole set of linked alleles. Case-control studies based on haplotypes are therefore much more powerful and informative, compared with studies based on single nucleotide polymorphisms (Joosten P.H. et al, 2001; Tregouet D.A. et al, 2002).
Due to the proximity of the (*H, L*), (*X, Y*) and exon 1 polymorphisms (Madsen H.O. et al, 1998), molecular haplotyping seemed a correct choice. PCR analysis revealed 16 MBL haplotype pairs. Of these, two (*HYA/HYA, LYD/LYD*), the most frequent, could be confirmed by family data.
When the subjects were classified as having two copies of the *HYA* haplotype (*HYA/HYA*), one (*HYA/−)* or none (*−/−*), the *HYA/HYA* subjects were represented only among controls: *HYA/HYA* subjects were 0.1% as likely as *−/−* subjects to be positive to the *B. abortus* (OR: 0.001; CI:0.0006–0.01847; *P*<10−7; Table 5).
The frequency of the *HYA* haplotype among controls (0.57) was also significantly higher than among cases (0.1; Fig. 3b).
The above results, an OR=0.009 characterizing the association between the *HYA/HYA* haplotype pair and brucellosis (Table 5) versus an OR=0.15 characterizingthe association between the *AA* genotype and the same disease (Table 4), highlight the importance of studying haplotypes rather than single alleles (Joosten P.H. et al, 2001; Tregouet D.A. et al, 2002).
The *LYD/LYD* subjects were instead more frequent among cases. They were approximately 10 times more likely to be positive to the *B. abortus* tests than *−/−*

subjects (OR=9.9; CI: 6.280− 15.857; $P<10^{-7}$; Table 6). The remaining haplotype pairs, much rarer, were not analyzed for association with the disease. In conclusion, the *HYA/HYA* haplotype pair is associated with resistance against *B. abortus* infection and the *LYD/LYD* haplotype pair with predisposition to the infection by the same pathogen. Both the promoter and exon 1 alleles of the human *mbl* gene differ from each other in one single base substitution (Garred P., 2003). If this holds also for the water buffalo *mbl* gene, then remarkably, the ability of a haplotype to promote protection or susceptibility depends upon one single nucleotide change, as in the case of the haplotype pairs *HYA/HYA* versus *HYA/ HYD*, *HYA/HYA* versus *LYA/LYA*, or *HYA/HYA* versus *HXA/HXA*.

Studies of the human MBL gene have clarified the chemical relationship between single point mutations occurring in this gene and the function of the controlled protein. In particular, it has been shown that single base substitutions caused by mutant alleles interfere with the correct folding of the three basic subunits of the MBL molecule. If this protein contains one defective chain, it becomes prone to degradation or unable to activate the complement (Garred P. et al, 2003). On the basis of this evidence, we propose that, as observed in humans, resistant water buffaloes have fully functional MBL protein capable of combating *B. abortus* infection effectively; susceptible animals, on the contrary, have partially inactive MBL protein, unable to carry out rapid opsonization of the pathogen.

So, we measured the concentration of MBL protein in the serum of resistant and susceptible subjects by flow cytometry. The *HYA/HYA* controls showed a median MBL level significantly higher, compared to the two *HYA/HYA* cases and the subjects (cases or controls) with a non-*HYA/HYA* haplotype pair (Fig. 4).

When carrying out genetic case- control studies, the biological plausibility of the candidate gene is a critical requisite. The association is likely to be meaningful (and reproducible) if there is evidence, from an animal model or a homologue gene, of a biological relation between trait and candidate gene (Wacholder S. et al, 2004).

So, we tried therefore to verify experimentally the biological plausibility of the correlation between *mbl* haplotypes and resistance to *B. abortus* infection.

It has been shown that MBL protein has an antibacterial activity independent by complement system (Fernandez- Prada C.M. et al, 2001).

To probe a possible difference in the biological activity of the protein between resistant (*HYA/HYA*) and susceptible (*LYD/LYD* or *LYA/LYD*) subjects, the *B. abortus* strain 2308 (10^5 CFU) was incubated *in vitro* in the presence of heat-inactivated (control) and non inactivated (activity) autologous serum from 10 water buffaloes of each group. For each animal, two blood samples collected at 2-week intervals were tested, each time in triplicate. Following incubation at 37° for 24 h, the content of each well was plated to count CFU. The serum from *HYA/HYA* animals displayed a significantly higher antibacterial activity compared with serum from *LYD/LYD* animals (Fig. 5). The evidence that heat inactivation destroys the antibacterial activity of the serum (Fig. 5) suggests that this activity is indeed mediated by MBL, a heat-labile lectin (Anders E.M. et al, 1994). Involvement of the MBL in the antibacterial activity was indicated also by the following observations: the antibacterial activity was inhibited by pre-incubation of the serum with 10 mM EDTA, 25 mM mannose or 50 mM N-acetyl-D-glucosamine (Fig. 6), known to specifically inhibit MBL (Anders E.M. et al, 1994).

Most importantly, the antibacterial activity was inhibited in the presence of mouse anti-human MBL or after passage of the serum through mannan-Sepharose column (Fig. 6).

One of the functions of the MBL protein is pathogen opsonization and phagocytosis (directly and by activating the antibody-independent pathway of the complement;Takahashi K. et al, 2005).
To determine whether *HYA/HYA* subjects are resistant to *B. abortus* infection because their phagocytes can internalize or process the pathogen more efficiently, monocytes were infected with organisms preincubated with heat- inactivated (control) and non inactivated (activity) autologous serum from 10 buffaloes of each group (*HYA/HYA, LYD/LYD*) at a MOI of 10:1 for different timing until 1h.
Nonadherent brucellae were removed by using gentamicin (40 μg/ml) for 30 min, and after the viable counts of organisms were determined to follow the kinetic of internalization or alternatively the plates were incubated again for 24h to study the killing of brucellae by monocytes.
We found that if monocytes were infected with *HYA/HYA* serum- treated brucellae, can internalize more quickly the pathogen (Fig.7) and rapidly destroy the pathogen (Fig.8) compared with LYD/LYD serum- treated brucellae.
More importantly, we observed a downregulation of inflammatory response in terms of tnf- α, il- 6 and il- 1β expression level, when monocytes were stimulated with *HYA/HYA* serum- treated brucellae (5 h at MOI =10), compared with *LYD/LYD* serum- treated brucellae (Fig. 9).
These evidences suggest why *HYA/HYA* animals are resistant and also why do not form anti-*B. abortus* antibodies upon contact with the pathogen: the ingested bacteria are rapidly destroyed by phagocytes, and the inflammatory signaling is consequently too short and weak to induce a systemic response (antibody production).
The evidence that genetic analysis and functional analysis yield fully concordant results lends biological significance to the protective role of the *HYA/HYA* genotype against *B. abortus* infection and the predisposing role of the *LYD/LYD* genotype.
In conclusion, the results reported here illustrate how the option to control animal diseases by genetic selection is realistic. With regard to water buffalo brucellosis, the positive selection of resistant subjects and the concurrent negative selection of the susceptible ones could rapidly increase the level of herd immunity. The relatively high initial frequency of the resistant animals would further facilitate this process. This approach may have a positive impact on the economics of the dairy industry and hopefully contribute to changing the culture of animal health control by slaughter.

1. INTRODUZIONE

1.1. Mozzarella di bufala e il Marchio D.O.P

La Mozzarella di Bufala Campana è un formaggio da tavola di pasta filata molle, la cui produzione puo' avvenire sia con latte sottoposto a trattamento termico sia con latte intero; tuttavia, il disciplinare contenuto nel DPR del 28/9/1979 prevede per la produzione di tale prodotto, l'utilizzo esclusivo di latte di bufala. Ed è proprio tale materia prima a fornire gli elementi di tipicità alla Mozzarella di Bufala Campana (Mauriello G. et al, 2003).
Il latte bufalino, infatti, ha sapore dolce, colore bianco opaco dovuto all'assenza di carotenoidi. Il pH oscilla tra il 6,6-6,8. Il grasso è tra il 6-9% con prevalenza dell'acido oleico tra gli acidi insaturi e del acido palmitico tra gli acidi saturi. Le sostanze azotate, variano dal 3,8-4%, da Albumina, Globulina, Proteosi-Peptoni 0,50-1%. Le sostanze azotate non proteiche variano tra lo 0,20-0,30%. Il lattosio varia tra il 4,5-5%.
Inoltre, in condizioni normali, nel latte di bufala sono presenti alcuni ceppi di lattobacilli (*Lactococcus lactis subsp. lactis, Lactobacillus delbrueckii subsp. lactis, Lactobacillus helveticus* e *Streptococcus thermophilus*) in concentrazioni superiori a quelle contenute ad esempio nel latte vaccino. L'attività metabolica di questi batteri è responsabile del sapore e dell'aroma tipici della Mozzarella di Bufala (Coppola S et al,2006) ed influisce notevolmente sul fenomeno di acidificazione della cagliata durante la trasformazione.
Questi diversi valori contribuiscono senz'altro alla tipicità del prodotto grazie alla diversa consistenza che esso viene, alla fine, ad assumere.
La Mozzarella di Bufala Campana ha ottenuto il riconoscimento del marchio D.O.P. il 12 Giugno 1996 per effetto del reg. Ce 1107/96.
Per DOP, secondo il regolamento CEE n. 2081/92 del 14 luglio 1992, s'intende un marchio di tutela giuridica che viene attribuito a quei prodotti agricoli alimentari le cui peculiari caratteristiche qualitative dipendono essenzialmente dal territorio in cui sono prodotti.
Per poter ricevere l'appellativo deve sussistere perciò la condizione per cui la produzione delle materie prime e la loro trasformazione ed elaborazione fino al prodotto finito devono essere effettuate nella regione delimitata di cui il prodotto porta il nome.
Per "ambiente geografico" il disciplinare intende non solo i fattori naturali (clima, caratteristiche ambientali), ma anche quelli umani (tecniche di produzione tramandate nel tempo, artigianalità, know-how), che, combinati insieme, consentono di ottenere un prodotto inimitabile al di fuori di una determinata zona produttiva.
Nell'ambito di tale processo di valorizzazione è chiaro che lo scopo di tutti i produttori è quello di fornire al consumatore un prodotto che abbia delle caratteristiche sensoriali e nutrizionali ottime e naturalmente queste caratteristiche sono tanto più conservate quanto è minore lo stress termico che subisce il latte. Al giorno d'oggi infatti la produzione di tale formaggio avviene ancora mediante l'utilizzo di latte crudo. Tuttavia accanto alle caratteristiche organolettiche e nutrizionali, esistono le condizioni essenziali di salubrità e non pericolosità del prodotto. E' fondamentale quindi mettere in atto tutte le procedure necessarie per ridurre il rischio microbiologico a un livello minimo accettabile.

1.2. Il Bufalo e la sua importanza economica

Negli ultimi anni, in Italia, l'allevamento del bufalo (*Bubalus bubalis*), proprio in virtù del'espansione del mercato dei prodotti alimentari da esso derivati, ha acquisito un'importanza economica via via crescente, tale da richiedere risorse tecnologiche avanzate per ottimizzarne la gestione.
Il bufalo appartiene all'ordine degli ungulati, sottordine artiodattili, gruppo ruminanti, famiglia cavicorni, sottofamiglia bovidi, genere bubalus. Ed è nel genere *Bubalus bubalus*, tipo River, che si inquadra la bufala allevata in Italia che fino a pochi anni fa era definita come bufalo di tipo mediterraneo e che oggi ha ricevuto la denominazione di "Bufala Mediterranea Italiana".
Tale traguardo è stato raggiunto grazie al lungo isolamento (almeno 16 secoli) ed alla mancanza di incroci con bufale appartenenti alla stessa razza o a razze diverse allevate in altri Paesi del mondo.
Negli ultimi cinquanta anni la specie bufalina è tra quelle che hanno dimostrato il maggior incremento numerico in Italia: dai 12.000 capi si è passati a circa 200.000 tra capi iscritti e non iscritti al Libro Genealogico. Durante questo intervallo di tempo il bufalo è passato da animale da lavoro, ad animale da latte dal quale si può ricavare un reddito sicuro.
E' interessante notare che tale incremento abbia interessato il bufalo, nonostante tale specie non sia certamente la più produttiva tra i ruminanti allevati, e soprattutto non abbia beneficiato, sino ad oggi, di sistematici piani di selezione genetica, né usufruito di genotipi importati da Paesi zootecnicamente all'avanguardia (come si è verificato ad esempio, per i bovini).
Parallelamente è stato necessario incrementare le attività di controllo dei Servizi Veterinari nell'ambito dei piani di eradicazione di brucellosi e tubercolosi.
In particolare, i programmi di eradicazione della brucellosi, basati essenzialmente sull'abbattimento degli animali infetti, pur essendo stati perpetuati per più di 20-30 anni sono risultati inefficaci a causa delle difficoltà esistenti nel distinguere sierologicamente gli animali vaccinati da quelli naturalmente infettati, dalle infezioni latenti, dall'incubazione prolungata del patogeno e dalla protezione incompleta fornita dai vaccini.
E' chiaro quindi, che altre strategie di controllo della malattia sono necessarie per la valorizzazione e la salvaguardia della specie bufalina e dell'industria lattiero-casearia che su questa specie basa la maggior parte dei suoi profitti.

1.3. Brucellosi

La Brucellosi è endemica in tutto il mondo, in particolare nei paesi mediterranei dell'Europa, nel nord e nell'est dell'Africa, in India, in Medio Oriente, Asia centrale, Messico, America centrale e meridionale.
Il serbatoio principale di questi microrganismi è rappresentato dagli animali domestici: bovini, suini, capre, pecore. Possono essere importanti diffusori di questa patologia anche gli animali selvatici, i quali mantengono un ciclo silvestre che può poi coinvolgere gli erbivori domestici portati al pascolo sugli stessi prati.
La malattia rappresenta da sempre uno dei più gravi problemi zooeconomici per gli ingenti danni provocati negli allevamenti di animali da riproduzione, a causa della perdita dei prodotti del concepimento, delle infezioni genitali, dei fenomeni di

ipofecondità o sterilità temporanea o permanente, della diminuzione della secrezione lattea, dell'aumentata incidenza delle malattie neonatali e del notevole deprezzamento commerciale dei soggetti colpiti.
Allo stesso tempo, la brucellosi costituisce un importante problema di sanità pubblica per le infezioni umane. Il contagio puo' avvenire indirettamente per contatto con animali e materiali infetti o, più frequentemente, indirettamente attraverso il consumo di alimenti contaminati (latte e latticini).
Nel bufalo, la brucellosi è sostenuta da *Brucella abortus*.

1.3.1. *Brucella abortus*

Brucella abortus appartiene al genere Brucella. E' un cocco Gram-negativo, pleiomorfo, non mobile e asporigeno. *B. abortus* è un batterio intracellulare facoltativo, è in grado cioè di resistere non solo al processamento da parte dei neutrofili dopo l'internalizzazione ma è anche in grado di replicare attivamente in vari tipi di cellule ad attività fagocitica professionale, come i macrofagi (Baldwin C. L. et al, 1994), e non (Detilleux, P. G. et al, 1990).
La nicchia ecologica preferita per le brucellae è il compartimento fagosomale all'interno dei macrofagi dell'ospite (Roop, R. M. et al, 2004). La capacità di questi microrganismi di instaurare e mantenere un'infezione cronica è strettamente correlata alla capacità degli stessi di sopravvivere e replicare all'interno della cellula ospite (Roop, R. M. et al, 2004). In seguito alla penetrazione attraverso le mucose, le brucellae migrano ai linfonodi, al fegato, alla milza, alle ghiandole mammarie all'interno dei fagociti. Ed è proprio attraverso le ghiandole mammarie che molte bufale infette liberano brucellae nel latte.

1.4. La risposta immuno- innata e il sistema del complemento

L'interazione tra il sistema immunitario e i patogeni consiste in un equilibrio dinamico tra i meccanismi messi in atto dall'ospite per eliminare l'infezione e le strategie del patogeno messe in atto per eludere la risposta immunitaria.
In tale contesto, un ruolo essenziale è svolto dai meccanismi effettori della risposta immuno-innata che costituisce appunto la prima linea difensiva. Pertanto è ormai chiaro che la sopravvivenza e la patogenicità di un microrganismo sono funzione della sua capacità di evadere o resistere ai meccanismi protettivi dell'immunità innata dell'ospite.
Le strategie attuate dal sistema innato si basano sostanzialmente sul riconoscimento di un ampio numero di strutture conservate espresse in maniera selettiva dai patogeni (PAMPs, pathogen-associated molecular patterns). Tale riconoscimento avviene grazie all'espressione di recettori presenti su numerosi popolazioni cellulari (patter-recognition receptor, PRR).
Questi recettori sono coinvolti nell'eliminazione dei patogeni, essendo responsabili dell'attivazione dei processi infiammatori e del reclutamento ed attivazione di cellule capaci di eliminare i microrganismi stessi.
Appartiene alla classe dei PRR, il sistema del complemento, una serie di circa trenta proteine del plasma che agiscono in cascata. Il sistema del complemento utilizza tre diverse strategie per il riconoscimento del patogeno, ciascuna delle quali dà origine una via di attivazione del complemento che culmina con il legame covalente delle proteine complementari alla superficie microbica. La via classica è innescata dagli anticorpi legati agli antigeni sulla superficie microbica. Le altre due vie, quella

alternativa e la via mediata dalla MBL, stimolano l'attivazione del complemento in assenza di anticorpi. La differenza sostanziale esistente tra le tre vie è rappresentata dunque, dall'evento di riconoscimento che avvia l'attivazione e le fasi immediatamente successive.
E' invece comune a tutte le vie una sequenza di reazioni che portano all'assemblaggio di una proteasi nota come C3 convertasi, che scinde C3 nei frammenti C3a e C3b. C3a agisce da mediatore dell'infiammazione e C3b si lega covalentemente alla superficie del patogeno.
Le proteine del complemento possono essere distinte in base alla propria funzione (Janeway et. al., 2005):
1. Opsonizzazione: C3b e C4b sono opsonine, proteine quindi in grado di riconoscere e legare PAMPs sulla superficie dei microrganismi. L'opsonizzazione del microrganismo ne facilita la fagocitosi da parte delle cellule ad attività fagocitica professionale.
2. Infiammazione: I frammenti C5a e, meno significativamente, C4a e C3a sono importanti attivatori dell'infiammazione in grado di indurre permeabilità vascolare, reclutamento e attivazione dei fagociti.
3. Lisi: C5b lega e recluta C6 e C7 alla superficie del microrganismo. C7 e successivamente C8 subiscono una variazione conformazionale tale da esporre domini idrofobici in grado di inserirsi nel bilayer lipidico. Il complesso C5b678 catalizza la polimerizzazione del component finale C9 che forma un poro transmembrana di circa 10nm di diametro, causando la lisi della cellula bersaglio. Questo assemblaggio macromolecolare prende il nome di Complesso di Attacco alla Membrana (MAC).
4. Eliminazione degli immunocomplessi: la solubilizzazione e la rimozione degli immunocomplessi in circolo avviene grazie al legame di C4b e C3b, a loro volta covalentemente legati agli immunocomplessi, a CR1, un recettore del complemento presente sugli eritrociti.In questo modo sono trasportati al fegato e alla milza dove vengono distrutti.

1.5. Mannose Binding Lectin

La Mannose-binding lectin (MBL) è una proteina del siero, calcio- dipendente in grado di riconoscere e legare i carboidrati presenti sulla superficie di un'ampia varietà di patogeni (virus, batteri, funghi, protozoi), attivando il sistema del complemento o agendo direttamente come opsonina.
Appartiene alla famiglia delle collectine e consiste di multimeri di una identica catena polipeptica di 32 kDa. Ciascuna catena comprende quattro distinte regione codificate dai quattro diversi esoni che compongono il gene *mbl2*.
Ciascuna catena presenta all' N-terminale una regione ricca in residui di Cisteina, quindi un dominio tipo collagene caratterizzato dalla presenza di circa 18-20 ripetizioni in tandem Glicina-X-Y e un dominio CRD (carbohydrate-recognition domain) al C- terminale. Ciascun dominio CRD lega ioni calcio in grado di stabilire legami di coordinazione con i gruppi idrossilici degli zuccheri (mannosio, glucosio, N-acetiglucosamina) presenti sulla superficie dei patogeni (Figura 1) (Eisen D.P. et al, 2003; Dommet R.M. et al, 2006; Garred P. 2008; Garred P. et al, 2006).
Mediante il dominio tipo collagene tre catene formano triple eliche stabilizzate da interazioni idrofobiche e da ponti disolfuro intracatena tra le regioni N-terminali. L'assemblaggio di tali strutture porta alla formazione di oligomeri di varie dimensioni. Tuttavia è stato osservato che i dimeri ed i trimeri non sono biologicamente attivi e,

per l'attivazione del complemento, è necessario almeno un tetrametro (Eisen D.P. et al, 2003; Ip W.K. et al, 2009; Garred P. 2008). La MBL promuove la fagocitosi dei patogeni, mediante l'attivazione del sistema del complemento in maniera indipendente dalla risposta anticorpale (Eisen D.P. et al, 2003; Garred P. 2008; Garred P. et al, 2006).
In circolazione, infatti, la MBL forma un complesso funzionale con le serinaproteasi associata alla MBL (MBL- associated serine protease - MASPs) 1, 2 e 3 che, dopo il legame con un microrganismo, divengono enzimaticamente attive. La MASP-2 è identica all'esterasi-C1 nella via classica del complemento ed è in grado di scindere C4 e C2, formando C4b2a. C4b2a agisce come una C3-convertasi scindendo C3, formando C3b ed inducendo in questo modo l'opsonizzazione del microrganismo.
E' stato dimostrato che tale processo ha un'effetto modulatore, dose dipendente, sul rilascio di citochine proinfiammatorie quali TNF-α, IL-1β e IL-6 a seguito dell'infezione (Jack D.L. et al, 2001).
Fernandez- Prada C.M. e collaboratori (2001) hanno mostrato, inoltre, che la MBL è anche in grado di svolgere un'attività antibatterica diretta, indipendente cioè dall'attivazione del complemento.

1.5.1. Polimorfismi nel gene *mbl* umano

Nel gene *mbl* sono state individuate sei mutazioni puntiformi che comportano variazioni nella quantità e/o nella funzionalità della proteina nel siero e si associano ad un'aumentata suscettibilità a molte malattie infettive (Garred P. et al, 1997; Neth O. et al, 2001).
La prima mutazione identificata al codone 54 dell'esone 1 del gene *mbl* determina la sostituzione di un residuo di Glicina con uno di Acido Aspartico (la variante è nota come Allele B). Il polimorfismo al codone 57 dell'esone 1 causa invece la sostituzione di un residuo di Glicina con un residuo di Acido Glutammico (la variante è nota coma Allele C).
Entrambe queste sostituzioni interrompono la sequenza ripetuta in tandem Glicina – X-Y impedendo, pertanto la formazione di interazioni idrofobiche intracatena e quindi l'assemblaggio degli oligomeri.
La mutazione che mappa nel codone 52 dell'esone 1, riconosciuta come variante D, determina la sostituzione di un residuo di Arginina con uno di Cisteina. L'introduzione di un ulteriore residuo di Cisteina a livello della regione N- Terminale causa la formazione di ponti disolfuro intracatena casuali, impedendo, anche in questo caso, la produzione di oligomeri ad alto peso funzionali (Madsen H.O. et al, 1994).
Successivamente, altri due siti polimorfici sono stati descritti nel gene *mbl*: entrambi localizzati nella regione promotrice e rispettivamente in posizione -550 (variante H/L) e -221 (variante X/Y) ed entrambi determinanti la sostituzione di una Guanina con una Citosina. Tali mutazioni determinano una diminuzione della concentrazione della proteina MBL nel siero (Madsen H.O. et al,1995).

1.6. Polimorfismo a singolo nucleotide

I polimorfismi di singola base (Single Nucleotide Polimorphisms, SNPs) costituiscono il tipo di variazione genetica più comune nei mammiferi; rappresentano, infatti, il 90% di tutti i polimorfismi e sono presenti nella popolazione con una frequenza allelica >

1%. Si tratta, nella maggior numero dei casi, di polimorfismi biallelici (ossia presenti in due forme alternative) e vengono ereditati in modo mendeliano.
Anche se generalmente, questo tipo di variazioni nel genoma sono neutre e non hanno pertanto effetto sulla regolazione dell'espressione di geni o sulla loro funzione, alcuni SNPs presenti nelle regioni codificanti (cSNPs) e regolatorie dei geni possono alterare la funzione genica, determinando variazioni nelle caratteristiche individuali come ad esempio, una differente suscettibilità ad una malattia.

1.6.1. Sistemi di rivelazione di SNP

In seguito alla scoperta degli SNPs, lo sviluppo di metodi di tipizzazione ad alta processività hanno consentito l'identificazione di migliaia di marcatori presenti sul genoma rapidamente e con bassi costi. Infatti, anche se gli SNPs sono biallelici e, quindi, meno informativi dei marcatori genetici usati attualmente (ad esempio le STR), essi sono presenti con una più alta densità nel genoma e sono associati ad un più basso rischio di errore nella genotipizzazione.
Attualmente vi sono molte tecniche di genotipizzazione ed in generale in ognuna si distinguono due fasi:
1) preparazione del campione (sintesi di prodotti alleli specifici);
2) separazione ed individuazione di tali prodotti.
La fase di preparazione del campione in genere si basa su procedure enzimatiche (5' nuclease assay, primer extension etc.) o su ibridazioni allele specifiche. Queste fasi possono essere poi accoppiate a diversi sistemi di analisi o interpretazione, come fluorescenza o determinazione della massa e quindi a diversi tipi di strumentazioni, quali: elettroforesi capillare, spettrometro di massa e microchip.
Tuttavia, è l'ibridazione con probe allele-specifici o ARMS (Amplification Refractory Mutation System) ad essere ampiamente usata nella maggior parte dei metodi per l'analisi degli SNPs. Si tratta di una reazione di PCR nella quale vengono utilizzati alternativamente due che differiscono per l'ultima base al 3'. Solo i primer il cui 3' termina con le corrette basi complementari all'interno della regione polimorfica sarà esteso con efficienza dalla polimerasi. Viene tipicamente eseguita una reazione di amplificazione in parallelo per ciascun allele, e la generazione di prodotti di PCR viene analizzata mediante elettroforesi su gel di agarosio (Newton, C.R. et al,1989) (Figura 2).

1.7. Studio Caso- Controllo

Per studio Caso- Controllo si intende un'analisi epidemiologica osservazionale, che misura l\esposizione pregressa ad un fattore di rischio separatamente in due gruppi diversi di soggetti, denominati appunto casi e controlli. Per tale motivo esso è noto anche come studio retrospettivo.
A partire dagli anni \60, lo studio caso-controllo si è diffuso enormemente,sino a diventare oggi, probabilmente, lo studio epidemiologico osservazionale più utilizzato (Parodi et al, 2004).
Generalmente, gli studi caso-controllo sono impiegati per valutare il ruolo di uno o più fattori di rischio nell'eziopatogenesi di una malattia, attraverso il calcolo dell'odds ratio (OR) e nella valutazione del ruolo di singoli fattori di rischio e della loro eventuale interazione. In questo caso, è previsto lo studio di due gruppi di soggetti: i malati (o le persone affette da una particolare condizione) che costituiscono i casi ed i controlli ovvero i soggetti con le stesse caratteristiche dei primi ma dai quali

differiscono solo per il fatto che non presentano la malattia. L'attendibilità e l'affidabilità dello studio dipendono evidentemente dalla corretta selezione dei casi e dei controlli, in altre parole dipendono da una accurata diagnosi.
Per la scelta dei controlli si utilizza la tecnica dell'appaiamento (matching): per ogni caso, si seleziona un controllo con le stesse caratteristiche (età, sesso). Questa scelta consente di ottenere sia una maggiore potenza sia un semplice ma efficace controllo del confondimento (l'alterazione dell'effetto di un fattore di rischio causato dalla presenza concomitante di un altro) (Barazzoni F. et al, 2003).
I risultati di uno studio caso-controllo possono essere descritti in una tabella 2 x 2 (tabella 1). Lo stimatore di gran lunga più utilizzato per descrivere il risultato di uno studio di associazione è l'Odds Ratio di esposizione. Esso può essere ricavato semplicemente dalla seguente formula:

$$OR = (a/b)/(c/d) = ad/bc$$

Risulta intuitivo che un OR uguale ad 1 indica l'assenza di associazione. Un valore inferiore ad uno indica un'associazione negativa (cioè il fattore protegge dalla malattia), mentre un OR maggiore di 1 indica l'esistenza di un' associazione positiva (il fattore può causare la malattia) (Parodi S. et al, 2004).
I corrispondenti intervalli di confidenza dell'OR possono essere invece calcolati attraverso la seguente formula:

$$95\%\ CI\ (OR) = OR\ e^{\pm 1{,}96\ E\ 1/a + 1/b + 1/c + 1/d}$$

(Motulski, H. et al,1995)

1.8 Scopo della tesi

La registrazione del marchio DOP rappresenta per la Mozzarella di Bufala Campana il riconoscimento di caratteristiche organolettiche e merceologiche derivanti da condizioni ambientali e da metodi tradizionali di preparazione proprie di alcune zone geografiche della Campania e del basso Lazio. Nell'ambito di tale processo di valorizzazione è necessario dunque preservare le caratteristiche di salubrità e non pericolosità del prodotto, assicurando al minimo il rischio microbiologico.
Brucella abortus, quale agente eziologico della brucellosi, costituisce la principale causa di zoonosi (di infezione cioè trasmissibile da animale a uomo) nei paesi del Mediterraneo. I piani di eradicazione che si sono susseguiti negli ultimi anni, pur basandosi sull'abbattimento dei capi infetti sono risultati fallimentari.
In tale contesto si inserisce l'approccio di migliorare la resistenza degli animali domestici alle infezioni mediante selezione genetica.
Nell'uomo alcune varianti alleliche del gene *mbl* rappresentano un fattore di rischio per infezioni virali, batteriche e fungine (Garred P. et al, 1997; Neth O. et al, 2001).
Durante il triennio di dottorato, è stata effettuata un'analisi genetica dell'associazione tra le varianti alleliche al locus *mbl* e la resistenza o suscettibilità alla brucellosi nel bufalo e parallelamente un' analisi funzionale della proteina codificata dal gene *mbl* per correlare il livello di proteina nel siero al genotipo malattia resistente allo scopo di proporre l'allevamento selettivo degli animali genotipo resistente come metodo di controllo della brucellosi nel bufalo.

2. MATERIALI E METODI

2.1. Disegno sperimentale

I soggetti inclusi nello studio appartengono a 4 allevamenti localizzati in provincia di Caserta (Sud Italia), un'area dove la brucellosi è endemica e dove pertanto è atteso che i geni in grado di conferire resistenza alla malattia siano soggetti a selezione.
Poiché sia i casi e sia i controlli, appartengono agli stessi allevamenti, è chiaro che tutti gli animali hanno lo stesso livello di esposizione alla malattia. Inoltre per evitare eventuali effetti di confondimento dovuti a sesso, età o vaccinazione sono stati inclusi nello studio solo animali di sesso femminile, in lattazione, di età compresa tra 1 e 10 anni e non vaccinati.
Poiché un'accurata diagnosi è fondamentale per la riproducibilità di uno studio caso controllo, la suddivisione dei soggetti in casi (infetti) e controlli (non infetti) si è avvalsa dell'utilizzo di più test diagnostici. Il siero dei soggetti studiati è stato infatti analizzato due volte, con un intervallo di un mese, per la ricerca di anticorpi anti- *B. abortus* mediante siero- agglutinazione rapida e citometria a flusso.
Contemporaneamente il latte è stato analizzato per la ricerca di *B. abortus* contaminanti mediante conteggio vitale su piastra e mediante identificazione del DNA batterico attraverso la reazione polimerasica a catena. Il genotipo *mbl* è stato quindi determinato in maniera random, senza conoscere cioè l'appartenenza dell'animale al gruppo caso o controllo.

2.2. Calcolo della dimensione del campione

La dimensione del campione necessario ad individuare con una potenza statistica dell' 90% ed una significatività α (a due code) dello 0.05, un OR uguale a 2, è stata calcolata usando il software Power and Sample Size Calculation e partendo da dati di frequenza degli alleli al gene *mbl* preliminarmente ottenuti da 100 casi e 147 controlli.
Il programma PS è scaricabile dal sito del Dipartimento di Biostatistica dell'Università di Vanderbilt, http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize.

2.3. Diagnosi sierologica

Lo stato sierologico degli animali è stato stabilito mediante Siero-Agglutinazione Rapida (SAR) (Alton G.G. et al, 1975), test, ufficialmente usato nei paesi dell'Unione Europea (Blasco J.M. et al, 1994) e mediante citometria a flusso.

2.3.1. Siero-Agglutinazione Rapida (SAR)

La Siero-Agglutinazione Rapida (SAR) prevede l'utilizzo di un antigene acido a pH 3,5 colorato con Rosa Bengala. L'antigene, unico su tutto il territorio nazionale, è stato fornito dall'Istituto Zooprofilattico Sperimentale per il Mezzogiorno.
La reazione di SAR è stata effettuata ponendo una goccia (30 μL) di antigene a fianco di una goccia (30 μL) di siero in esame su una piastra bianca; i due reagenti sono stati mescolati manualmente oppure servendosi di un oscillatore a movimenti rotatori per almeno 4 minuti; in mancanza di agglutinanti la reazione è stata

considerata negativa mentre in presenza di qualsiasi grado di agglutinazione la reazione è stata considerata positiva (Ciuchini F. et al, 2005).

2.3.2. Test citofluorimetrico

Una sospensione cellulare di *B. abortus* 2308, in fase esponenziale, è stata diluita ad una concentrazione pari a 10^8 cellule/ml. Le cellule sono state fissate in paraformaldeide al 4% in PBS per 20 minuti. Tale trattamento, come dimostrato da test preliminari, non interferisce con il legame antigene- anticorpo.
A $5 \cdot 10^6$ cellule batteriche fissate, sono stati aggiunti 100 μl di siero da testare in diverse diluizioni e il campione è stato quindi incubato per 12 ore a +4°C.
Dopo centrifugazione a 8000 g per 5 minuti, i campioni sono stati quenciati in 2% di milk blocking solution per 30 minuti (50 μl per tubo) a temperatura ambiente, quindi centrifugati e lavati con PBS, infine incubati per 2 ore con anticorpo fluoresceinato anti- IgG di bufalo prodotto in topo diluito 1:1000 (50 μl per tubo) e infine analizzati attraverso un citofluorimetro a flusso Coulter (Coulter, Miami, FL, USA).

2.4. Isolamento di *B. abortus* da latte

Il latte è stato campionato prelevandone almeno 20 mL per ogni quadrante della mammella.
Dopo l'arricchimento, che prevede un'incubazione di 16 ore a 37°C in atmosfera contenente il 10% di CO_2, i campioni sono stati centrifugati a 2000 g per 15 minuti. Successivamente la panna e il deposito sono stati diluiti in PBS e piastrati in duplicato utilizzando il terreno Brucella medium base (Oxoid) supplementato con antibiotici (polimixina B, bacitracina, acido nalidixico, nistatina, vancomicina) e 5% di horse serum.
Le piastre sono state incubate a 37°C in atmosfera contenente il 10% di CO_2 ed esaminate a giorni alterni, fino a 10 giorni, per la comparsa di colonie.

2.5. Diagnosi biomolecolare

Prevede l'utilizzo della PCR qualitativa per evidenziare la presenza di DNA della brucella nel latte.

2.5.1. Estrazione del DNA batterico da latte

Il metodo di estrazione utilizzato prevede l'uso del protocollo messo a punto da Leal-Klevezas e collaboratori. A ciascun campione di latte (400 µl), sono stati aggiunti 400 µl di soluzione di lisi (2% Triton X-100, 1% sodio dodecil solfato (SDS), NaCl 100 mM, Tris-HCl 10 mM [pH 8.0]) e 10 µl di proteinasi K (10 mg/ml).
Dopo mescolamento, i campioni sono stati incubati per 30 minuti a 50°C, trattati con 400 µl di fenolo saturo, mescolati nuovamente e centrifugati a 8000 g per 5 minuti. Alla fase acquosa, opportunamente trasferita in un nuovo tubo, è stato aggiunto un ugual volume di una miscela cloroformio- alcol isoamilico (24:1); quindi i campioni sono stati vortexati e centrifugati a 8000 g per 5 minuti. Alla fase acquosa, trasferita in un nuovo tubo, sono stati aggiunti 200 µl di Ammonio Acetato 7.5 M e incubati in ghiaccio per 10 minuti. Successivamente I campioni sono stati centrifugati a 8000 g per 5 minuti, e la fase acquosa nuovamente trasferita in un nuovo tubo. Dopo aver aggiunto due volumi di etanolo al 95%, i campioni sono stati incubati a -20°C per 12

ore. Quindi il DNA è stato recuperato centrifugando i campioni a 8000 g per 5 minuti, lavato in 1 ml di etanolo 70%, seccato e risospeso in 20 µl di TE buffer (Tris-HCl 10 mM [pH 8.0]), EDTA disodio 1 mM. I campioni sono sati conservati a -20°C se non processati immediatamente.

2.5.2. Quantificazione del DNA

La concentrazione del DNA è stata analizzata mediante lettura allo spettrofotometro alle lunghezze d'onda di 260 e 280 nm (OD= 1 corrisponde approssimativamente a 50 µg di Dna a doppio filamento). Un rapporto tra i valori dell'assorbanza a 260 nm e 280 nm di 1.8- 2.0 è indice di elevato grado di purezza del DNA rispetto alle proteine.

2.5.3. PCR Brucella genus- specifica

La reazione di PCR è avvenuta in 25 µl di volume finale contenenti approssimativamente 50 ng di DNA, $MgCl_2$ 1.5 mM, ciascun primer alla concentrazione di 0.4 µM, dNTP 0.2 mM, Polimerasi GoTaq Flexi DNA (Promega) 1 U in 5 µl di reaction buffer (Promega) 5X.
Il profilo termico prevede:

3 minuti at 95°C
15 secondi a 95°C
15 secondi a 60°C } 40 cicli
45 secondi a 72°C
7 min a 72°C

Sono stati usati i primer: reverse, CGCGCTTGCCTTTCAGGTCTG e forward, TGGCTCGGTTGCCAATATCAA, in grado di amplificare un prodotto di 223 bp sulla sequenza genica comune a tutte le biovars di *B. abortus* (Queipo-Ortuno et al. 1997).
Ogni campione è stato testato in duplicato e in ciascuna reazione è stato incluso un controllo negativo (contenente tutti i reagenti, eccetto il templato) e un controllo positivo (contenente tutti i reagenti ed una sospensione cellulare di *B. abortus* pari a 15 cellule/ ml).

2.6. Estrazione di DNA da sangue

Il metodo di estrazione utilizzato prevede l'uso della tecnica del Salting Out per la precipitazione e l'allontanamento delle proteine. A ciascun campione di sangue periferico (1 ml), contenente EDTA come anticoagulante, sono stati aggiunti cinque volumi di tampone di lisi per gli eritrociti (NH_4Cl 1.5 M). I campioni sono stati successivamente sottoposti a centrifugazione a 2000 g per 10 minuti al fine di ottenere la separazione dei globuli bianchi dai detriti dei globuli rossi e dal siero. Al termine della centrifugazione, si sono separate due fasi distinte: una superiore contenente la parte sierica del sangue e i detriti cellulari, una inferiore contenente i globuli bianchi. Dopo aver rimosso il sopranatante, i leucociti sono stati raccolti e risospesi nuovamente in una soluzione di NaCl 0.15 M per pulire il sedimento. Tale operazione di lavaggio è stata ripetuta due o tre volte fino ad ottenere un pellet pulito.

Tale pellet è stato poi risospeso in 2.5 ml di una soluzione di lisi per globuli bianchi (Tris- HCl 0.17 M [pH 8.4], EDTA 0.5 M, NaCl 6 M, SDS 0,1%) e incubato per 30 minuti in un apparato termostatato a 37°C. Successivamente per eliminare tutte le proteine presenti sono stati aggiunti proteinasi K (2 µg/ml) e SDS (1,3%) e lasciati agire per 10 minuti a 60°C. In seguito per precipitare il DNA sono stati aggiunti 1,5 ml di NaCl 6M ed i campioni sono stati poi centrifugati a 2000 g per 15 minuti. Il sovranatante è stato quindi trasferito in un nuovo tubo da 50 ml dove sono stati poi aggiunti 2 volumi di etanolo assoluto freddo. Quindi miscelando delicatamente è stato ottenuto un precipitato di DNA che, con l'aiuto di una pipetta Pasteur, è stato trasferito in una nuova provetta. Il DNA è stato quindi lavato più volte in etanolo 70% al fine di rimuovere eventuali tracce di sali e successivamente asciugato, prima di essere risospeso in 400 µl di Tris-HCl 10 mM [pH 8.0], ed EDTA 1 mM (TE) in tubi eppendorf da 1.5 ml e conservato alla temperatura di 4°C.

2.7. Genotipizzazione del promotore del gene *mbl*

I polimorfismi al promotore sono stati determinati usando primer complementari alla sequenza bovina del gene *mbl* (accession number NM_174107). I Primer sono stati disegnati usando il software DNASIS (Genetic Analysis, Hitachi, Olivet Cedex, France). La reazione di PCR è avvenuta in 25 µl di volume finale contenenti approssimativamente 75 ng di DNA genomico, $MgCl_2$ 1.5 mM, ciascun primer alla concentrazione di 0.4 µM, dNTP 0.2 mM, Polimerasi GoTaq Flexi DNA (Promega) 1 U in 5 µl di reaction buffer (Promega) 5X. Il profilo termico prevede:

3 minuti at 95°C
30 secondi a 95°C
30 secondi a 62°C } 40 cicli
60 secondi a 72°C
5 min a 72°C

I prodotti di PCR sono stati risolti mediante elettroforesi su gel d'agarosio al 2% (con un voltaggio pari a 90 V per 60 minuti). Il gel, colorato con bromuro d'etidio veniva visualizzato agli UV.
Il genotipo del promotore veniva determinato sulla base dell'assenza o presenza di un prodotto di 373 bp, quando venivano usate appropriate combinazioni di primer (Tabella 2).
Sono stati usati i primer (5′ to 3′): primer A,CTTACCCAGGCAAGCCGGTC; primer B, CTTACCCAGGCAAGCCGGTG; primer C, CTGGAAGACTATAAACATGCTGTCG; primer D, CTGGAAGACTATAAACATGCTGTCC).

2.8. Genotipizzazione dell'esone 1 del gene *mbl*

I polimorfismi all'esone 1 sono stati determinati usando primer complementari alla sequenza umana del gene *mbl* (accession number AL_583855). I primer sono stati disegnati usando il software DNASIS (Genetic Analysis, Hitachi, Olivet Cedex, France). La reazione di PCR è avvenuta in 25 µl di volume finale contenenti approssimativamente 75 ng di DNA genomico, $MgCl_2$ 1.5 mM, ciascun primer alla concentrazione di 0.4 µM, dNTP 0.2 mM, Polimerasi GoTaq Flexi DNA (Promega) 1 U in 5 µl di reaction buffer (Promega).
Il profilo termico prevede:

2 minuti at 95°C
30 secondi a 95°C
45 secondi a 58°C } 35 cicli
45 secondi a 72°C
5 min a 72°C

I prodotti di PCR sono stati risolti mediante elettroforesi su gel d'agarosio al 2% agarose (con un voltaggio pari a 90 V per 100 minuti). Il gel, colorato con bromuro d'etidio veniva visualizzato agli UV.
Il genotipo del promotore veniva determinato sulla base della presenza di un prodotto di 128 bp (Allele *D*, *A*), 135 bp (Allele *B*, *A*) o 143 bp (Allele *C*, *A*) quando venivano usate appropriate combinazioni di primer (Tabella 3).
I primer usati sono (5′ to 3′): primer E, ACAGCATCTTGTGCAGACAC; primer F, TCTCCCTTGGCACCATGACA; primer G, TCTCCCTTGGCACCATGACG; primer H, TCCCTTTTCTCCCTTGTCAT; primer I, TCCCTTTTCTCCCTTGTCAC; primer L, CCTGGTTCTC CCTTTGCTT; primer M, CCTGGTTCTCCCTTTGCTC.

2.9. Quantificazione della proteina MBL nel siero

Il test citofluorimetrico è stato condotto in tubi di polietilene opportunamente pretrattati con una soluzione fisiologica tamponata (PBS) al 2% di milk blocking solution, allo scopo di rendere inerte il polietilene e di evitare interazioni con il latex.
Circa 40000 particelle di latex da 10 μm di diametro (Poliscience), sono state sospese in 900 μl di Borato Buffer 0.1 M (pH 8.5), e incubate con 100 μl di anticorpo anti- MBL (rabbit) (100 μl diluito1: 50) per 12 ore a +4°C. Dopo il quenching in 2% di milk blocking solution, le particelle sono state incubate in successione con campioni di siero individuali (100 μl diluiti 1:20 in PBS contenente $CaCl_2$ 50 mM), con un anticorpo anti- MBL (rabbit) (100 μl diluito1: 50), con un anticorpo fluoresceinato anti-rabbit (100 μl diluito 1:200) e infine analizzate attraverso un citofluorimetro a flusso Coulter (Coulter, Miami, FL, USA).
Nella curva standard, i campioni di siero sono stati sostituiti con quantità note di MBL ricombinante (rhMBL) (Enzon Pharmaceuticals Inc, NJ) (100 μl da 20-500 ng/tubo) diluite in siero di bufalo depletato di MBL. Il limite di detection del test corrisponde a 40 ng/ml.

2.10. Ceppi batterici e condizioni di crescita

Brucella abortus 2308 è il ceppo batterico utilizzato in tutti gli esperimenti descritti in questo lavoro. I batteri sono stati cresciuti in terreno TSB (Triptone Soya Broth, Oxoid), in agitazione e a 37°C (10% CO_2). In tutti gli esperimenti, i batteri sono stati raccolti al raggiungimento della fase esponenziale (OD_{600} nm = 0.8) mediante centrifugazione e, nel caso degli esperimenti di infezione o stimolazione, prima dell'utilizzo, incubati con sieri provenienti da animali geneticamente resistenti o da animali geneticamente suscettibili per 1 ora a 37°C (5% CO_2). I controlli corrispondevano a campioni di siero pretrattati a 56°C per 30 minuti per inattivare la MBL (Anders E.M. et al. 1994).

2.11. Deplezione di MBL da siero

Il Mannosio (Sigma) è stato accoppiato ad una colonna CNBr-activated Sepharose 4B-CL (Pharmacia) utilizzando 8 mg di mannosio per ml di beads. Il siero è stato quindi incubato con un uguale volume di beads in presenza di $CaCl_2$ 0.5 mM per 4 ore in ghiaccio e frequente agitazione. Le beads sono state quindi rimosse mediante centrifugazione e il siero depletato, ora diluito 1:2, è stato conservato a - 70 °C fino all'utilizzo.

2.12. Test di attività antibatterica

La sospensione cellulare di *B. abortus* 2308 è stata diluita in PBS, ad una concentrazione pari a $1 \cdot 10^6$ cellule/ ml. 100 μl di tale inoculo sono stati posti in una piastra di polistirene a 96 pozzetti e incubati con campioni individuali di siero (100 μl diluiti 1:2) provenienti da animali resistenti (*HYA/HYA*) o suscettibili (*LYD/LYD*). La piastra è stata incubata per 24 ore a 37°C (10% CO_2). Il contenuto di ciascuna piastra è stato quindi diluito e piastrato per valutare la percentuale di inibizione della crescita. Ogni campione è stato testato in triplicato.

2.12.1. Specificità dell'attività della MBL

Per dimostrare che l'osservata attività antibatterica è stata mediata da MBL, sono stati effettuati test di attività utilizzando sieri pretrattati con specifici inibitori della MBL (EDTA 10 mM, Mannosio 25 mM, N-Acetil-D-Glucosammina 50 mM), con un anticorpo anti- MBL (25 μl diluiti 1:500) o con sieri depletati di MBL mediante il passaggio attraverso una colonna di Sepharosio- mannosio (Anders E.M. et al. 1994). Gli zuccheri o l'anticorpo sono stati aggiunti ai campioni di siero 30 minuti prima dell' addizione di *B. abortus*.

2.13. Isolamento e coltura di monociti da sangue di bufalo

La purificazione di monociti da sangue intero di bufalo è stata effettuata utilizzando il reagente Lympholyte®- Mammal (Cederlane Labs), seguendo le istruzioni del produttore. In breve, il sangue è stato diluito con un ugual volume di terreno RPMI. Utilizzando tubi da centrifuga da 14 ml, a 3 ml di Lympholyte®- Mammal sono stati aggiunti 3 ml di sangue diluito. Quindi i campioni sono stati centrifugati per 20 minuti a 8000 g a temperatura ambiente. Usando una pipetta Pasteur, le cellule all'interfaccia sono stata rimosse e trasferite in un nuovo tubo. Le cellule sono state quindi diluite in RPMI e centrifugate a 8000 g per 10 minuti per pellettare i linfociti.
Le cellule sono state contate utlizzando la camera di Burker e quindi piastrate in RPMI ad una concentrazione pari a $1 \cdot 10^6$ cellule/ ml. Dopo un'ora di incubazione a 37°C (5% CO_2) le cellule sono state lavate con RPMI e infine incubate in RPMI a cui era stato precedentemente addizionato FCS all'1% a 37°C (5% di CO_2).

2.14. Cinetica di infezione *in vitro*

I monociti bufalini in piastra sono stati infettati con una sospensione cellulare di *B. abortus* 2308, precedentemente pretrattata, ad una molteplicità di infezione (MOI) pari a 10. La piastra è stata poi centrifugata (750 g per 5 minuti) in modo da facilitare il contatto delle cellule con i batteri e poi incubate a 37°C (5% di CO_2).

Ad intervalli di 20, 40 e 60 minuti, le cellule sono state lavate con RPMI e quindi incubate in RPMI addizionato con FCS 1% e Gentamicina 40 μg / ml per 30 minuti a 37°C (5% CO_2), allo scopo di abbattere la carica batterica extracellulare.
Le cellule sono state lisate con una soluzione di Tryton all'1 % e i campioni sono stati diluiti serialmente per effettuare il conteggio vitale su piastra dei batteri intracellulari.

2.15. Saggio di infezione *in vitro*

I monociti bufalini in piastra sono stati infettati con una sospensione cellulare di *B. abortus* 2308, precedentemente pretrattata, ad una molteplicità di infezione (MOI) pari a 10. La piastra è stata poi centrifugata (750 g per 5 minuti) in modo da facilitare il contatto delle cellule con i batteri e poi incubate per 60 minuti a 37°C (5% CO_2).
Le cellule sono state quindi lavate con RPMI e quindi incubate in RPMI addizionato con FCS 1% e Gentamicina 40 μg /ml per 30 minuti a 37°C (5% CO_2), allo scopo di abbattere la carica batterica extracellulare.
Le cellule sono state quindi lavate con RPMI ed incubate per 24 ore in RPMI addizionato con FCS all'1% a 37°C (5% CO_2).
Le cellule sono state lisate con una soluzione di Tryton all'1 % e i campioni sono stati diluiti serialmente per effettuare il conteggio vitale su piastra dei batteri intracellulari.

2.16. Stimolazione di citochine pro infiammatorie *in vitro*

I monociti bufalini in piastra sono stati infettati con una sospensione cellulare di *B. abortus* 2308, precedentemente pretrattata, ad una molteplicità di infezione (MOI) pari a 10 e quindi incubati a 37°C (5% CO_2) per 5 ore.
Al termine dell'incubazione, l'RNA totale è stato estratto per valutare il livello di espressione dei geni codificanti le principali citochine proinfiammatorie: TNF- α, IL- 6 e IL- 1β.

2.17. Estrazione dell'RNA totale

L'RNA totale è stato estratto utilizzando il reagente TRIZOL® (Invitrogen), seguendo le istruzioni del produttore. Brevemente, le cellule in piastra sono state lisate aggiungendo 1 ml di TRIZOL®. Il lisato è stato incubato per 5 minuti a temperatura ambiente per consentire la completa dissociazione dei complessi nucleo proteici.
Al lisato sono stati aggiunti 0.2 ml di cloroformio per 1 ml di TRIZOL®, agitato vigorosamente e quindi incubato per 3 minuti a temperatura ambiente.
Dopo centrifugazione a 12000 g per 15 minuti a 4°C, la fase acquosa è stata recuperata e da essa l'RNA è stato precipitato aggiungendo alcol isopropilico per ciascun ml di TRIZOL® usato per l'omogenizzazione iniziale. Il campione è stato quindi incubato a temperatura ambiente per 10 minuti e quindi centrifugato a 12000 g per 10 minuti a 4°C. L' RNA precipitato è stato lavato con etanolo al 75%.
Dopo centrifugazione a 7500 g per 5 minuti a 4°C, l'RNA è stato disciolto in acqua RNAsi-free.

2.17.1. Purificazione dell'RNA

Il clean- up dell'RNA è stato effettuato utilizzando l'RNeasy ® Mini Kit Qiagen, seguendo le istruzioni del produttore. Brevemente, il campione di RNA (100 μl) è stato mescolato con 350 μl di Buffer RLT, e quindi 250 μl di etanolo al 100%. Il

campione è stato quindi trasferito ad una colonna e centrifugato a 8000 g per 15 secondi. Alla colonna sono stati aggiunti 500 μl di buffer RPE e quindi è stata centrifugata a 8000 g per 15 secondi: questa operazione è stata ripetuta due volte allo scopo di lavare la membrana della colonna a cui è stato legato l'RNA.
Infine alla colonna sono stati aggiunti 50 μl di acqua RNAsi- free per eluire L'RNA mediante centrifugazione per 1 minuto a 8000 g.

2.17.2. Quantificazione e Integrità dell'RNA

La concentrazione dell'RNA è stata determinata misurando l'assorbanza del campione al Nanodrop 8000, assumendo che l''assorbanza di 1 unità a 260 nm corrisponda a 44 μg di RNA/ ml.
Per verificare l'integrità dell' RNA ottenuto, sono stati caricati 10 μl del campione su un gel d'agarosio allo 0.7% (p/v) colorato con Bromuro d' etidio. Dopo la corsa elettroforetica, il gel è stato visualizzato ai raggi UV. L'RNA ribosomale deve apparire come una banda netta e il rapporto tra l'rRNA 18S e l'RNA 28S deve essere approssimativamente pari a 2:1.

2.18. Sintesi del cDNA

Per la retrotrascrizione di ciascun campione di RNA, 2 μg di RNA sono trasferiti in tubo Eppendorf da 0.2 ml, e sottoposti ad un ciclo di reazioni che porta alla sintesi del cDNA.
I campioni di RNA (in un volume finale di 10 μl) sono incubati a 65°C per 5 minuti e poi raffreddati rapidamente fino a raggiungere una temperatura di 4°C; questo trattamento elimina gli aggregati e le strutture secondarie dell'RNA che potrebbero interferire con la sintesi del cDNA. A questo punto, i campioni sono aggiunti ad una miscela di reazione (in un volume finale di 10 μl) contenente 4 μl di Buffer 5x, 1 μl di dNTP 10 mM, 2 μl di DTT 0,1 M, 1 μl di oligo dT 30 μM, 1 μl di RNasina e 1μl di Superscript II (Invitrogen) 200 U/μl. I campioni sono stati incubati in apparato a controllo termico ciclico a 42°C per 60 minuti, portato a 95°C per 5 minuti ed infine a 4°C. Il trattamento a 95°C denatura gli ibridi RNA-cDNA ed inattiva l'enzima. Dopo il trattamento denaturante, la miscela di reazione viene centrifugata e conservata a –20°C per il successivo utilizzo.

2.19. PCR Quantitativa

La reazione di PCR real-time quantitativa è avvenuta nell'apparecchiatura iCYCLER SYSTEM (Biorad), su un'aliquota, pari ad 1/20, del prodotto della reazione della RT, usando la SYBR green master mix (Stratagene).
La reazione è stata condotta in un volume finale di 20 μl, contenenti 1 μl di ciascun primer 10μM, 10 μl di SYBR green master mix. E' stato eseguito il seguente programma di amplificazione:

10 minuti a 95°C
15 secondi a 95°C } 45 cicli
45 secondi a 60°C }

Sono stati usati i primer per il *tnf- α:* reverse, GTCTGGGCCATAGAACTGATG e forward, TCTCAGCCTCTTCTCATTCCT; per *il- 6*, CAGTTTGGTAGCATCCATCAT e

forward, AAAGAGTTGTGCAATGGCAATT; per *IL-1*β, CTTGCCGCGAGGCTTTCTC e forward, TGGTCCTCATCTGGAAGATCA.
Ogni campione di cDNA è stato analizzato in triplicato mentre un campione privo di cDNA è stato incluso come controllo negativo. I risultati sono stati analizzati con Cycler iQ analysis software (Biorad): i livelli di espressione relativa dei geni in esame sono stati calcolati con il metodo dell'analisi comparativa del ciclo soglia (C_T) con il gene di riferimento *gapdh.*

2.20. Influenza del genotipo *mbl* sulla produzione di latte

La produzione di latte è stata determinata utilizzando i dati di campionamento effettuati ogni mese dall'Associazione Nazionale Allevatori Specie Bufalina ANASB. La produzione di latte individuale è stata normalizzata (alla resa di latte di una bufala alla sua quinta lattazione, munta due volte al giorno, in un periodo di lattazione di 270 giorni) usando il software PUMA (www.aia.it). Le differenze in termini di produzione di latte tra i diversi genotipi è stata analizzata mediante il test t di Student.

2.21. Statistica

Tutti i dati sono presentati come media ± deviazione standard o mediana ± deviazione standard. Le variabili continue sono state comparate mediante il software Graph Pad Prism usando il test t di Student. Le variabili categoriche sono state comparate mediante il software Graph Pad Prism usando il test χ^2 (o test di Fischer).
L'Odd Ratio e gli intervalli di confidenza (CI) sono stati calcolati come descritti nell'introduzione, in accordo al Motulski, 1995.
Un valore di $P< 0.05$ è considerato significativo.

3. RISULTATI

3.1. Dimensione del campione: casi e controlli

Dati preliminari ottenuti da 100 casi e 147 controlli, hanno consentito di calcolare, utilizzando il software Power and Sample Size Calculation, la dimensione del campione. Per individuare con una potenza statistica dell' 90% ed una significatività α (a due code) dello 0.05, un OR uguale a 2, risultava necessario includere almeno 254 casi e 254 controlli. Pertanto, per assicurare un'adeguata potenza, in questo studio, sono stati analizzati 335 casi e 335 controlli.
La riproducibilità di uno studio di associazione si basa anche su un'accurata diagnosi, cioè su una accurata suddivisione dei soggetti in casi e controlli (Zondervan K.T. et al, 2002). Poiché non esiste un unico test in grado di diagnosticare specificamente la brucellosi (Godfroid J. et al, 2002), si è proceduti parallelamente con una diagnosi sierologia e biomolecolare e allo stesso tempo mediante isolamento della brucella da latte. Queste tecniche hanno permesso di suddividere gli animali in casi, se positivi a tutti i test e in controlli, se negativi a tutti i test.

3.2. Identificazione di alleli al gene *mbl*

L'analisi mediante PCR ha identificato quattro alleli all'esone 1 del gene *mbl* (*A, B, C, D*), due alleli (*H, L*) in posizione −550 e due alleli in posizione −221 (*X, Y*) al promotore del gene.
Poiché è noto che il genotipo dell'esone 1 può influenzare la concentrazione della proteina MBL nel siero e quindi la sua capacità di opsonizzare i patogeni nell'uomo (Hibberd M.L. et al, 1999; Kilpatrick D.C. et al, 2002; Madsen H.O. et al, 1998), è stato investigato se gli animali con due copie dell'allele (*AA*), con un allele mutato (*AB, AC, AD*; nell'insieme indicati come animali *AO*) o con due alleli mutati (*BB, CC, DD, BC, BD, CD*; nell'insieme indicati come animali *OO*) avessero diverse frequenze di distribuzione nei casi e nei controlli.
Gli animali *AA* sono risultati più frequenti tra i controlli, mentre gli animali *OO* più frequenti tra i casi che tra i controlli: (OR: 0.15; CI: 0.09824–0.2417; $P<10^{-7}$; Tabella 4). La frequenza dell'allele *A* tra i controlli (0.67) risulta essere infatti significativamente più elevata che tra i casi (0.42; Fig. 3a). Questi dati forniscono una prima evidenza che il genotipo *AA* conferisca resistenza all'infezione da *B. abortus.*

3.3. Effetto degli aplotipi *mbl* sull'infezione da *B. abortus*

Gli alleli al promotore in posizione −550 (*H, L*) e in posizione −221 (*X, Y*) sono ereditati sullo stesso cromosoma (*in cis*) in forma cioè di aplotipi (Madsen H.O. et al,1998). Gli aplotipi contengono l'informazione relativa ad un intero set di alleli legati tra loro. Gli studi caso-controllo basati su aplotipi risultano molto più informativi, se confrontati con studi basati su un singolo polimorfismo (Joosten P.H. et al, 2001; Tregouet D.A. et al, 2002). Pertanto, poiché prossimi ai polimorfismi all'esone 1, si è scelto di includere nello studio anche i polimorfismi (*H, L*), (*X, Y*).
L'analisi mediante PCR ha rivelato 16 coppie di aplotipi al gene *mbl*. Di questi due, (*HYA/HYA*, *LYD/LYD*), i più frequenti sono stati confermati anche da dati familiari. I soggetti *HYA/HYA* risultano rappresentati, con solo due eccezioni, solo tra i controlli (OR: 0.001; CI: 0.0006– 0.01847; $P < 10^{-7}$; Tabella 5).

La frequenza dell'aplotipo *HYA* tra i controlli (0.57) è, infatti, significativamente più alta della frequenza osservata tra i casi (0.1; Fig. 3b).
Il risultato ottenuto, cioè un OR di associazione tra la coppia di aplotipi *HYA/HYA* e la brucellosi (Tabella 5), pari a 0.001, se confrontato con l'OR di associazione tra il genotipo *AA* e la stessa malattia (Tabella 4), pari a 0.15, conferma l'importanza di studiare aplotipi piuttosto che singoli alleli (Joosten P.H. et al, 2001; Tregouet D.A. et al, 2002).
D'altra parte, i soggetti *LYD/LYD* sono più rappresentati tra i casi. Infatti, essi risultano approssimativamente 10 volte più frequenti tra i casi rispetto ai soggetti *-/-* (OR= 9.9; CI: 6.280− 15.857; $P < 10^{-7}$; Tabella 6).
Le restanti coppie di aplotipi, più rari, non sono stati analizzati per l'associazione alla malattia.
In conclusione, l'analisi genetica ha evidenziato che la coppia di aplotipi *HYA/HYA* è associata alla resistenza all'infezione sostenuta da *B. abortus,* mentre la coppia di aplotipi *LYD/LYD* è associata alla predisposizione all'infezione sostenuta dallo stesso patogeno.
Gli alleli al promotore e all'esone 1 del gene *mbl* differiscono tra loro sostanzialmente per una singola sostituzione nucleotidica. E' da sottolineare quindi, la capacità dell'aplotipo di promuovere la protezione o la suscettibilità alla malattia anche attraverso una singola variazione come nel caso delle coppie di aplotipi *HYA/HYA* rispetto a *HYA/HYD*, *HYA/HYA* rispetto a *LYA/LYA*, o *HYA/HYA* rispetto a *HXA/HXA*.

3.4. Effetto del genotipo *mbl* sulla produzione di latte

E' stato dimostrato che la resistenza ai patogeni può comportare un costo in termini di fitness (Woolhouse, M. et al, 2002). Poiché è chiaro che per un allevatore il tratto più importante è la quantità di latte prodotta per singolo animale, è stato necessario valutare se l'aplotipo *HYA* influenzasse negativamente tale resa. Tuttavia, nessuna differenza è stata trovata tra la produzione di latte di animali geneticamente resistenti e geneticamente suscettibili ($t_{0.05}$ 0.10; 22 gradi di libertà; P = 0.92).
Questo dato indica pertanto, che un'eventuale selezione per la resistenza alla brucellosi non influenzerebbe minimamente l'efficienza della produzione di latte dell'animale, carattere di profitto e quindi imprescindibile per l'allevatore.

3.5. Quantificazione della proteina MBL nel siero di bufalo

Nell'uomo, è stato dimostrato che i polimorfismi al gene *mbl* comportano una diminuzione della concentrazione della proteina nel siero (Garred P. et al, 2003).
E' stato verificato quindi se anche nel bufalo i polimorfismi avessero lo stesso effetto. I dati ottenuti mediante citometria a flusso, usando un saggio tipo sandwich, indicano che la mediana del livello di MBL nel siero di animali con genotipo *HYA/HYA* è significativamente più alta, comparata a quella dei due unici soggetti con genotipo *HYA/HYA* ma infetti, e ai soggetti (casi e controlli) con un aplotipo non-*HYA/HYA* (Figura 4).
Questo risultato costituisce una prima evidenza di una piena concordanza tra l'analisi genetica e quella funzionale e allo stesso tempo contribuisce ad affermare il ruolo protettivo dell'aplotipo *HYA/HYA* contro l'infezione da *B.abortus* e il ruolo dell'aplotipo *LYD/LYD* nella predisposizione alla malattia.
Infine questo risultato ci permette di speculare che la resistenza alla brucellosi mostrata dagli animali privi dell'aplotipo *HYA* (una o due copie) potrebbe essere

dovuta alla presenza di uno o più geni in grado, con meccanismi diversi da quelli previsti dal gene *mbl*, di proteggere dalla malattia.

3.6. Plausibilità biologica

Molti studi caso-controllo risultano dei falsi positivi poichè a fronte dell'analisi genetica, non viene investigato il significato biologico dell'associazione stessa (Lander E.S. et al, 1994; Wacholder S. et al, 2004). In questo studio, quindi, è stata verificata sperimentalmente la plausibilità biologica dell'associazione tra gli aplotipi *mbl* e la resistenza all'infezione da *B.abortus.*

3.6.1. Attività antibatterica mediata da MBL

Per la proteina MBL è stata dimostrata un'attività antibatterica indipendente dal complemento (Fernandez- Prada C. M. et al, 2001).
Quindi, per testare una possibile differenza in termini di attività antibatterica della proteina tra soggetti resistenti (*HYA/HYA*) e suscettibili (*LYD/LYD)*, una sospensione cellulare di *B. abortus* 2308 (10^6 cellule/ ml) è stata incubata in vitro in presenza di siero autologo inattivato al calore (controllo) e non inattivato (attività). Per ciascun animale, sono stati raccolti due campioni di sangue ad intervalli di due settimane e ognuno testato in triplicato. Dopo incubazione a 37° per 24 ore, è stato effettuato un conteggio vitale su piastra. Il siero prelevato da animali *HYA/HYA* ha mostrato un'attività antibatterica diretta significativamente più alta rispetto a quella esplicata dal siero di animali con genotipo *LYD/LYD* o *LYA/LYD* (Figura 2).
L' evidenza che il trattamento al calore inibisce l'attività antibatterica del siero (Figura 5) suggerisce che tale attività sia mediata proprio da MBL, una lectina labile al calore (Anders E. M. et al, 1994).
Il reale coinvolgimento della proteina MBL nel mediare l'attività antibatterica è evidente inoltre dalle seguenti osservazioni: l'attività antibatterica è inibita quando il siero è preincubato con EDTA 10 mM, con Mannosio 25 mM o con N-Acetil-D-glucosammina 50 mM (Figura 6), tutti inibitori noti dell' MBL (Anders E. M. et al, 1994). Inoltre, l'inibizione dell'attività antibatterica si osserva anche in presenza di anticorpi anti- human MBL o dopo deplezione della MBL mediante cromatografia d'affinità con una colonna coniugata al mannosio (Figure 6).

3.6.2. Effetto della proteina MBL sulla fagocitosi di *B. abortus* in monociti bufalini

Una delle principali funzioni svolte dall' MBL consiste nell'opsonizzazione del patogeno, diretta o mediata dal complemento (Jack D.L. et al, 2001; Takahashi K. et al, 2005). Tale processo facilita la fagocitosi e la degradazione del patogeno all'interno del macrofago. E' stato quindi utilizzato un modello *ex vivo*, per verificare se i soggetti *HYA/HYA* risultavano in grado di contrastare l'infezione da *B.abortus* rispetto agli animali suscettibili (*LYD/LYD*), perché in grado di internalizzare più velocemente il patogeno.
Una sospensione cellulare di *B. abortus* 2308, pretrattata con un siero autologo proveniente da animali *HYA/HYA* o *LYD/LYD* inattivato al calore (controllo) e non inattivato (attività) è stata utilizzata per infettare, ad una MOI pari a 10, monociti isolati da sangue di bufalo.

Ciascun campione è stato testato in triplicato. Ad intervalli di 20, 40 e 60 minuti, i monociti sono stati lisati ed è stato effettuato un conteggio vitale su piastra per quantificare il numero di batteri intracellulari. Come mostrato in figura 8, a 20 e 40 minuti dall'infezione, l'opsonizzazione delle brucellae con un siero proveniente da un animale geneticamente resistente aumenta significativamente la velocità di internalizzazione del batterio stesso (*P*= 0.003).
Il trattamento al calore inibisce la capacità di un siero *HYA/HYA* di accellerare il processo di internalizzazione del patogeno (Figura 7), suggerendo, pertanto, che tale attività sia mediata da MBL, che è labile al calore (Anders E.M. et al, 1994).

3.6.3. Effetto della proteina MBL sulla sopravvivenza di *B. abortus* in monociti bufalini

E' stato quindi esaminato l'effetto di un siero *HYA/HYA*, ad alto contenuto di MBL,e di un siero *LYD/LYD*, a basso contenuto di MBL, sul processamento delle brucellae da parte dei monociti. La sopravvivenza intracellulare dei batteri è stata investigata usando un saggio di esclusione con gentamicina, quantificando il numero dei batteri internalizzati dopo 24 ore di incubazione. Ogni campione è stato testato in triplicato.
Il numero di batteri intracellulari risultava ridotto del 40 % in presenza di un siero *HYA/ HYA* (*P*= 0.0025) (Figura 8).
Tale attività non viene esplicata da un siero proveniente da un animale geneticamente suscettibile *LYD/LYD* (Figura 9). L'evidenza che il trattamento al calore inibisce la riduzione osservata con un siero HYA/HYA indica ancora una volta che tale attività è mediata da MBL (Figura 8) (Anders E.M. et al, 1994).
Questi risultati, combinati con quelli che confermano il ruolo della proteina MBL nello stimolare la fagocitosi delle brucellae (Figura 7), contribuiscono a dare un significato biologico alla resistenza genetica degli animali *HYA/HYA*.

3.6.4. Modulazione della risposta immunitaria alla *B.abortus* mediata da MBL

E' noto inoltre che l'MBL sia un potente regolatore della risposta infiammatoria attraverso la modulazione del rilascio di citochine infiammatorie (Ghezzi M.C. et al, 1998, Jack D.L. et al, 2001, Nadesalingam J. et al, 2005), la cui produzione è fondamentale per la promozione della successiva risposta adattiva richiesta per il pieno controllo dell'infezione.
E' stato quindi investigato se la resistenza degli animali *HYA/HYA* alla *B. abortus* sia dovuta alla capacità di questi ultimi di elicitare una risposta infiammatoria bilanciata in termini di produzione di TNF-α, IL-6 e IL-1β.
I monociti, purificati da campioni di sangue bufalino, sono stati stimolati con *B. abortus* preincubata con sieri, inattivati al calore (controllo) e non inattivati (attività), provenienti da animali *HYA/HYA* e *LYD/LYD*. Dopo 5 ore si è proceduti all'estrazione dell'RNA per la relativa quantificazione delle citochine suddette mediante PCR quantitativa.
Tale analisi, ha evidenziato che il pretrattamento del batterio con un siero "ad alto contenuto" di MBL induce una significativa (*P*< 0.05) "downregolazione " della risposta proinfiammatoria in termini di espressione dei geni codificanti per le principali citochine quali TNF-α, IL-6 e IL-1β (Figura 9), rispetto al pretrattamento del patogeno con un siero "a basso contenuto" di MBL, proveniente cioè da animali *LYD/LYD*. Tale ipomodulazione è proprio indotta dalla proteina MBL poiché un siero

HYA/HYA inattivato a 56°C non è in grado di esplicare lo stesso effetto (Figura 9) (Anders E.M. et al, 1994).
Questi risultati sono pertinenti con le caratteristiche della brucellosi, dove sia l'attivazione che le relative proporzioni delle citochine pro e anti - infiammatorie possono essere critiche per l'outcome della malattia: una produzione incontrollata di citochine potrebbe infatti portare allo shock settico.
E' ormai chiaro, che la maggior parte delle conseguenze di un'interazione ospite-patogeno sia mediata dai Toll like Receptors (Ishii K.J. et al, 2008). E' possibile quindi speculare che i nostri risultati siano consistenti con un'eventuale coinvolgimento dell'MBL nel modulare la risposta infiammatoria alla *B.abortus* a partire proprio da uno o più recettori appartenenti a questa classe.

4. DISCUSSIONE

La denominazione di Origine Protetta “Mozzarella di Bufala Campana” è stata riconosciuta con Regolamento CE n. 1107/96. L’industria costruita intorno a questo prodotto, solo nella regione Campania, coinvolge l'attività di centoquaranta caseifici, con la produzione di ventotto milioni di chilogrammi ogni anno di mozzarella di bufala Dop, di cui il 65 per cento viene commercializzato fuori regione.
Numerosi sono gli sforzi sostenuti per la valorizzazione del prodotto e la tutela del consumatore per garantire le proprietà organolettiche ma soprattutto di sicurezza e salubrità del prodotto, in un’area dove è noto, la brucellosi è endemica.
L’eradicazione della brucellosi in Italia è, anche se risultato inefficace negli ultimi trenta anni, ancora affidata all’abbattimento dei capi infetti.
Questo lavoro descrive, invece, una strategia alternativa per il controllo della brucellosi nel bufalo, proponendo l’allevamento selettivo degli animali con un genotipo malattia resistente. Anche negli allevamenti bufalini dove la propagazione dell’infezione da *B. abortus* è particolarmente estesa, si osserva che circa il 20% dei soggetti rimane negativo ai test sierologici e quindi presumibilmente è non infetto. E’ proprio questa osservazione a suggerire che la variazione genetica può svolgere un ruolo fondamentale nella resistenza alla brucellosi.
E d’altra parte è noto che la genetica dell’ospite può influenzare l’infezione da *B. abortus* nel bufalo (Borriello et al., 2006; Capparelli R. et al, 2007). Questi autori hanno dimostrato, ad esempio, che la variante allelica *BB* al locus *nramp* è associata alla resistenza alla brucellosi. Gli animali con genotipo *BB* esprimono più alti livelli di messaggero *nramp* e quindi più alti livelli di proteina, e possono pertanto controllare la replicazione del patogeno più efficacemente (Borriello G. et al, 2006).
Del resto, la ricerca di alleli in grado di influenzare la suscettibilità ai patogeni non è nuova: negli ultimi anni, infatti, l’identificazione di polimorfismi genetici ha prodotto numerose informazioni sull'associazione di varianti alleliche con malattie infettive e non. Gli studi caso- controllo costituiscono un valido strumento nell’identificazione di associazioni tra i geni dell’ospite e resistenze alle malattie, partendo da un disegno sperimentale concettualmente semplice: la frequenza dell’allele che conferisce resistenza nel gruppo dei casi (infetti) viene confrontata con la frequenza nel gruppo dei controlli (non infetti). E’ atteso, naturalmente, che la variante allelica in grado di conferire resistenza presenti una frequenza di distribuzione sensibilmente più alta tra i controlli.
In questo studio è stata descritta l’identificazione di un altro gene di resistenza alla brucellosi nel bufalo, il gene *mbl*, codificante una delle proteine chiave dell’immunità innata (Ip W.K. et al, 2009).
Il disegno sperimentale di uno studio caso- controllo si basa su una preliminare e corretta suddivisione dei soggetti in infetti (casi) e non infetti (controlli). Questo è uno step critico per la riproducibilità di uno studio di associazione (Zondervan K.T. et al, 2006) e poiché nessun test in uso attualmente è in realtà in grado di distinguere la *B. abortus* da altri batteri con cui essa condivide epitopi antigenici (*Yersinia pestis* ad esempio), in questo studio la diagnosi si è avvalsa di una combinazione di test sierologici e biomolecolari.
Per quanto riguarda l’analisi genetica, mediante PCR sono stati individuati nel bufalo, quattro alleli all’esone 1 del gene *mbl* (*A, B, C, D*), due alleli (*H, L*) in posizione −550 e due alleli in posizione −221 (*X, Y*) al promotore del gene.
Lo studio di aplotipi con una forte relazione causale al tratto che viene analizzato, anziché di semplici alleli, è una caratteristica comune a molti studi caso- controllo

rivelatisi informativi e riproducibili (Risch N.J. et al, 2005). Questa informazione ci ha spinto ad analizzare per la resistenza alla *B.abortus* non solo le varianti all'esone 1 del gene *mbl* ma anche i polimorfismi al promotore, i quali essendo prossimi vengono ereditati come aplotipi.
I dati mostrano una più alta prevalenza dei soggetti *HYA/HYA* tra i controlli ($P<10^{-7}$; Tabella 5). In contrasto, i soggetti *LYD/LYD* ($P=10^{-7}$; Tabella 6) presentano una più alta prevalenza tra i casi. Gli studi condotti sul gene *mbl* umano hanno ampiamente definito la relazione chimica tra i singoli polimorfismi del gene *mbl* e la funzione della proteina: in particolare è stato dimostrato che ogni singola sostituzione causata da gli alleli mutanti interferiscono con il corretto folding delle tre subunità base della proteina. Se la proteina contiene una catena mutata, essa diviene più sensibile alla degradazione o incapace di attivare il complemento (Garred P. et al, 2003). Sulla base di questa evidenza, abbiamo speculato che i bufali resistenti hanno una proteina MBL pienamente funzionale e sono pertanto capaci di combattere l'infezione da *B. abortus* efficacemente; gli animali suscettibili, al contrario, hanno una proteina MBL parzialmente attiva, incapace di condurre una rapida opsonizzazione del patogeno.
Molti studi di associazione risultano dei falsi positivi e non riproducibili poiché privi di uno studio che dia un significato biologico all'analisi genetica (Lander E.S. et al, 1994; Wacholder S. et al, 2004). La plausibilità biologica del gene candidato diviene quindi un parametro critico per la validità di uno studio di associazione.
In questo studio la funzione della MBL è stata investigata in soggetti *HYA/HYA* (geneticamente resistenti) e in soggetti *LYD/LYD* (geneticamente suscettibili) usando approcci indipendenti. Analogamente a quanto osservato nell'uomo (Capparelli R. et, 2009), i sieri provenienti da animali *HYA/HYA* presentano una concentrazione della proteina MBL maggiore di quella osservata in sieri provenienti da soggetti *LYD/LYD* (Figura 4).
Il siero proveniente da bufali geneticamente resistenti (*HYA/HYA)* hanno mostrato *in vitro* una più elevata attività battericida comparati al siero proveniente da soggetti suscettibili (*LYD/LYD*) (Figura 5).
Quando i monociti bufalini sono infettati *in vitro* con brucellae opsonizzate con sieri provenienti da animali *HYA/HYA,* a differenza di quanto si verifica con sieri provenienti da animali *LYD/LYD*, si osserva un'accelerazione del processo di internalizzazione (Figura 7) e un maggior processamento del patogeno (Figura 8). Inoltre l'opsonizzazione delle brucellae con un siero proveniente da un animale geneticamente resistente ipomodula la risposta infiammatoria in termini di citochine proinfiammatorie (Figura 9).
L'evidenza che il siero degli animali *HYA/HYA* favorisce rapidamente la degradazione del patogeno (Figura 7 e 8), è in grado di spiegare perché la quasi totalità degli animali geneticamente resistente rimane sieronegativa (status accertato mediante siero- agglutinazione rapida o citometria a flusso), in altre parole perché la maggior parte degli animali *HYA/HYA*, pur essendo esposta al patogeno non produce anticorpi anti- brucellae: i batteri internalizzati sono rapidamente processati dai fagociti e il segnale infiammatorio è troppo breve e debole per innescare una risposta sistemica.
In conclusione, l' associazione qui riportata sembra avere tutte le caratteristiche per essere affidabile: la rilevanza biologica del gene candidato, la potenza statistica (ottenuta rispettando il calcolo della dimensione del campione), un attento controllo degli effetti di confondimento e una adeguata selezione dei controlli, ottenuta assicurando lo stesso livello di esposizione, cioè includendo nei due gruppi, animali

provenienti dagli stessi allevamenti e quindi sottoposti alla stessa probabilità di contrarre l'infezione.
Tuttavia, poichè è noto che la proteina MBL interagisce con numerosi patogeni (Eisen D.P. and Minchinton R.M., 2003; Koch A. et al, 2001; Zhang H. et al, 2005), non è possibile formalmente escludere che l'elevata frequenza degli animali *HYA/HYA* qui osservata potrebbe essere stata guidata anche da altri patogeni. Inoltre, l'evidenza che, anche se sporadicamente è possibile individuare animali *HYA/HYA* geneticamente resistenti ma infetti, sottolinea la possibilità che la resistenza alla *B.abortus* sia poligenica.
Infatti, anche se certamente esistono geni dell'immunità innata caratterizzati da una diretta corrispondenza tra genotipo e fenotipo, tuttavia, con ogni probabilità a rappresentare la maggioranza sono quelli, la cui espressione è influenzata da altri geni. I geni che regolano la risposta immuno- innata, infatti, sono sottoposti ad uno stringente controllo e il delicato bilancio tra controllo del patogeno e danno tissutale è spesso il risultato di una complessa interazione tra geni (Dyment D.A. et al, 2005; Gregersen J.W. et al, 2006).
E' necessario inoltre, anche tenere presente le influenze di natura non genetica: ad esempio la protezione contro l'HIV mediata dall'HLA non è efficace quando l'infezione avviene attraverso l'allattamento al seno (Carrington and O'Brien 2003).
In maniera analoga l'immunità fornita dall'aplotipo *HYA/HYA* potrebbe essere insufficiente a prevenire l'infezione se l'ospite è esposto al patogeno attraverso una via non convenzionale. Cio' potrebbe spiegare la presenza di quei pochi soggetti *HYA/HYA* infetti.
In conclusione, i risultati riportati durante il triennio di dottorato, illustrano come l'opzione di controllare l'infezione da *B.abortus* mediante selezione genetica sia realistica e in particolare, proprio riguardo alla brucellosi nel bufalo, come la selezione positiva dei soggetti resistenti e la parallela selezione negativa dei soggetti suscettibili potrebbe rapidamente incrementare il livello di immunità dell'allevamento. Tale processo risulterebbe facilitato, d'altra parte, dalla relativa alta frequenza degli animali resistenti, osservata almeno in questo studio.
Questo approccio potrebbe avere un impatto positivo sull'economia dell'industria lattiero- casearia nata intorno al bufalo e potrebbe contribuire a cambiare la radicata idea che il controllo della salute dell'animale possa passare solo ed esclusivamente attraverso l'abbattimento.

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6. TABELLE

	Esposizione		Totale
	+	-	
Casi	A	b	a+b
Controlli	C	d	c+d
	a+ c	b+d	

Tabella 1: Risultato di uno studio caso-controllo con fattore di esposizione a due livelli (presente o assente) rappresentato in una tabella di contingenza.

Genotipo	Coppia di Primer				lunghezza (bp)
	B^a+C^b	B^a+D^b	A^a+C^b	A^a+D^b	
HX	+	-	-	-	373
HY	-	+	-	-	373
LX	-	-	+	-	373
LY	-	-	-	+	373

[a]Forward
[b]Reverse

Tabella 2: Coppie di primer usate per la genotipizzazione dei polimorfismi al promotore del gene *mbl* in 4 singole reazioni di PCR.

Genotipo	Coppie di primer						Lunghezza (bp)
	E^a+F^b	E^a+G^b	E^a+H^b	E^a+I^b	E^a+L^b	E^a+M^b	
AA							128/135/143
AB	-	+	+	+	-	+	128/135/143
AC	-	+	-	+	+	+	128/135/143
AD	+	+	-	+	-	+	128/135/143
BB	-	+	+	-	-	+	128/135/143
BC	-	+	+	+	+	+	128/135/143
BD	+	+	+	+	-	+	128/135/143
CC	-	+	-	+	+	-	128/135/143
CD	+	+	-	+	+	+	128/135/143
DD	+	-	-	+	-	+	128/135/143

[a]Forward
[b]Reverse

Tabella 3: Coppie di primer usate per la genotipizzazione dei polimorfismi all'esone 1 del gene *mbl* in distinte reazioni di PCR.

Genotipo *mbl*	Casi	Controlli	OR	*P*
AA	70	159		
AO	145	134		
OO	120	42	0.15	$< 10^{-7}$
Totale	335	335		

Tabella 4: Distribuzione dei genotipi all'esone 1 del gene *mbl* tra i casi e i controlli.

Aplotipo	Casi	Controlli	OR	*P*
HYA/HYA	2	115		
HYA/-	68	151		
-/-	265	69	0.001	$< 10^{-7}$
Totale	335	335		

Tabella 5: Distribuzione dell'aplotipo *HYA* tra i casi e i controlli.

Aplotipo	Casi	Controlli	OR	P
LYD/LYD	49	2		
LYD/-	131	26		
-/-	155	307	9.9	$< 10^{-7}$
Totale	335	335		

Tabella 6: Distribuzione dell'aplotipo *LYD* tra i casi e i controlli.

7. FIGURE

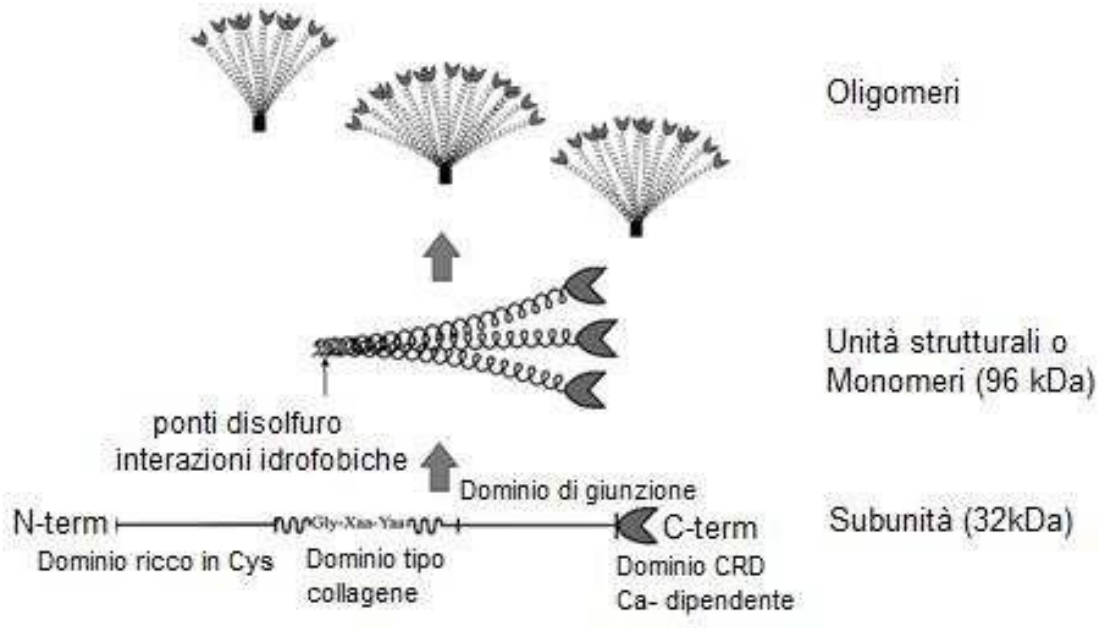

Figura 1: Caratteristiche della subunità della proteina MBL ed assemblaggio degli oligomeri ad alto peso (adattato da Eisen D.P. et al, 2003) (CRD: carbohydrate-recognition domain).

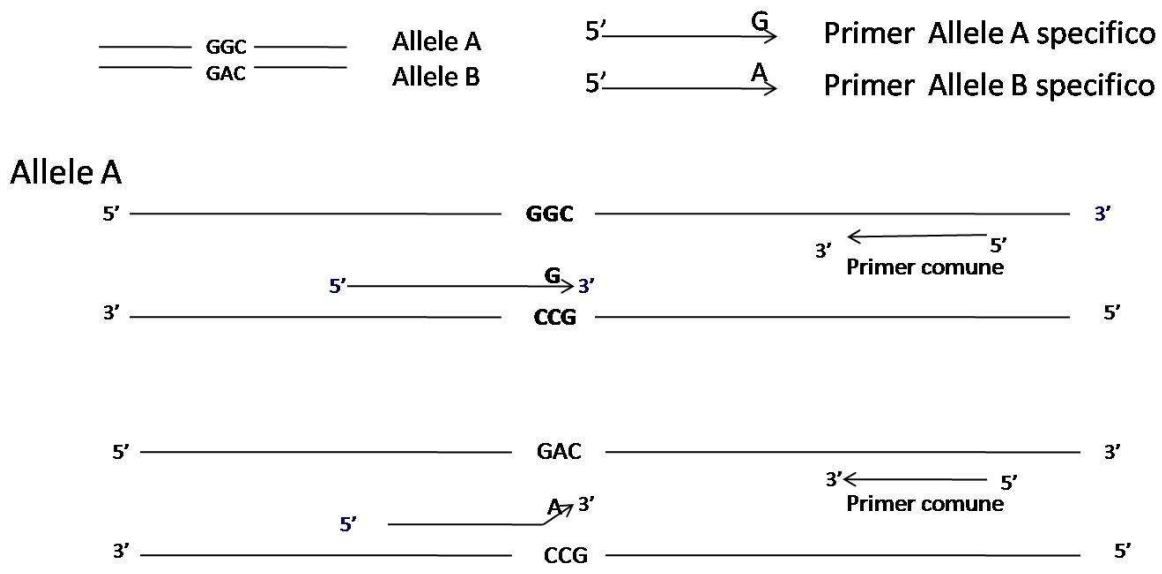

Figura 2: PCR allele specifica o ARMS: se si dispone di un primer corrispondente alla sequenza wt (Allele A), la presenza di una mutazione ne impedirà l'amplificazione e l'assenza dell'amplificato indicherà la presenza della mutazione. Viceversa se si dispone di un primer il cui 3' sia complementare al polimorfismo, l'amplificazione avrà luogo solo quando il DNA è mutato (Allele B).

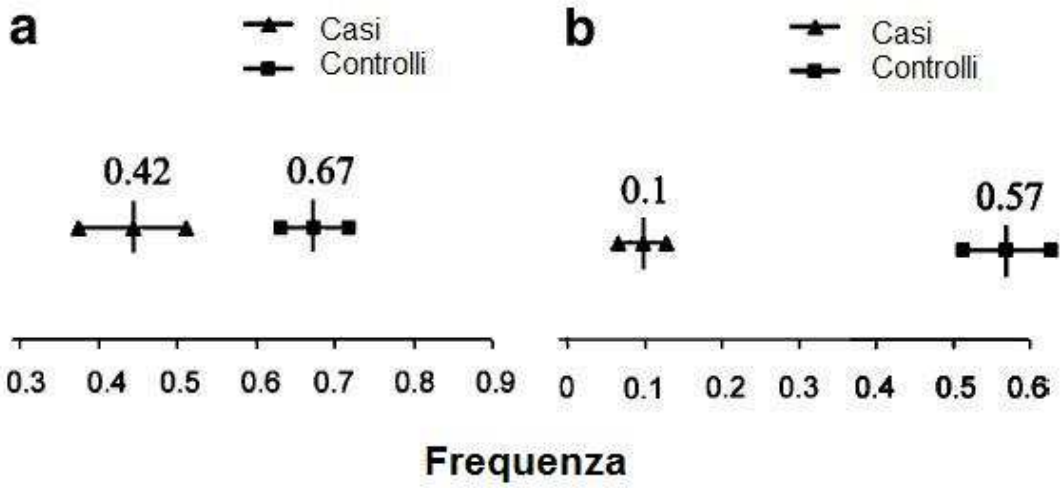

Figura 3: Frequenza dell'allele *A* e dell'aplotipo *HYA* nei casi e nei controlli. La frequenza (F ± C I) dell'allele A (a) è stata calcolata usando la formula F = (*AA* + 0.5×*AO*)/N. La frequenza (F ± C I) dell'aplotipo *HYA* (b) è stata calcolata usando la formula F = (*HYA/HYA* + 0.5×*HYA*/-)/N.
HYA/- : animali con un'unica copia dell'aplotipo *HYA*.

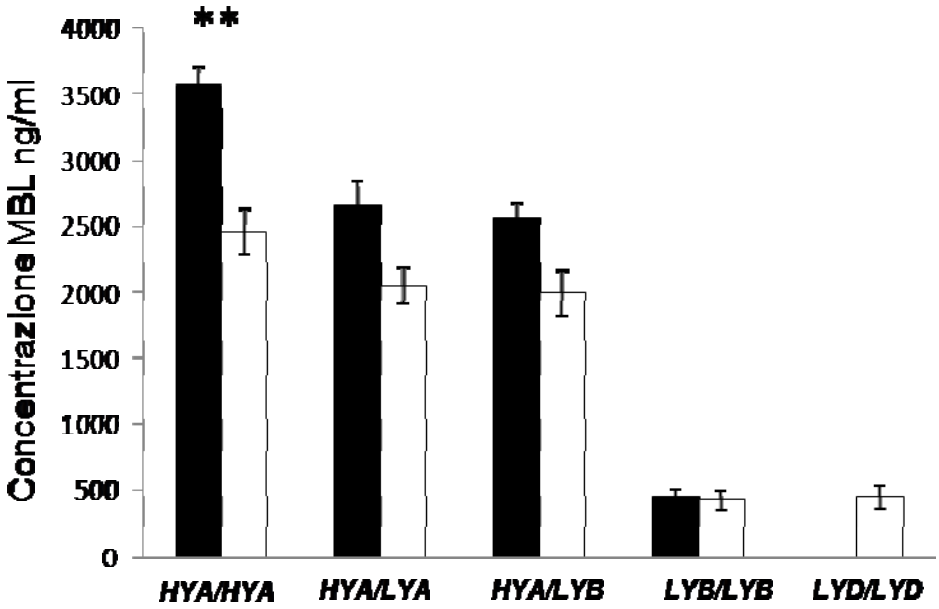

Figura 4: Concentrazione della proteina MBL nel siero di animali con differente aplotipo. Gli istogrammi rappresentano la media ± deviazione standard di due casi (□) e 30 controlli (■) *HYA/HYA*; 20 casi (□) e 20 controlli (■) *HYA/LYA*; 20 casi (□) e 20 controlli (■) *HYA/LYB*, 10 casi (□) e 10 controlli (■) *LYB/LYB* e 15 casi (□) *LYD/LYD*. ** indica una differenza altamente significativa.

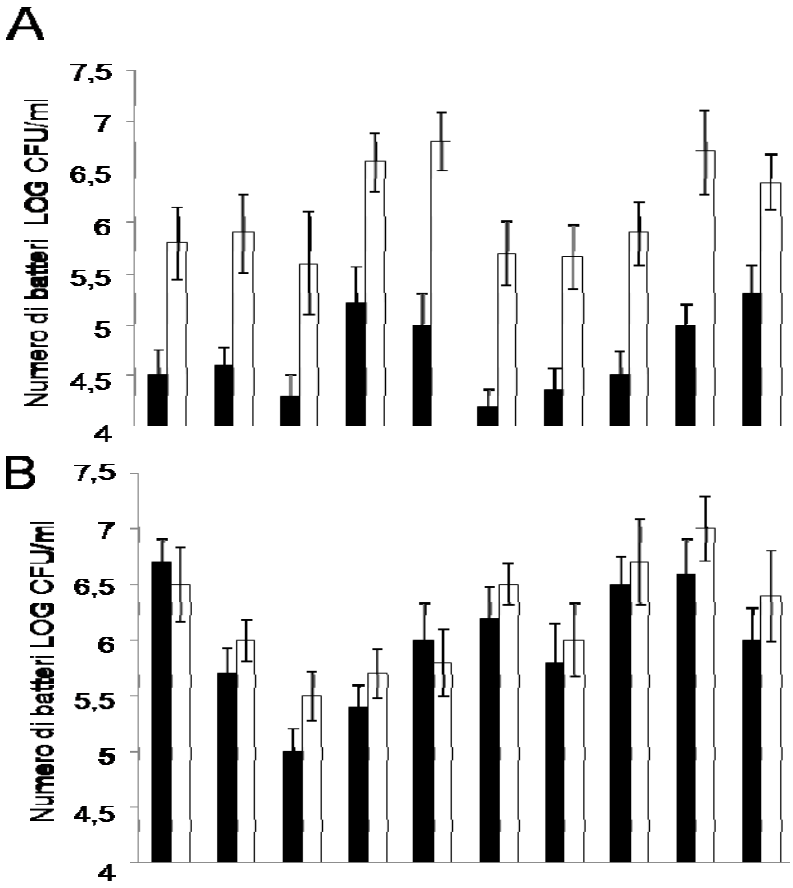

Figura 5: Attività antibatterica esercitata da soggetti resistenti *HYA/HYA* e suscettibili *LYD/LYD*. La differenza in CFU/ml tra campioni di siero non inattivati (■) e campioni di sieri inattivati al calore (□) è altamente significativa ($P< 0.0035$) nel caso di soggetti resistenti (A), ma non nel caso di soggetti suscettibili (B). Ciascun istogramma rappresenta la media ± deviazione standard di due campioni di siero, ciascuno testato in triplicato.

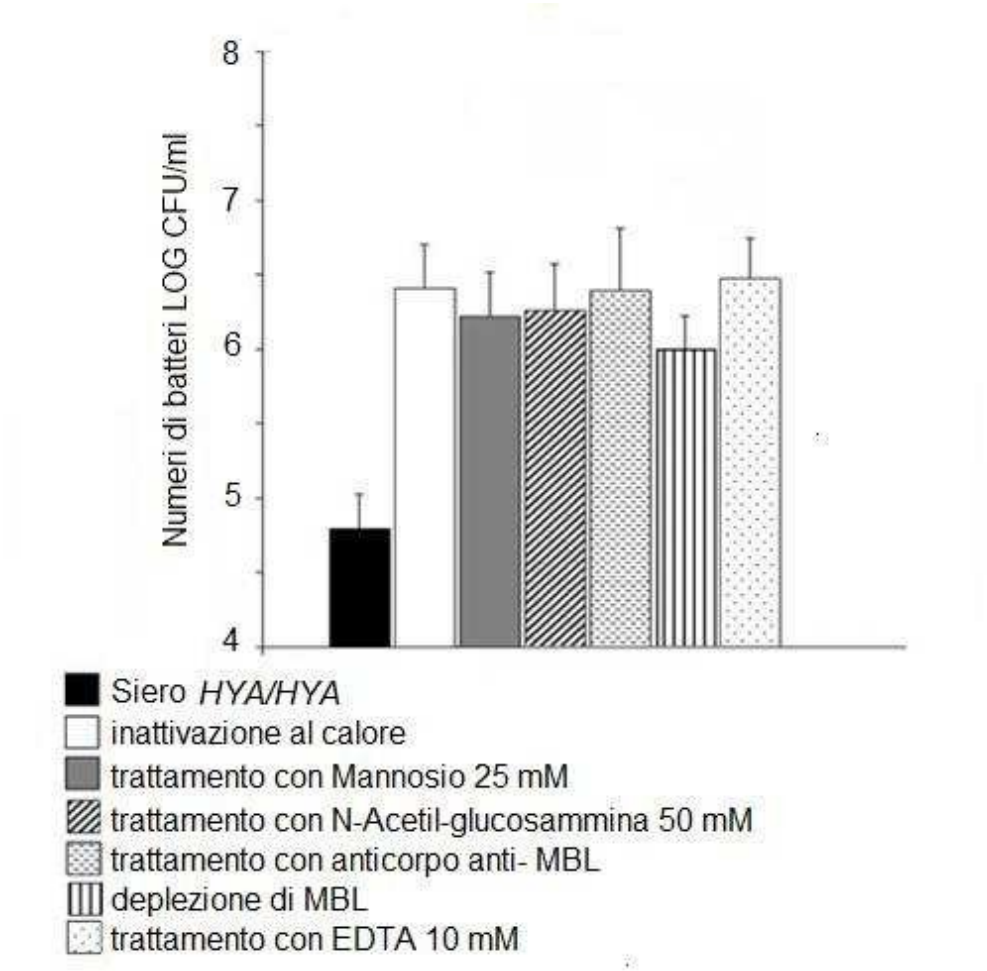

Figura 6: Neutralizzazione dell'attività antibatterica del siero proveniente da animali *HYA/HYA*. La differenza in CFU/ml tra sieri trattati e non trattati è altamente significativa ($P< 0.0001$). Gli istogrammi rappresentano la media ± deviazione standard di 10 campioni di siero per gruppo.

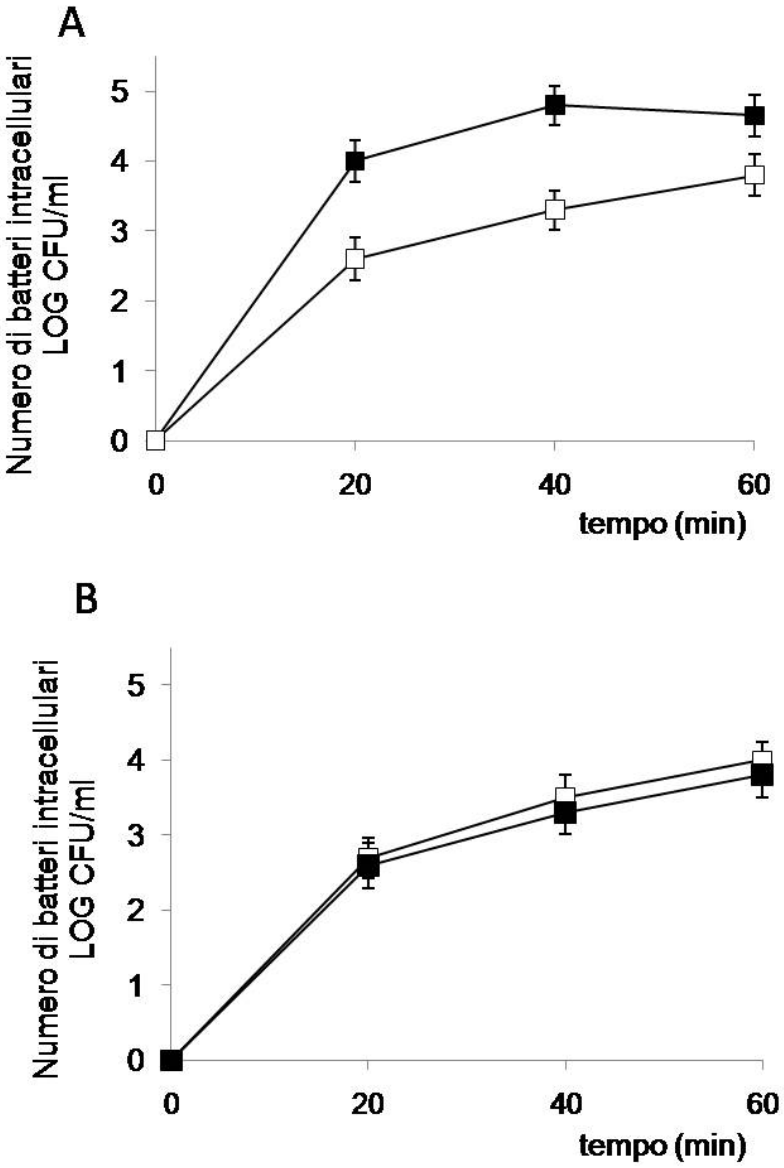

Figura 7: Internalizzazione di *B.abortus* pretrattata con sieri provenienti da animali *HYA/HYA* (A) e *LYD/LYD* (B). Il numero di batteri intracellulari è stata determinato 20, 40 e 60 minuti dopo l'infezione. La differenza in CFU/ml tra campioni di siero non inattivati (■) e campioni di sieri inattivati al calore (□) a 20 e 40 minuti dall'infezione è altamente significativa (*P*= 0.003) nel caso di soggetti resistenti (A), ma non nel caso di soggetti suscettibili (B). Ciascun istogramma rappresenta la media ± deviazione standard di cinque campioni di siero, ciascuno testato in triplicato.

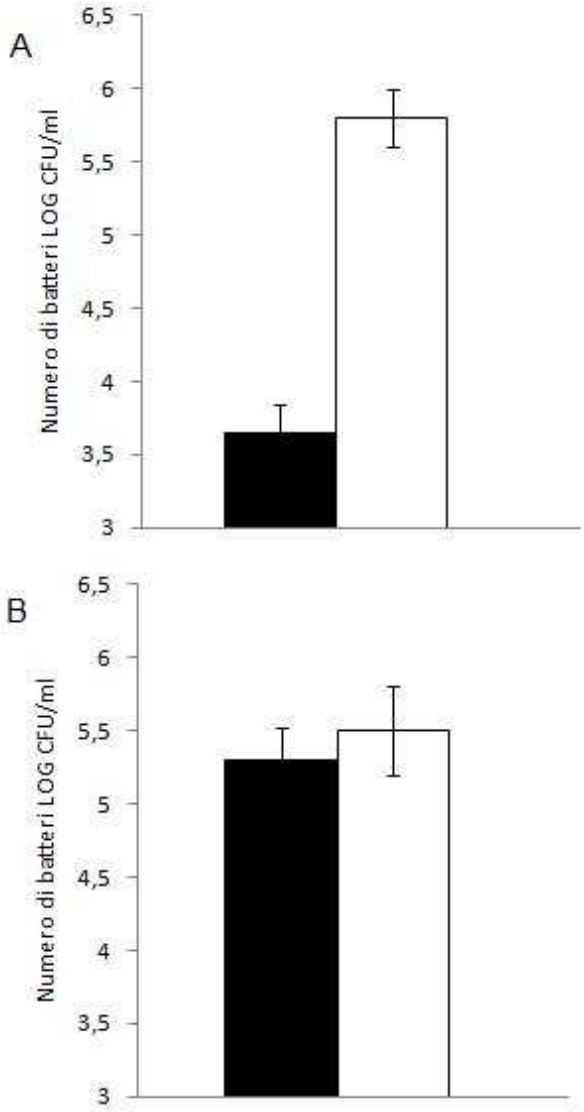

FIgura 8: Sopravvivenza intracellulare di *B. abortus* pretrattata con sieri provenienti da animali *HYA/HYA* (A) e *LYD/LYD* (B). Il numero di batteri intracellulari è stata determinato 24 ore dopo l'infezione. La differenza in CFU/ml tra campioni di siero non inattivati (■) e campioni di sieri inattivati al calore (□) è altamente significativa (*P*= 0.0025) nel caso di soggetti resistenti (A), ma non nel caso di soggetti suscettibili (B). Ciascun istogramma rappresenta la media ± deviazione standard di cinque campioni di siero, ciascuno testato in triplicato.

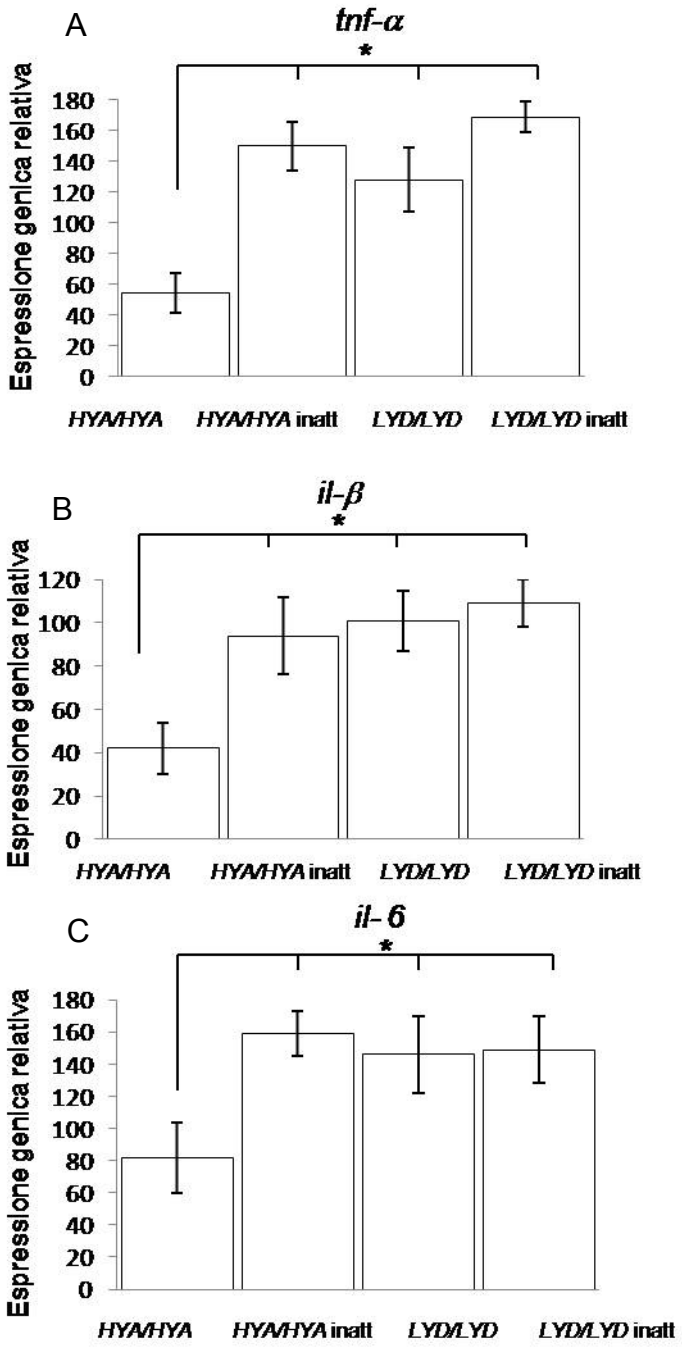

Figura 9. Livello di espressione dei geni *tnf-* α (A), *il-1*β (B) e *il-6* (C) in monociti stimolati con *B. abortus*, pretrattata con sieri provenienti da animali *HYA/HYA o LYD/LYD* (attività) o con sieri inattivati al calore (controllo).
I risultati si riferiscono a 3 esperimenti indipendenti, ognuno dei quali è stato condotto in triplicato. * indica una differenza significativa.

Pubblicazioni

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Comunicazioni

1. Parlato M., Borriello G., Iannnaccone M., Palumbo D., Capparelli R. Studio del gene *mbl* (mannose binding lectin) in relazione alla resistenza all'infezione da *B.abortus* nel buffalo (Bubalo bubalis). Giornate Scientifiche. Università degli Studi di Napoli "Federico II" 20-21 Settembre 2007, p.247.

2. Parlato M., Borriello G., Amoroso M.G., Ventimiglia I., Palumbo D., Iannaccone M., Capparelli R., Iannelli D. Modello sperimentale di terapia fagica contro *S. aureus* nel topo. Giornate Scientifiche. Università degli Studi di Napoli "Federico II", 20-21 Giugno 2006, p.221.

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Experimental Phage Therapy against *Staphylococcus aureus* in Mice †

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The present study describes a bacteriophage (M^{Sa}) active against *Staphylococcus aureus*, including methicillin-resistant staphylococcal strains. When inoculated into mice simultaneously with *S. aureus* A170 (10^8 CFU/mouse), phage (10^9 PFU) rescued 97% of the mice; when applied to nonlethal (5 10^6 CFU/mouse) 10-day infections, the phage also fully cleared the bacteria. The phage M^{Sa}, delivered inside macrophages by *S. aureus*, kills the intracellular staphylococci in vivo and in vitro. The phage can also prevent abscess formation and reduce the bacterial load and weight of abscesses. These results suggest a potential use of the phage for the control of both local and systemic human *S. aureus* infections.

Staphylococcus aureus is an extremely flexible organism: it can be a commensal but also a dangerous pathogen, causing skin abscesses, wound infections, endocarditis, osteomyelitis, pneumonia, and toxic shock syndrome (12, 20). *S. aureus* can also adapt to live inside cells, where it finds protection from host defense mechanisms and antibiotics (9). The number of staphylococcal infections continues to increase—in parallel with the increased use of intravascular devices (20)—while the treatment of these infections becomes ever more difficult because of the emergence of staphylococcal strains resistant to multiple antibiotics, including vancomycin (33, 34). In the United States and the United Kingdom, 40 to 60% of nosocomial *S. aureus* strains are multidrug resistant (19). The mortality rate due to methicillin-resistant versus methicillin-sensitive *S. aureus* infections is almost threefold higher (19). This context explains the urgency in developing new antibacterial agents. The present paper describes the isolation of a phage active against local and systemic infections of *S. aureus*. The phage is also active against intracellular staphylococci and methicillin-resistant staphylococcal strains. The lethal effect of bacteriophages on their bacterial hosts has been known since their discovery (25). However, the advent of antibiotics adversely affected the use of phages as antibacterial agents (25, 32). Rigorous studies conducted with animals (4, 25, 30) and the high incidence of antibiotic-resistant bacteria have resurrected phage therapy (25, 32).

MATERIALS AND METHODS

Bacteria. The study included 20 *S. aureus* strains isolated from patients hospitalized at the Medical School of the University of Naples Federico II. Specimens were streaked on Baird-Parker agar base supplemented with egg yolk (Oxoid, Milan, Italy). Single colonies were amplified in Luria-Bertani (LB) broth (Oxoid). Strains were confirmed as *S. aureus* by the coagulase test, microscopic observation, and PCR assay of the *S. aureus*-specific gene *clfA* (21). For in vivo and in vitro experiments, bacteria were grown in LB medium (Difco, Becton Dickinson, Sparks, MD) at 37°C, harvested while in exponential growth phase (optical density at 600 nm, 1.5 to 1.8), centrifuged (8 10^3 *g* for 10 min), washed with saline (0.15 M NaCl), and resuspended in saline (10^6 to 10^9 CFU/ml). The bacterial genes *eta*, *etb*, *tst*, *lukS-PV–lukF-PV*, *lukE-lukD*, *lukM*, and *sea* (coding for the exfoliative toxin A, exfoliative toxin B, toxic shock syndrome toxin 1, Panton-Valentine leukocidin components S and F, the leukotoxin LukE-LukD, the leukotoxin LukM, and enterotoxin A, respectively) were detected by PCR (2, 13, 24).

Phage isolation. Bacteria were grown in LB broth. When cultures reached the exponential growth phase (optical density at 600 nm, 1.5 to 1.8), mitomycin C (final concentration, 1 g/ml) was added to the cultures. Following incubation with mitomycin C for 30 min, bacteria were washed with LB broth and incubated again for 4 h at 37°C. The supernatants were filtered through a 0.45- m membrane and screened for the presence of phages by the spot test (1). Supernatants positive in the spot test were tested again by a plaque-forming assay (1). Individual plaques were expanded in LB broth (2 ml) containing the sensitive bacterial host (10^6 CFU). Phage purification was carried out as described previously (29). Phage DNA was isolated as described previously (3). The phage genes *eta*, *tst*, *lukSPV-lukFPV*, and *sea* were detected by PCR (2, 13, 24).

Adsorption rate, latent period, and phage burst size. The adopted procedures were those described previously (1). Briefly, to measure the adsorption rate, 1 ml phage M^{Sa} (1.5 10^3 PFU/ml) and 1 ml *S. aureus* A170 (5 10^8 CFU/ml) were mixed and the number of free phage particles was determined after treatment with chloroform (200 l). To determine the latent period and burst size, *S. aureus* A170 bacteria (5 10^8 CFU/ml) were incubated with phage M^{Sa} (3 10^3 PFU/ml) for 5 min, washed with cold LB broth to remove free phage particles, and then resuspended in fresh medium. The cell suspension was periodically titrated for newly produced phage on an *S. aureus* A170 lawn.

Phage selection. A phage strain able to persist in mouse blood was isolated as described previously (8). Briefly, four mice were injected intraperitoneally with phage W^{Sa} (10^8 PFU/mouse) and, at 12-h intervals, with four doses of the sensitive bacterial host *S. aureus* A170 (10^7 CFU/dose). Blood samples were collected periodically from the orbital plexuses of the mice. The phage mutant isolated following this procedure (M^{Sa}) persisted in the circulation for 21 days.

Mice. Experiments were carried out on female BALB/c mice (aged 8 to 10 weeks) at the animal facility of the University of Naples. Phage (10^6 to 10^9 PFU in 200 l saline) and bacteria (10^6 to 10^9 CFU in 200 l saline) were inoculated by the route (intravenous or subcutaneous) indicated for each experiment. To induce abscesses, mice were inoculated subcutaneously on both flanks with 10^7 CFU *S. aureus* A170 suspended in 50 l saline and, concurrently or 4 days later, with phage (10^9 PFU suspended in 50 l saline). Organs (hearts, kidneys, and spleens) and abscesses were dissected and weighed. One gram of each sample was homogenized in 1 ml saline and serially diluted in distilled water. CFU were evaluated by plating each dilution on Baird-Parker agar plates, and PFU were evaluated by plating each dilution on a lawn of *S. aureus* A170. Animal experiments were approved by the Animal Care Committee of the University of Naples.

***S. aureus* A170 transformation.** The plasmid pSK236 was introduced into *S. aureus* A170 by electroporation. The plasmid contains the construct *sarA-gfp-uvr*, constitutively expressing green fluorescent protein (GFP) (16). Standard conditions for electroporation were as follows: 150 ng plasmid and 10^9 CFU bacteria in a 50- l volume, 2.5 kV/cm, 800 , and 25 F. Following electroporation, the

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transformed bacteria (GFP-expressing *S. aureus*) were incubated for 90 min at 37°C in 1 ml brain heart infusion medium (Oxoid) supplemented with 0.3 M sucrose. Bacteria were then spread on brain heart infusion agar plates supplemented with 15 g/ml chloramphenicol.

Intracellular killing activity of phage P1. Peritoneal mouse macrophages were distributed in 24-well plates (10^5 cells/well), incubated overnight (37°C, 5% CO_2) in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (Sigma, Milan, Italy), and then infected with *S. aureus* A170 (10^4 bacteria/well). The plates were centrifuged (750 *g*, 5 min) to facilitate cell contact and then incubated for 1 h at 37°C in 5% CO_2. Extracellular bacteria were killed with gentamicin (12.5 g/well for 1 h). Cells were washed with DMEM and incubated in DMEM containing 5% fetal calf serum at 37°C in 5% CO_2. Following 3 h of incubation, cells were infected with phage M^{Sa} (5 10^6 PFU/well), incubated for another 45 h, and lysed with Tween 20 (final concentration, 0.03%) at different time points to recover intracellular bacteria. Each lysate was then serially diluted in saline and plated on Baird-Parker agar. Intracellular staphylococci recovered after 48 h of incubation were stained with 30 nM SYTO9 (Molecular Probes, Eugene, OR) and 15 M propidium iodide (Molecular Probes) for 15 min in the dark and then analyzed by flow cytometry, using a Coulter Epics Elite flow cytometer (Coulter, Miami, FL) and a LIVE-DEAD assay kit (Molecular Probes).

Antibodies. Antibodies against phage W^{Sa} (R W^{Sa}) were raised in Fischer 344 rats. Each rat received two intraperitoneal injections (10^7 PFU in 500 l phosphate-buffered saline [PBS] emulsified with an equal volume of complete Freund's adjuvant) at an interval of 2 weeks. The source of antibodies against phage M^{Sa} (M M^{Sa}) was the serum of a mouse with M^{Sa} circulating in its blood for 20 days. Goat anti-rat immunoglobulin G (IgG) labeled with fluorescein isothiocyanate (G $RIgG^{FITC}$) and goat anti-mouse IgG labeled with fluorescein isothiocyanate (G $MIgG^{FITC}$) were purchased from Sigma. R W^{Sa} and M M^{Sa} were diluted 1:100. G $RIgG^{FITC}$ and G $MIgG^{FITC}$ were diluted 1:600. The volume of antibodies used in each assay was the same (100 l/tube) for the four reagents.

Phage neutralization test. Phage M^{Sa} (10^5 PFU suspended in 100 l PBS) was incubated with M M^{Sa} (diluted 4 10^{-2}, 2 10^{-2}, and 10^{-2} in PBS) for 3 h at room temperature. Bacteria (10^5 CFU in 1 ml tryptic soy broth) were added to the antibody-coated phage particles, and the mixture was incubated for 3 h at 37°C. CFU and PFU numbers were then counted.

Flow cytometry. Latex particles (4 10^4/tube) with a diameter of 10 m (Polysciences, Warrington, PA) were incubated overnight with phage M^{Sa} or W^{Sa} (10^6 PFU/tube) suspended in 1 ml of 0.1 borate buffer, pH 8.5. Particles were washed with PBS, quenched with 2% milk blocking solution (Kierkegaard & Perry Laboratory, Gaithersburg, MD), washed again with PBS, and incubated for 3 h with M M^{Sa} or R W^{Sa}. The latex particles were then washed and incubated (1 h) with G M^{FITC} or G R^{FITC}. Samples were analyzed on a Coulter Epics Elite flow cytometer. In the analysis, a gate was set around the latex particles on the basis of their forward and side scattering characteristics. Standard markers were set by testing a negative control (where the M M^{Sa} or R W^{Sa} reagent was replaced with PBS). For each sample, data for 3,000 events were analyzed.

Real-time reverse transcription-PCR (RT-PCR). Total RNA was isolated from the mouse kidney by using Trizol reagent (Invitrogen, Milan, Italy) and was reverse transcribed using ImProm-II reverse transcriptase (Promega, Madison, WI) and oligo$(dT)_{18}$ according to the manufacturer's protocol. Real-time PCR was performed on cDNA, using Brilliant SYBR green master mix (Stratagene, La Jolla, CA) and a Bio-Rad iCycler instrument (Bio-Rad, Hercules, CA). Reactions were performed in 25 l in triplicate with the following thermal profile: 95°C for 10 min and 45 cycles of 15 s at 95°C and 45 s at 60°C. The PCR primers (500 nM each) were as follows: GAPDH forward, 5 TTCACCACCA TGGAGAAGGC 3 ; GAPDH reverse, 5 GGCATGGACTGTGGTCATGA 3 (26); IL-6 forward, 5 AAAGAGTTGTGCAATGGCAATT 3 ; IL-6 reverse, 5 CAGTTTGGTAGCATCCATCAT 3 ; TNF- forward, 5 TCTCAGCCTCTTC TCATTCCT 3 ; and TNF- reverse, 5 GTCTGGGCCATAGAACTGATG 3 . Specificity was verified by melting curve analysis and agarose gel electrophoresis. Relative expression levels were calculated by the comparative cycle threshold (C_T) method.

Statistical analysis. Survival rates of mice were analyzed using Fisher's exact test. Bacterial counts, abscess weights, and interleukin-6 (IL-6) levels were analyzed using Student's *t* test.

RESULTS

Phage isolation. Following incubation with mitomycin (1 g/ml for 30 min), the 20 strains of *S. aureus* included in this study yielded five phages. The phage displaying the largest host range (lysing 7 of the 19 strains tested) was isolated from *S. aureus* strain A171. This phage was designated W^{Sa} ("W" is for wild; "Sa" is for *S. aureus*) and further characterized with regard to the toxin genes *sea*, *lukSPV-lukFPV* (both present), *eta*, and *tst* (both absent).

Selection of a phage persisting in the mouse circulation. One of the limitations of phage therapy is the rapid clearance of phages by the reticuloendothelial system (14, 25, 35). In vivo amplification of phages in the presence of the bacterial host has been shown to favor the emergence of rare phage mutants able to persist in the mouse circulation (8). Therefore, four mice were injected intraperitoneally with the wild phage W^{Sa} (10^8 PFU/mouse) and, at 12-h intervals, with four doses of the sensitive host *S. aureus* A170 (individual dose, 10^7 CFU/mouse). This bacterial strain was chosen for the in vivo experiments since it is positive for only two toxins (exfoliative toxin B and Panton-Valentine leukocidin). Preliminary experiments established that repeated doses of 10^7 CFU of *S. aureus* A170 given intraperitoneally did not harm the mice. The procedure allowed the isolation of a phage strain that persisted in the blood for 21 days. This phage strain was designated M^{Sa} ("M" is for mutant; "Sa" is for *S. aureus*) and expanded in vitro from a single plaque. Two mice were given 10^9 PFU of phage W^{Sa} intravenously, and two more mice were given 10^9 PFU of phage M^{Sa}. The phage particles present in the spleen, liver, and blood were counted periodically. While phage M^{Sa} remained in the circulation for 20 to 25 days, phage W^{Sa} was almost completely trapped inside the spleen and liver within 2 days. The experiment demonstrates that the capacity acquired by phage M^{Sa} to persist in the mouse circulation is stable. The mice maintained phage M^{Sa} in vivo without displaying visible long-term negative effects. Phage M^{Sa} was further characterized with regard to the adsorption rate (1.89 10^{-9} ml/min), latent period (30 min), and burst size (80 PFU).

Phages W^{Sa} and M^{Sa} are serologically distinct. The two phages were tested with antibodies against phage W^{Sa} prepared in the rat (R W^{Sa}) and with the antibodies present in the serum from one of the mice, which had phage M^{Sa} circulating in its blood for about 20 days (M M^{Sa}). The rat antibodies reacted with both phages, but more strongly with phage M^{Sa} than with phage W^{Sa}, with the latter being the phage used for immunization. The mouse antibodies reacted with phage M^{Sa} only (Fig. 1). Presumably, phage M^{Sa}, driven by the immune system, lost one or more of the surface antigens present on the wild phage W^{Sa}, becoming antigenically distinguishable. Incubation of phage M^{Sa} with an excess of mouse anti-M^{Sa} antibodies did not interfere with the phage's capacity to lyse *S. aureus* A170 cells (Table 1). This result supports the conclusion that the antibodies elicited by phage M^{Sa} in the mouse are nonneutralizing and explains the coexistence in the mouse circulation of phage M^{Sa} and corresponding antibodies. Phages can easily induce neutralizing antibodies (32). The characteristic of phage M^{Sa} of not inducing this class of antibodies is therefore biologically relevant and demonstrates how selection can improve the antimicrobial properties of phages.

Phage treatment of systemic infections. *S. aureus* is the major cause of septicemia, invasive endocarditis, and septic arthritis in humans (12). To investigate whether phage M^{Sa} could protect mice from *S. aureus* A170 when the pathogen entered

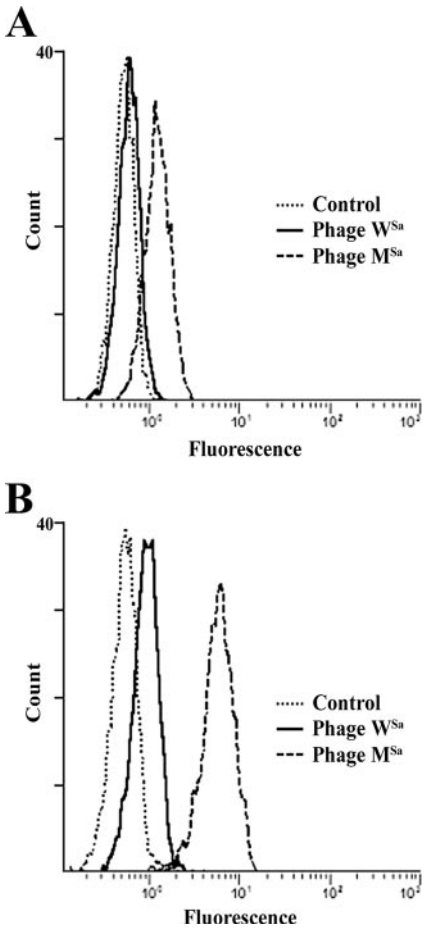

FIG. 1. Phages W^{Sa} and M^{Sa} are serologically distinct. (A) M M^{Sa} antibodies react with the mutant phage M^{Sa} but not with the wild phage W^{Sa}. The peak of phage W^{Sa} coincides with the peak of the negative control. (B) R W^{Sa} antibodies react with the heterologous antigen (phage M^{Sa}) more strongly than with the homologous one (phage W^{Sa}).

the bloodstream, four groups of mice (five mice per group) were injected intravenously with a dose of *S. aureus* A170 (10^8 CFU/mouse) which killed 90 to 100% of mice within 4 days. One group served as an untreated control, and the remaining three groups were given the phage M^{Sa} (10^7, 10^8, or 10^9 PFU/mouse) intravenously immediately after infection. All mice of the control group and the group treated with the lowest phage

TABLE 1. Phage M^{Sa} is not neutralized by circulating mouse antibodies (M M^{Sa})[a]

M M^{Sa} dilution	Count (log CFU or PFU/ml)							
	Bacteria				Phage			
	t_0		t_3		t_0		t_3	
4 10^{2}	4.95	0.23	2.94	0.31	5.12	0.20	7.70	0.16
2 10^{2}	5.1	0.29	2.81	0.19	5.23	0.31	7.35	0.15
1 10^{2}	4.92	0.19	2.75	0.23	5.21	0.21	7.77	0.23

[a] Preincubation with antibodies did not inhibit the killing capacity of phage, as demonstrated by the decreasing number of CFU and the increasing number of PFU at 3 h.

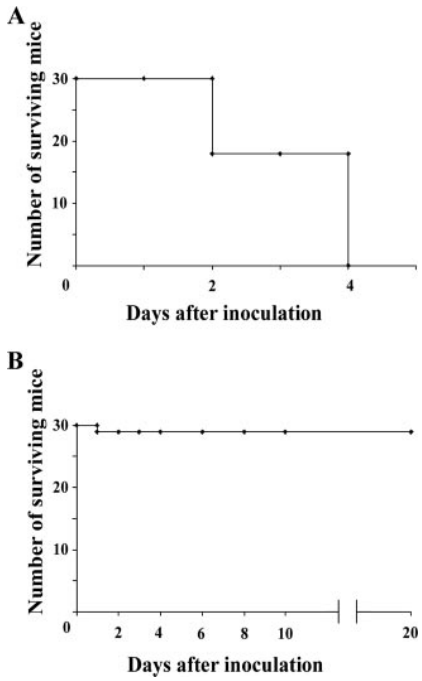

FIG. 2. Phage M^{Sa} given concurrently with *S. aureus* A170 rescues 93% (29/30 mice) of mice artificially infected with a lethal dose of the pathogen. (A) Mice infected with *S. aureus* A170 (10^8 CFU/mouse). (B) Mice infected with *S. aureus* (10^8 CFU/mouse) and treated immediately after with phage M^{Sa} (10^9 PFU/mouse).

dose (10^7 PFU/mouse) died within 4 days (10/10 mice). The mice treated with the intermediate dose (10^8 PFU/mouse) were only partially protected (2/5 mice survived). The mice treated with the highest dose (10^9 PFU/mouse) were all protected from death (5/5 mice). Subsequent experiments were carried out using 10^8 CFU of *S. aureus* A170 and 10^9 PFU of phage M^{Sa} per mouse (standard schedule). Cumulative data from six independent experiments carried out according to the standard schedule showed that while 100% (30/30 mice) of the mice of the control group died within 4 days, only 3% (1/30 mice) of the treated mice died (P 0.0001) (Fig. 2). The clinical advantage of phage treatment was also evident by quantifying the bacterial load 4 days after phage injection. Although all *S. aureus* A170-infected mice not given phage displayed a high bacterial load, no bacteria were isolated from phage-treated mice (Table 2 and Fig. 3).

The occurrence of a host threshold for reproduction is common among microbial predators (36). To establish the threshold density of *S. aureus* A170 required for the replication of

TABLE 2. Mice infected with *S. aureus* A170 (10^8 CFU/mouse) and treated concurrently with phage M^{Sa} (10^9 PFU/mouse) become sterile 4 days after phage treatment

Mouse group	Bacterial count (mean log CFU/g SD)[a]							
	Kidney		Heart		Spleen		Lung	
Untreated	7.06	0.34	2.99	0.18	3.70	0.20	3.35	0.19
Treated	0		0		0		0	

[a] Organs were homogenized in saline, diluted in distilled water, and plated.

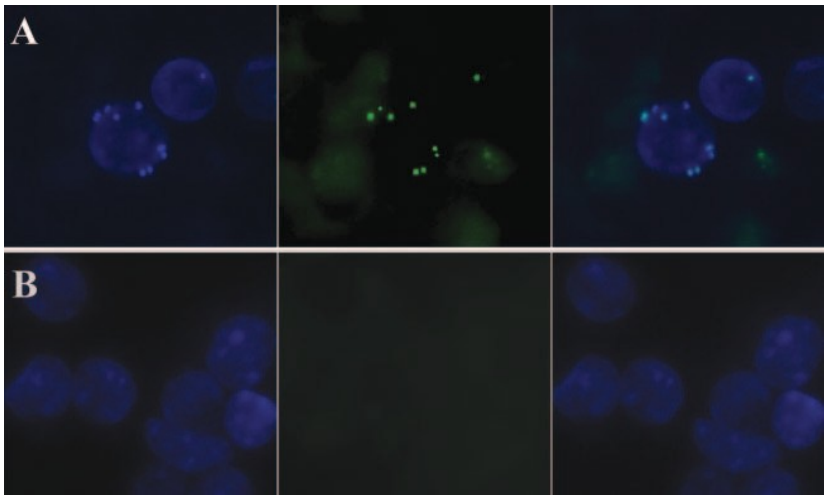

FIG. 3. In vivo bactericidal activity of phage M^{Sa}. (A) Fluorescence microscopy pictures of kidney cells recovered from mice 4 days after intravenous infection with 10^8 CFU of GFP-expressing *S. aureus*. (B) Kidney cells from mice infected concurrently with 10^8 CFU of GFP-expressing *S. aureus* and 10^9 PFU of phage M^{Sa}. Cells were counterstained with 4 ,6 -diamino-2-phenylindole (DAPI). (Left) Cells analyzed with a 340- to 380-nm filter (DAPI). (Center) Cells analyzed with a 450- to 490-nm filter (GFP). (Right) Overlay. Magnification, 1,000 (oil immersion).

phage M^{Sa}, mice were infected concurrently with bacteria and phage M^{Sa} according to the standard schedule (10^8 CFU and 10^9 PFU/mouse). Circulating bacterial and phage titers were measured daily for 20 days. The density of *S. aureus* A170 at which phage replication began was approximately 10^4 CFU/ml and was the same for the spleen, heart, and kidneys (Fig. 4). In the blood, *S. aureus* A170 did not reach this threshold (perhaps it was forced to move out of the bloodstream in search of refuge), and in the absence of adequate bacterial density, phage expansion did not occur (Fig. 4). The threshold host density observed in this study is surprisingly close to that reported for several gram-negative and gram-positive bacteria and lytic as well as lysogenic phages (36), providing further support to the suggestion that the threshold for replication might be similar for most phage-host combinations (36).

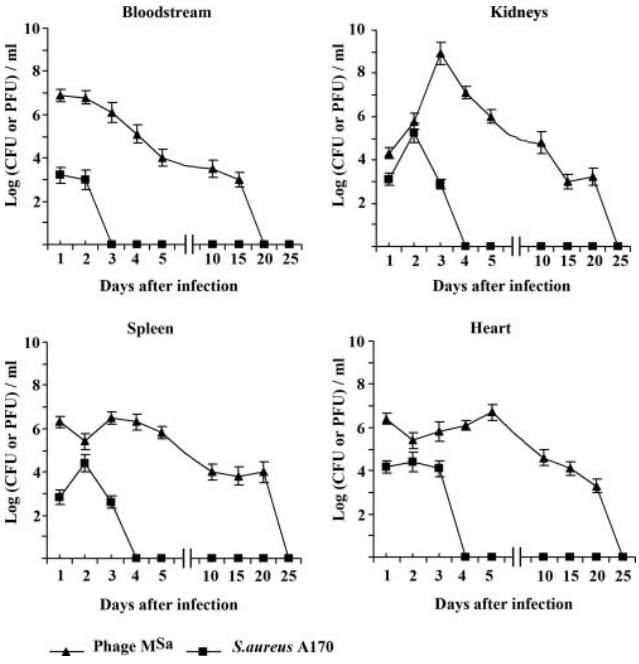

FIG. 4. In vivo phage replication occurs when the bacterial density reaches a threshold of approximately 10^4 CFU/ml. Mice were treated concurrently with 10^8 CFU *S. aureus* A170 and 10^9 PFU phage M^{Sa}. The organs were dissected in saline, diluted in distilled water, and plated. In the bloodstream, bacteria did not reach the threshold for phage replication. The bacterial density thresholds were approximately the same for the various organs.

Phage treatment can drastically reduce inflammation caused by *S. aureus* infection. To determine whether phage treatment, by killing bacteria, could modulate inflammation, the levels of the proinflammatory cytokines IL-6 and tumor necrosis factor alpha (TNF-) were analyzed by real-time RT-PCR 24 h after intravenous infection of mice (3 mice/group) with M^{Sa} alone (10^9 PFU/mouse), *S. aureus* alone (10^8 CFU/mouse), or M^{Sa} (10^9 PFU/mouse) and *S. aureus* (10^8 CFU/mouse) concurrently. Three control mice provided the average basal levels of the cytokine genes. Analysis was carried out on kidney cells, which are heavily colonized by *S. aureus* (Table 2). M^{Sa}, given concurrently with *S. aureus*, reduced the IL-6 expression level induced by *S. aureus* infection about fivefold (P 0.001) (Fig. 5). Twenty-four hours after infection, the TNF- expression level did not vary significantly among experimental groups.

Phage activity against methicillin-resistant *S. aureus* strains. Given the high incidence of methicillin-resistant *S. aureus* strains (12, 19), it seemed worth investigating whether phage M^{Sa} was also active against this class of bacteria. Two groups of mice (10 mice/group) were infected intravenously with the methicillin-resistant A352 strain of *S. aureus* (10^8 CFU/mouse). One group of animals served as untreated controls; the second group was given the phage M^{Sa} (10^9 PFU/mouse) intravenously immediately after infection. The survival rate was 20% (2/10) for the control group and 100% (10/10) for the treated group (Fig. 6) (P 0.0007). Thus, phage M^{Sa} repre-

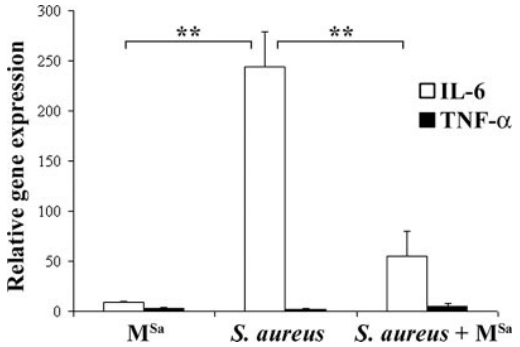

FIG. 5. Expression levels of IL-6 and TNF- genes in the kidneys of mice treated with phage M^{Sa} alone, *S. aureus* alone, or both. Expression levels were measured by real-time RT-PCR 24 h after treatment. Expression levels of the IL-6 and TNF- genes are given relative to the average levels measured in three control mice. Results refer to three independent experiments, with each one carried out in triplicate. Significant results (at a *P* value of 0.001) are marked with asterisks.

sents an antimicrobial potentially effective against human infections with methicillin-resistant *S. aureus*.

Phage treatment is also effective when phage are administered 10 days after bacterial infection. To investigate the effects of delaying phage treatment until after *S. aureus* A170 exposure, mice were infected intravenously with the lowest dose of *S. aureus* A170 not cleared by innate immunity (5 10^6 CFU/mouse) and then divided into two groups of 10 animals each. One group was given phage M^{Sa} (10^9 PFU/mouse) 10 days after infection. The second group served as an untreated control. The reason for using a low infection dose was twofold, namely, to approximate human *S. aureus* infection under natural conditions, which is typically started by a small initial inoculum; and to avoid death by an overresponse of the immune system (17, 18) (rather than by the bacteria directly), which would obscure the real contribution of phage. Twenty days after infection with *S. aureus* (10 days after M^{Sa} treatment), the spleens, kidneys, hearts, and blood of the control mice (10/10 mice) were still infected; the same organs of the mice treated with phage (10/10 mice) were sterile (Fig. 7). A repeat experiment with 20 more animals (10 phage-treated mice and 10 control mice) confirmed the above results. The experiment demonstrates clearly that by using the proper infection model, phages can be shown to be highly effective, even when administered 10 days after bacterial infection.

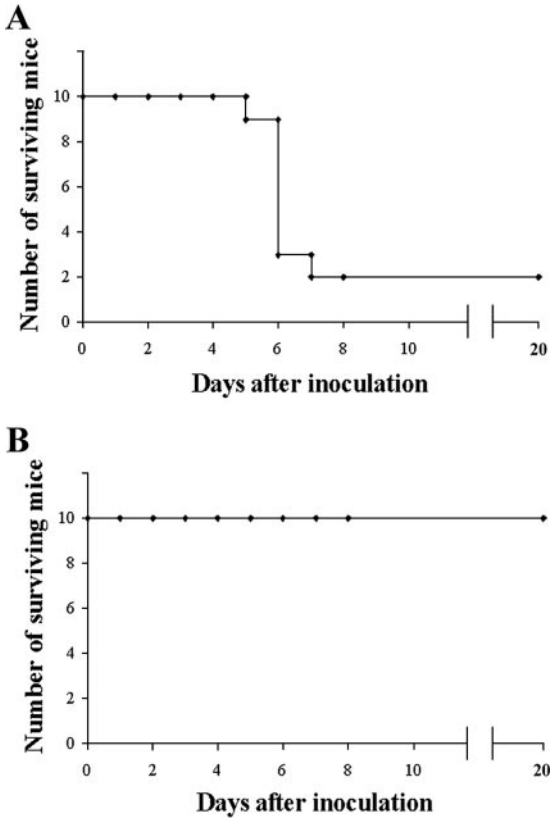

FIG. 6. Phage M^{Sa} is active against the methicillin-resistant strain *S. aureus* A352. (A) Mice infected with *S. aureus* A352 (10^8 CFU/mouse). (B) Mice infected with *S. aureus* A352 (10^8 CFU/mouse) and treated immediately after with phage M^{Sa} (10^9 PFU/mouse).

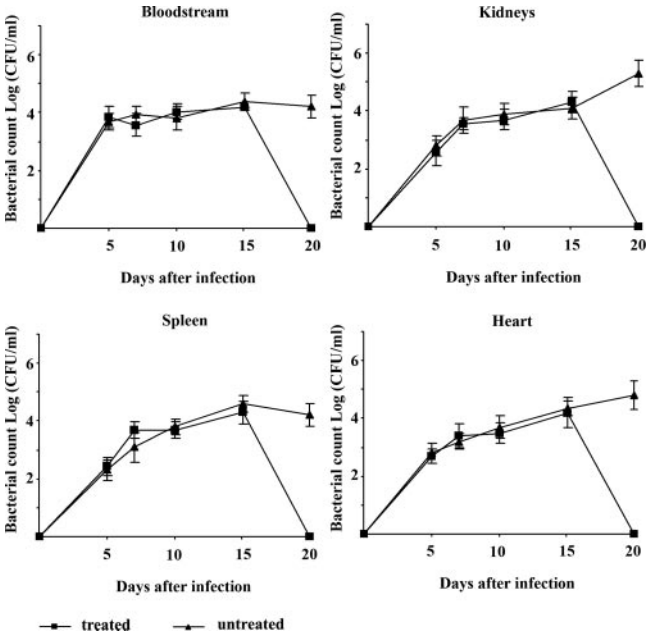

FIG. 7. Using mouse sterilization as a criterion for measuring phage activity, M^{Sa} was shown to be active when administered 10 days after infection. Mice received 5 10^6 CFU *S. aureus* A170 and, 10 days later, 10^9 PFU phage M^{Sa}. The experiment included 20 phage-treated and 20 untreated animals.

Phage treatment of local infections. *S. aureus* accounts for a large proportion of the morbidity and mortality due to surgical wound infections (12, 15). Phage M^{Sa} was therefore tested for the capacity to control local infections. The first experiment was designed to test the efficacy of M^{Sa} given concurrently with *S. aureus*. Two groups of mice (5 mice/group) received either *S. aureus* alone (10^8 CFU/mouse; untreated group) or *S. aureus* (10^8 CFU/mouse) and, immediately after, phage M^{Sa} (10^9 PFU/mouse) (treated group) subcutaneously on both sides of the abdomen.

The second experiment was designed to test the efficacy of phage therapy once the abscesses became well established.

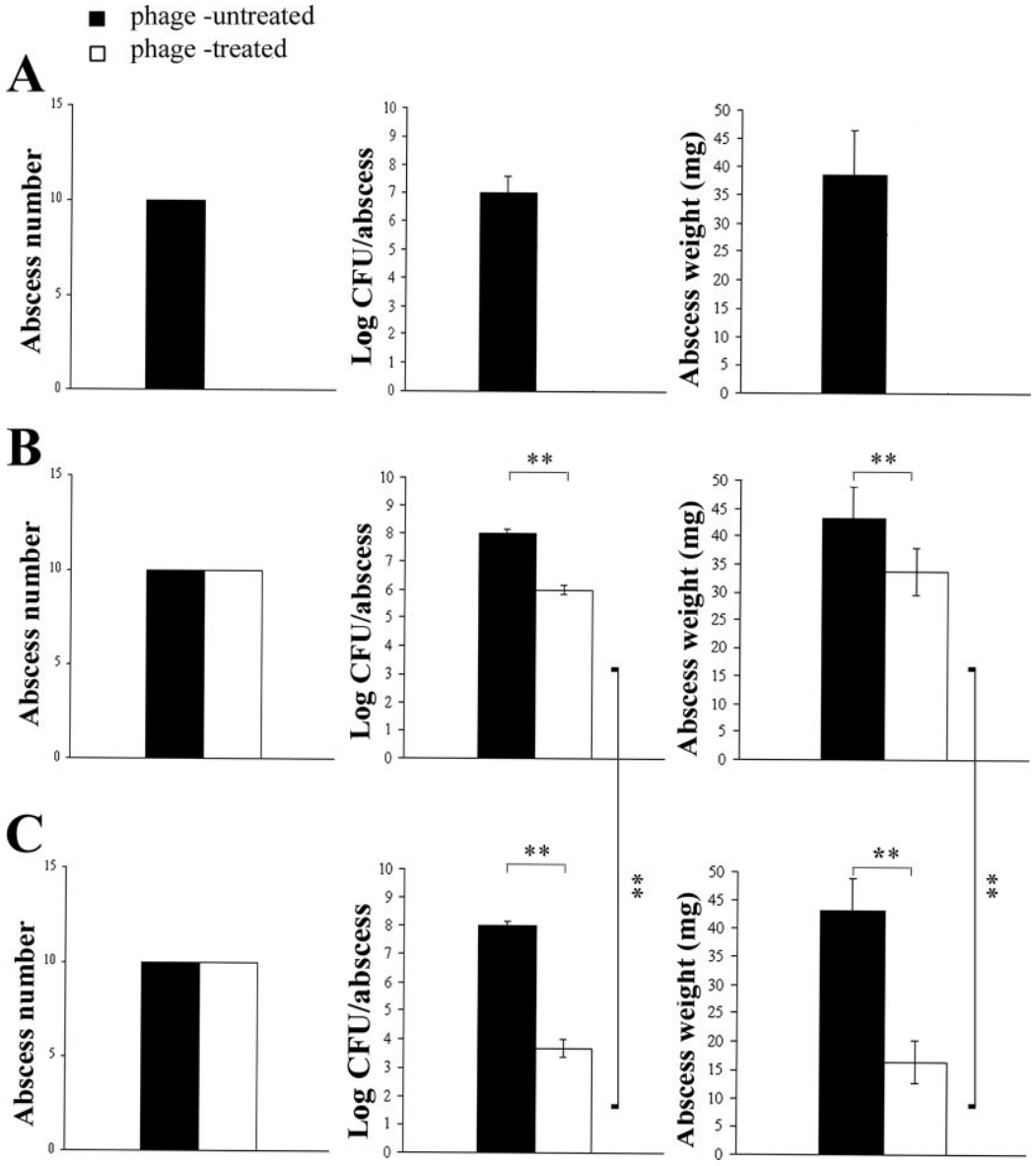

FIG. 8. Effect of phage M^{Sa} on abscess formation. (A) One single dose of phage M^{Sa}, given concurrently with *S. aureus*, inhibits abscess formation. (B) One single dose of phage M^{Sa}, given 4 days after *S. aureus*, cannot prevent abscess formation but significantly reduces the bacterial load and the weight of abscesses. (C) Multiple doses of phage M^{Sa}, given 4 days after *S. aureus*, are more effective than a single dose in reducing the bacterial load and the weight of abscesses. Significant results (at a P value of 0.001) are marked with asterisks.

Phage M^{Sa} (10^9 PFU/mouse) was therefore administered 4 days after mouse infection with *S. aureus* (10^8 CFU/mouse).

The third experiment was designed to test the efficacy of multiple doses of phage M^{Sa}. Mice therefore received four daily doses of phage M^{Sa} (individual dose, 10^9 PFU/mouse), starting 4 days after *S. aureus* infection (10^9 CFU/mouse).

The parameters used to evaluate the efficacy of phage therapy were the same for the three experiments and included prevention of abscess formation and reductions in the bacterial load (CFU/abscess) and the weight of abscesses. These parameters were evaluated 4 days after phage administration.

Figure 8 illustrates the results of the three experiments. Given concurrently with bacteria, phage M^{Sa} inhibited abscess development (P 0.0001). Furthermore, one single dose or multiple doses of phage M^{Sa} given 4 days after bacterial administration did not influence the incidence of abscesses (as expected); however, the phage significantly reduced both the bacterial load and the weight of abscesses (P 0.001). Multiple doses were more effective than single doses in reducing both the bacterial load and the weight of abscesses (P 0.001). In view of the adverse conditions present in the abscesses, i.e., low pH, slow bacterial growth, and enzymes protecting bacteria (23, 31), the above results look encouraging.

Phage activity against intracellular *S. aureus*. The data reported in Table 2 prove that 4 days after phage administration, mice are free of the bacteria used to infect them. In view of the

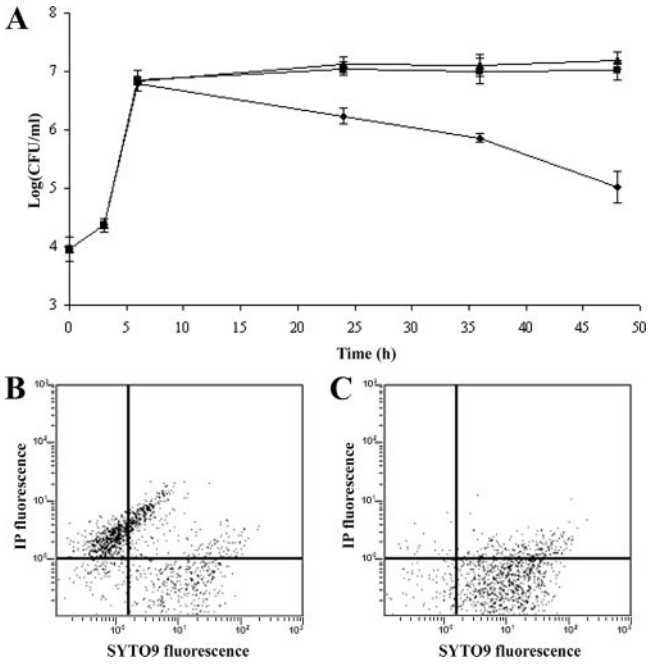

FIG. 9. M^{Sa} kills intracellular *S. aureus* A170. (A) Numbers of live bacteria recovered in the 0- to 48-h time interval from mouse peritoneal macrophages (10^5/well) infected with *S. aureus* A170 (10^4 CFU/well) and subsequently treated with phage M^{Sa} alone (f), infected with *S. aureus* A170 preincubated with phage M^{Sa} ({), or left untreated (Œ). (B) Flow cytometric analysis of dead and live intracellular staphylococci recovered from mouse peritoneal macrophages infected with *S. aureus* A170 and subsequently treated for 48 h with *S. aureus* A170 preincubated with phage M^{Sa}. (C) Flow cytometric analysis of dead and live intracellular staphylococci recovered from mouse peritoneal macrophages infected with *S. aureus* A170 and subsequently treated for 48 h with phage M^{Sa}. When used concurrently, propidium iodide stains dead bacteria and SYTO9 stains the bacteria which are alive.

evidence that *S. aureus* can adopt an intracellular lifestyle (9, 28), the complete eradication of infecting staphylococci supposes that phage M^{Sa} might kill both extracellular and intracellular staphylococci. To test this hypothesis, mouse peritoneal macrophages (10^5/well) were incubated in vitro with *S. aureus* A170 (10^4 CFU/well), and infection was allowed to occur for 1 h. The monolayer was then incubated with gentamicin for 1 h (to kill the extracellular bacteria), washed with DMEM (to remove the excess antibiotic), and further incubated for 3 h. Phage M^{Sa} (10^6 PFU/well), alone or adsorbed to *S. aureus* (10^4 CFU), was then added to the individual wells. The control wells were left without phage or phage-infected bacteria. Following incubation (24 to 48 h), macrophages were lysed and the number of surviving intracellular bacteria was determined by plating the lysate on Baird-Parker agar. The results (Fig. 9A) demonstrate that phage M^{Sa} alone cannot penetrate inside cells. Instead, when delivered inside cells by *S. aureus*, M^{Sa} efficiently killed intracellular bacteria at 24 and 48 h (Fig. 9A). Flow cytometry, by directly measuring the proportions of dead and live bacteria, confirmed the killing activity of phage M^{Sa} against intracellular bacteria. About 70% of the intracellular bacteria isolated from macrophages treated in vitro with phage-infected bacteria for 48 h were dead (Fig. 9B), while the intracellular bacteria isolated from macrophages treated with phage M^{Sa} alone were almost all alive (Fig. 9C). Thus, in vitro (Fig. 9) and in vivo (Table 2) results concur in demonstrating that phage M^{Sa}, once shuttled inside the host cells by *S. aureus*, also kills intracellular staphylococci.

Evolution of phage resistance. A concern often expressed about phage therapy is that bacteria can become refractory to phages. This study provides data relevant to this point. Different organs (spleen, kidneys, and heart) and the blood of more than 50 mice infected with *S. aureus* A170 and treated with phage M^{Sa} concurrently (Fig. 4) or 10 days after infection (Fig. 7) were all found to be sterile when the animals were sacrificed at the end of the experiment. In vivo, phage-resistant staphylococci might be cleared rapidly by the immune system. The frequency of phage-resistant bacteria was therefore also measured in vitro. Five large bacterial cultures (10^8 CFU/ml) in rich (LB) medium were infected with phage W^{Sa} (10^9 PFU/ml) and incubated at 37°C overnight. The observed frequency of phage-resistant bacteria was 1.3 10^{-8} 4.16 10^{-9}. The above results confirm previous studies (8, 11, 32) indicating that phage resistance is a rare event, perhaps rarer than antibiotic resistance (11, 32).

DISCUSSION

Treatment of staphylococcal infections with antibiotics is becoming increasingly difficult in view of the widespread presence of *S. aureus* strains resistant to multiple antibiotics. The present study highlights phage therapy as a possible solution. Given concurrently with a lethal dose of *S. aureus* A170, phage M^{Sa} rescued 97% of mice and completely eradicated bacteria in vivo within 4 days of phage treatment (Table 2 and Fig. 3). The phage was active against systemic (Fig. 2), local (Fig. 8), and intracellular (Fig. 9) bacterial infections. Most importantly, the phage lysed methicillin-resistant staphylococci (Fig. 6). Mortality is certainly an important criterion to judge the efficacy of a treatment, yet the present study also provides additional evidence, as follows: phage M^{Sa} was well tolerated by the animals, it drastically reduced inflammation (Fig. 5), and it did not stimulate the production of neutralizing antibodies. In all experiments, bacterial rebound or adverse effects due to rapid bacterial lysis (10) were not observed. Phage-treated mice, in fact, remained healthy 20 days after infection (Fig. 2), when the experiment was ended. The above results become more convincing when examined in the context of numerous reports documenting phage efficacy in vivo against several bacterial species (4, 28), including *S. aureus* (22, 37).

We also attempted to test the efficacy of phage M^{Sa} when bacterial infection was already established. To demonstrate that phage can be effective several days after experimental infection, we shifted the criterion for measuring phage efficacy from the survival rate for animals treated with a lethal bacterial dose to the sterilization rate for animals infected with a low bacterial dose. In this regard, our study differs significantly from previous ones on phage therapy. We infected mice with the smallest dose of *S. aureus* A170 not cleared by the innate immunity of the mouse (5 10^6 CFU/mouse) and then determined how many days after bacterial exposure phage therapy was successful. By these means, we mimicked human staphylococcal infection more closely and, at the same time, avoided sepsis, which could mask phage therapy effectiveness. Given 10

days after artificial infection, phage M^{Sa} sterilized the totality (20/20 mice) of the phage-treated mice, while the control mice (20/20 mice) were still infected (Fig. 7). The above experiment clearly demonstrates that phages are not temporally constrained in their ability to kill bacteria. This is perhaps the most significant contribution of this article to the application of phage therapy in a clinical context. Also, it supports earlier observations on the limits of some experimental infection designs and the opportunity to exploit long-term infections for treatment with phages (6).

In the case of systemic infections, just one single phage injection was sufficient for effective recovery. In the case of abscesses, clearance of bacteria required repeated doses. Both outcomes can be interpreted as direct consequences of the phage property of replicating only when the bacterial density is above a threshold. This threshold was reached in the course of systemic infections (Fig. 4), but presumably not in abscesses, which contain large numbers of slowly growing or static bacteria (31). Thus, systemic infections offer an example of active phage therapy (with in vivo phage multiplication), and abscesses give an example of passive therapy (without in vivo phage multiplication) (27). Clear cases of both active and passive therapy have been found (27).

Although traditionally considered an extracellular pathogen, *S. aureus* can internalize and survive inside mouse macrophages infected in vivo (Fig. 9). Phage M^{Sa} added alone to the infected macrophages did not reduce the number of intracellular bacteria (Fig. 9A) (very likely because phage particles could not penetrate inside the cells). However, *S. aureus* previously infected with M^{Sa} particles significantly reduced the number of intracellular bacteria (Fig. 9A). Flow cytometry confirmed this result (Fig. 9B and C). The simplest explanation for these results is that M^{Sa} is delivered to bacteria residing within macrophages by phage-infected staphylococci. If we assume that the same phenomenon also takes place in vivo (if we assume that M^{Sa} infects the extracellular bacteria and that these, in turn, carry phage particles inside the cells), we can understand how 4 days of phage treatment of mice resulted in complete sterility (Table 2). We are not the first to describe a phage active against an intracellular pathogen. Broxmeyer et al. (5) showed that phage TM4 can kill intracellular bacilli (*Mycobacterium tuberculosis* or *Mycobacterium avium*) in vitro. They proposed the use of attenuated strains of mycobacteria as delivery systems. Perhaps an animal model of infection might have shown these authors that TM4, like M^{Sa}, works equally well if given alone, shuttled inside the cells by extracellular mycobacteria.

One of the limitations of phages as antimicrobials is their rapid uptake by the reticuloendothelial system (14, 25, 35). This problem, originally solved by Merril et al. (25), was addressed again recently, with the devising of a protocol which permitted isolation of a phage mutant (specific for *Escherichia coli* O157:H7) persisting in the mouse circulation for over 1 month (8). The protocol also proved successful in the present study and very likely is of general applicability. The opportunity to easily select long-lasting phages expands phage use to the prophylaxis of bacterial infections (27, 30); more importantly, it demonstrates how phages can be manipulated by selection to suit a specific purpose. At the same time, given the complex phage pharmacodynamics (7, 27) and the rapidity with which the persisting phage evolved in this study, we cannot exclude that the wild phage W^{Sa} might have performed equally well in treating infections.

Rapid clearance in the spleen, an inability to kill intracellular bacteria, and stimulation of neutralizing antibodies are the recurrent objections to the use of phages against bacteria (14, 32, 35). As discussed above, M^{Sa} is free of these shortcomings and, in addition, lyses methicillin-resistant strains of *S. aureus*. The evidence that M^{Sa} can lyse only 7 of the 19 *S. aureus* strains tested does not seriously limit its potential use as an antimicrobial agent. In addition to M^{Sa}, we have isolated four more phages, which lyse the remaining 12 strains. More generally, the number of phage species is astonishingly high. Therefore, if a bacterial strain develops resistance or is naturally resistant to a bacteriophage, a new one can be isolated in a few days. In conclusion, given the cost in terms of mortality and morbidity imposed by the widespread presence of multidrug-resistant *S. aureus* strains (19) and the present lack of an effective solution to the problem, investigating the potential of M^{Sa} in the management of human diseases associated with *S. aureus* infections seems a reasonable suggestion.

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ORIGINAL PAPER

Mannose-binding lectin haplotypes influence Brucella abortus infection in the water buffalo (Bubalus bubalis)

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Abstract A case-control study established that the haplotype pair HYA/HYA at the MBL (mannose binding lectin) locus of water buffalo is associated with resistance to Brucella abortus infection ($P<10^{-7}$) and the haplotype pairs LYD/LYD with susceptibility to the same pathogen ($P<10^{-7}$). The subjects included in the present study were tested twice—at a 1-month interval—for the presence of anti-B. abortus antibodies in the serum by agglutination, complement fixation and flow cytometry. Cases (335 subjects) included animals consistently positive to all these tests; controls (335 subjects) comprised animals exposed yet negative by the same tests. The serum from genetically resistant subjects displayed in vitro significantly higher antibacterial activity compared to the serum from genetically susceptible subjects, lending biological significance to the results from the association study. Inhibition of the antibacterial activity following heat treatment of the serum, addition of specific MBL inhibitors (EDTA, mannose, N-acetyl-D-glucosamine) or anti-human MBL antiserum provide convincing evidence that the antibacterial activity present in the serum results from the interaction between MBL and B. abortus. A replication study (comprising 100 cases and 100 controls) confirmed the results from the original study.

Keywords Brucella abortus · Mannose binding lectin · Water buffalo

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Introduction

Water buffalo (Bubalus bubalis) is an economically important resource for the dairy industry in southern Italy, where brucellosis remains the most common disease affecting this livestock species. The disease, caused by Brucella abortus, results in heavy economic losses for the dairy industry (due to culling of infected animals, decreased milk production, and abortions). More importantly, brucellosis constitutes a serious public health risk, with the majority of human infections being caused by the consumption of contaminated milk products. The incidence of human brucellosis is especially high in the Middle East, central Asia, the Balkans, southern Italy, Greece, Spain, Mexico and Peru (Pappas et al. 2006). Reports of Brucella infection of prosthetic implants and devices in humans have increased in the past decade (Dhand and Ross 2007).

Host genetic factors are known to influence B. abortus infection in water buffalo (Borriello et al. 2006; Capparelli et al. 2007). The subjects with the BB genotype at the Nramp1 locus (Nramp1BB) remain seronegative despite

prolonged exposure to the pathogen (Capparelli et al. 2007). The monocytes from Nramp1BB subjects express a higher level of the Nramp1 messenger and thus can control more efficiently bacterial replication during the early stages of infection (Capparelli et al. 2007). Experiments in vitro have shown that the monocytes from Nramp1BB subjects are also resistant to B. melitensis infection (Borriello et al. 2006).

The present report describes the identification in the water buffalo of a second gene conferring resistance to B. abortus infection, the mannose-binding lectin gene (MBL). The mannose-binding lectin (MBL) is an acute phase protein, which binds mannose and N-acetyl-D-glucosamine sugars, expressed on a wide range of pathogens, including gram-positive and gram-negative bacteria, yeast, parasites, and viruses (Takahashi et al. 2005). On binding to its ligands, MBL activates the complement in an antibody-independent manner via MBL-associated serine protease (MASP)-1 and MASP-2. MASP-2 cleaves C4 and C2 (Thiel et al. 1997). Additionally, MBL opsonizes bacteria using the C1q receptor on macrophages without the involvement of complement (Nepomuceno et al. 1997), regulates the inflammatory cytokine release by phagocytic cells (Jack et al. 2001; Kilpatrick 2002), and inhibits virus infectivity (Wakamiya et al. 1992).

In humans, the exon 1 of the MBL2 gene (MBL1 is a pseudogene) contains four alleles (A, B, C, D). Additional polymorphisms have also been described at nucleotide −550 (H, L), −221 (X, Y), and +4 (P, Q) of the promoter (Madsen et al. 1998). In humans, certain MBL genotypes represent a risk factor for bacterial (Koch et al. 2001; Hibberd et al. 1999), fungal (Eisen and Minchinton 2003), and viral (Garred et al. 1997; Thio et al. 2005; Zhang et al. 2005) infections. As a continuation of previous studies (Borriello et al. 2006; Capparelli et al. 2007), the hypothesis that the MBL gene polymorphism influences B. abortus infection was tested in water buffalo. The availability of herds with high incidence of brucellosis and the presence, within each herd, of highly exposed yet noninfected animals represented material particularly suitable for the study of the association between host genetics and B. abortus infection in a natural population. Analysis of 335 cases (subjects which tested positive for the presence of anti B. abortus antibodies in the serum by agglutination, complement fixation and flow cytometry tests) and 335 controls (exposed subjects, which resulted non-infected by the same tests) demonstrated that in water buffalo, the MBL haplotype pair HYA/HYA is associated with resistance to B. abortus infection and the haplotype pair LYD/LYD with susceptibility. These results were confirmed by an independent study conducted on 100 cases and 100 controls.

Materials and methods

Study design

The interaction between the MBL gene and B. abortus was investigated in water buffaloes from four herds located in the province of Caserta (southern Italy), an area where brucellosis is endemic, and genes conferring resistance against the disease are expected to be subject to selection. Accurate diagnosis is fundamental for meaningful results from association studies (Healy 2006; Zondervan et al. 2002). Since no single test is fully specific for brucellosis (Godfroid et al. 2002; Munoz et al. 2005), a variety of tests was used. The subjects were tested twice—at a 1-month interval—for the presence of anti-B. abortus antibodies in the serum by agglutination, complement fixation and flow cytometry). Cases included animals consistently positive to all these tests; controls included animals exposed yet negative by the same tests. The animals included in the study were different from those in previous studies (Borriello et al. 2006; Capparelli et al. 2007). Cases and controls were sampled from the same source population (the four herds mentioned above). Thus, controls had the same opportunity as cases of becoming infected and being included in the study. To exclude confounding effects due to vaccination, sex or age, cases and controls were all unvaccinated lactating cows of 1–10 years age. In addition to the MBL locus, cases and controls were also tested for polymorphism at the Nramp1, and eight microsatellite marker loci (Capparelli et al. 2007). Animals with the Nramp1BB genotype, resistant to B. abortus infection (Capparelli et al. 2007), could have confounded the interpretation of the results and were therefore excluded. MBL genotypes were determined blindly (without knowing the case or control status of the subjects).

Sample size calculation

Preliminary data from 100 cases and 147 controls showed that the frequencies of the water buffalo MBL-O allele were 0.46 and 0.17, respectively. Based on this information, it was calculated that a sample size of 254 cases and 254 controls would provide 90% power to detect an odds ratio (OR) of 2 with a two-sided α of 0.05. To ensure adequate power, 335 cases and 335 controls were enrolled.

MBL genotyping

Genomic DNA was extracted from blood samples with the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Promoter polymorphisms were detected using primers complementary to the bovine MBL gene (accession

Table 1 Primer pairs used for water buffalo MBL promoter genotyping in four PCR reactions

Allele pair	Primer pair				Size (bp)
	B^a+C^b	B^a+D^b	A^a+C^b	A^a+D^b	
HX	+	−	−	−	373
HY	−	+	−	−	373
LX	−	−	+	−	373
LY	−	−	−	+	373

[a] Forward
[b] Reverse

number NM_174107). Primers were designed with the DNASIS software program (Genetic analysis, Hitachi, Olivet Cedex, France). Reactions were performed in 25 µl volume containing approximately 75 ng of genomic DNA, 1.5 mM MgCl2, 0.4 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) in 5 µl 5× reaction buffer (Promega). The thermal profile included one denaturation cycle (3 min at 95°C), 40 amplification cycles (30 s at 95°C, 30 s at 62°C, 60 s at 72°C) and one elongation cycle (5 min at 72°C). PCR products were resolved by electrophoresis in 2% agarose (voltage 90 V for 60 min). The gels were stained with ethidium bromide, and visualized with UV light. Promoter genotypes were determined by the presence or absence of a 373 bp band when using appropriate primer combinations (Table 1). Primer sequences (5′ to 3′) were as follows: primer A, CTTACCCAGGCAAGCCGGTC; primer B, CTTACCC AGGCAAGCCGGTG; primer C, CTGGAAGACTATA AACATGCTGTCG; primer D, CTGGAAGACT ATAAAC ATGCTGTCC). Exon 1 genotypes were detected using primers complementary to the human MBL gene (accession number AL583855). Reactions were carried out as described above, except for the thermal profile, which included one denaturation cycle (2 min at 95°C), 35 amplification cycles (30 s at 95°C, 45 s at 58°C, 45 s at 72°C) and one elongation cycle (5 min at 72°C). PCR products were resolved by electrophoresis in 2% agarose (voltage 90 V for 180 min). Genotypes were determined by the presence of a 128 (alleles D, A), 135 (alleles B, A), or 143 (alleles C, A) bp band when using appropriate primer combinations (Table 2). Primer sequences (5′ to 3′) were as follows: primer E, ACAG CATCTTGTGCAGACAC; primer F, TCTCCCTTGGCAC CATGACA; primer G, TCTCCCTTGGCACCATGACG; primer H, TCCCTTTTCTCCCTTGTCAT; primer I, TCCCTTTTCTCCCTTGTCAC; primer L, CCTGGTTCTC CCTTTGCTT; primer M, CCTGGTTCTCCCTTTGCTC.

Identification of anti-B. abortus antibodies by flow cytometry

B. abortus 2308 was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Preliminary tests demonstrated that paraformaldehyde treatment did not interfere with antibody binding to B. abortus. Bacteria (5×10^6 CFU) and the serum sample to be tested (100 µl serum diluted 10^{-2} with PBS or 100 µl blood stored at −20°C, also diluted 10^{-2} with PBS) were mixed in Eppendorf tubes and incubated overnight at +4°C. Tubes were centrifuged (8,000 rpm for 5 min), washed with PBS, quenched with 2% milk powder (Sigma; 50 µl/tube; 30 min at room temperature) and incubated (2 h) in succession with mouse anti water buffalo immunoglobulin preparation diluted 2×10^{-3} (50 µl/tube) and goat anti-mouse IgG labeled with fluorescein diluted 2×10^{-3} (Sigma; 50 µl/tube). The tubes were washed with PBS and analyzed by flow cytometry. In specificity controls, goat anti-mouse IgG labeled with fluorescein failed to bind in wells without the mouse anti water buffalo immunoglobulin preparation.

Table 2 Primer pairs used for water buffalo MBL exon 1 genotyping in six PCR reactions

Genotype	Primer pair						Size (bp)
	E^a+F^b	E^a+G^b	E^a+H^b	E^a+I^b	E^a+L^b	E^a+M^b	
AA	−	+	−	+	−	+	128/135/143
AB	−	+	+	+	−	+	128/135/143
AC	−	+	−	+	+	+	128/135/143
AD	+	+	−	+	−	+	128/135/143
BB	−	+	+	−	−	+	128/135/143
BC	−	+	+	+	+	+	128/135/143
BD	+	+	+	+	−	+	128/135/143
CC	−	+	−	+	+	−	128/135/143
CD	+	+	−	+	+	+	128/135/143
DD	+	−	−	+	−	+	128/135/143

[a] Forward
[b] Reverse

MBL antibacterial activity

Individual wells of a 96 well plate were filled with B. abortus 2308 (10^5 CFU suspended in 100 µl PBS) and individual serum samples (100 µl diluted 1:2) from resistant (HYA/HYA) or susceptible (LYD/LYD) subjects. The plate was incubated for 24 h at 37°C (5% CO_2). The content of each well was properly diluted (10^{-1} to 10^{-4}) with PBS and plated on tryptose soy agar plates. Each sample was tested in triplicate. Controls (also set up in triplicate) included wells with individual serum samples (100 µl diluted 1:2) pre-treated at 56°C for 30 min to inactivate MBL (Anders et al. 1994).

Specificity of MBL activity

To further demonstrate that the observed antibacterial activity was mediated by MBL, inhibition experiments were carried out on serum samples pretreated with specific MBL inhibitors (10 mM EDTA, 25 mM mannose or 50 mM N-acetyl-D-glucosamine), mouse anti-human MBL antiserum (25 µl diluted 5×10^{-2}) or depleted of MBL by passage through mannan-Sepharose column (Anders et al. 1994). Sugar or antiserum was added to the individual serum samples 30 min prior to the addition of B. abortus.

Detection of microsatellite markers

Subjects included in the association study were all typed for eight microsatellite marker loci. Markers were amplified using the primer pairs listed in (Capparelli et al. 2007). PCR program included an initial step of 10 min at 95°C; 30 cycles of 15 s at 95°C, 1 min at 57°C, 1 min at 72°C; a final extension step of 10 min at 72°C. PCR products were separated by capillary electrophoresis on ABI PRISM 310 analyzer (Applied Biosystems, Foster City, CA). Results were analyzed with GeneScan 3.1.2 and Genotyper 2.5.2 programs (Applied Biosystems).

Other procedures

Agglutination and complement fixation tests were carried out as described (Alton et al. 1975). Odds ratio, 95% confidence interval (CI) of the odds ratio, Fisher's exact test and Student's t test were calculated as described (Motulski 1995). Hardy-Weinberg equilibrium was calculated using the χ^2 test (Cavalli-Sforza and Bodmer 1971) for $k(k-1)/2$ degrees of freedom, where k is the number of alleles (Weir 1996). The influence of MBL haplotypes on milk yield was calculated as described (Capparelli et al. 2007).

Table 3 Genotype distribution of exon 1 water buffalo MBL among brucellosis cases and controls

MBL genotyping	Cases	HWE χ^2	Controls	HWE χ^2	OR	P value
AA	70		159			
AO	145		134			
OO	120	4.4	42	2.6	0.15	$< 10^{-7}$
Total	335		335			

HWE Hardy–Weinberg equilibrium

Results

Identification of water buffalo MBL alleles

PCR analysis identified four alleles in the exon 1 of the MBL gene (A, B, C, D), two alleles (H, L) at position −550 and two more at −221 (X, Y) of the MBL promoter. Because of the known influence of the exon 1 genotype on pathogen opsonization in humans (Hibberd et al. 1999; Kilpatrick 2002; Madsen et al. 1998), the primary analysis investigated whether animals with two copies of the wild allele (AA), with one mutant allele (AB, AC, AD; collectively referred to as AO animals) or with two mutant alleles (BB, CC, DD, BC, BD, CD; collectively referred to as OO animals) were present at different frequencies in cases and controls. The AA animals were more frequent among controls, while the OO animals were more frequent among cases than controls (OR: 0.15; CI: 0.09824–0.2417; $P < 10^{-7}$; Table 3). The frequency of the allele A among controls (0.67) was significantly higher than among cases (0.42; Fig. 1a). These results provided the first line of evidence that the AA genotype conferred resistance against B. abortus infection.

Cases and controls were separately tested for Hardy–Weinberg equilibrium at the MBL locus and eight additional polymorphic microsatellite marker loci. Genotypic frequencies at the MBL locus were in equilibrium among controls,

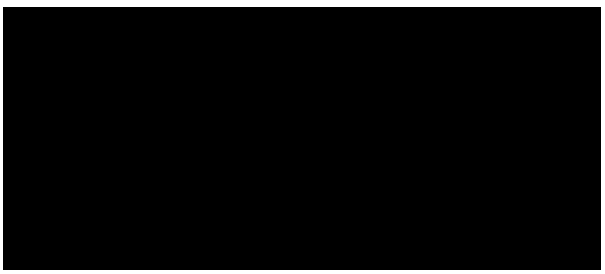

Fig. 1 Water buffalo MBL A allele and MBL HYA haplotype frequencies in brucellosis cases and controls. Values are frequencies ± confidence interval (CI). The frequency (F) of the A allele (a) was calculated using the formula $F=(AA+0.5\times AO)/N$ and values reported in Table 3. The frequency (F) of the HYA haplotype (b) was calculated using the formula $F=(HYA/HYA+0.5\times HYA/-)/N$ and values reported in Table 4. HYA/−: animals with one copy of the haplotype HYA

but not among cases, as expected in case of association between the candidate gene and brucellosis (Table 3). Genotypic frequencies at the marker loci were instead in equilibrium among cases as well as among controls (data not shown), excluding the presence of stratification within the population being studied (Pritchard and Rosenberg 1999).

Effect of water buffalo MBL haplotypes on B. abortus infection

The −550 (H, L) and the −221 (X, Y) promoter alleles are inherited on the same chromosome (in cis) with the structural alleles in the form of haplotypes (Madsen et al. 1998). Haplotypes contain the information relative to the whole set of linked alleles. Case-control studies based on haplotypes are therefore much more powerful and informative, compared with studies based on single nucleotide polymorphisms (Hill 1996; Hoogendoorn et al. 2003; Joosten et al. 2001; Tregouet et al. 2002). Due to the proximity of the (H, L), (X, Y) and exon 1 polymorphisms (Madsen et al. 1998), molecular haplotyping seemed a correct choice. PCR analysis revealed 16 MBL haplotype pairs. Of these, two (HYA/HYA, LYD/LYD), the most frequent, could be confirmed by family data.

When the subjects were classified as having two copies of the HYA haplotype (HYA/HYA), one (HYA/−) or none (−/−), the HYA/HYA subjects were represented only among controls: HYA/HYA subjects were 0.1% as likely as −/− subjects to be positive to the B. abortus (OR: 0.001; CI: 0.0006–0.01847; $P<10^{-7}$; Table 4). The P value ($P<10^{-7}$) places the study in the first-class of the Better Associations for Disease and Genes (BADGE) system (Manly 2005). First-class associations are expected to be reproducible even in the absence of other evidence supporting the association (Manly 2005). The frequency of the HYA haplotype among controls (0.57) was also significantly higher than among cases (0.1; Fig. 1b).

The above results—an OR=0.009 characterizing the association between the HYA/HYA haplotype pair and brucellosis (Table 4) versus an OR=0.15 characterizing the association between the AA genotype and the same disease (Table 3)—highlight the importance of studying haplotypes rather than single alleles (Hill 1996; Hoogendoorn et al. 2003; Joosten et al. 2001; Tregouet et al. 2002).

Table 4 Distribution of the water buffalo MBL haplotype HYA among brucellosis cases and controls

Haplotype pair	Cases	HWE χ^2	Controls	HWE χ^2	OR	P value
HYA/HYA	0		115			
HYA/−	70		151			
−/−	265	4.6	69	2.2	0.001	$< 10^{-7}$
Total	335		335			

HWE Hardy–Weinberg equilibrium

Table 5 Distribution of the water buffalo MBL LYD haplotype among brucellosis cases and controls

Haplotype pair	Cases	HWE χ^2	Controls	HWE χ^2	OR	P values
LYD/LYD	49		2			
LYD/−	131		26			
−/−	155	5.7	307	2.9	9.9	$< 10^{-7}$
Total	335		335			

HWE Hardy–Weinberg equilibrium

The LYD/LYD subjects were instead more frequent among cases. They were approximately 10 times more likely to be positive to the B. abortus tests than −/− subjects (OR=9.9; CI: 6.280− 15.857; $P<10^{-7}$; Table 5). The remaining haplotype pairs, much rarer, were not analyzed for association with the disease. In conclusion, the HYA/HYA haplotype pair is associated with resistance against B. abortus infection and the LYD/LYD haplotype pair with predisposition to the infection by the same pathogen.

Both the promoter and exon 1 alleles of the human MBL gene differ from each other in one single base substitution (Garred 2003). If this holds also for the water buffalo MBL gene, then remarkably, the ability of a haplotype to promote protection or susceptibility depends upon one single nucleotide change, as in the case of the haplotype pairs HYA/HYA versus HYA/ HYD, HYA/HYA versus LYA/LYA, or HYA/HYA versus HXA/HXA.

Reproducibility of the association

A central problem of the association studies is lack of replication of the results (Ioannidis et al. 2001; Lohmuller et al. 2003). Therefore, the study was repeated on an independent population sample of 100 cases and as many controls. This new study confirmed results from the previous one (Tables 6 and 7).

Table 6 Replication study

Haplotype pair	Cases	HWE χ^2	Controls	HWE χ^2	OR	P
HYA/HYA	0		36			
HYA/−	36		40			
−/−	64	4.6	24	3.5	0.0058	$< 10^{-7}$
Total	100		100			

Distribution of the water buffalo MBL HYA haplotype among brucellosis cases and controls.
HWE Hardy–Weinberg equilibrium

Table 7 Replication study

Haplotype pair	Cases	HWE χ^2	Controls	HWE χ^2	OR	P value
LYD/LYD	8		0			
LYD/−	72		10			
−/−	20	21	90	0.28	75	10^{-7}
Total	100		100			

Distribution of the water buffalo MBL LYD haplotype among brucellosis cases and controls.
HWE Hardy–Weinberg equilibrium

Selection against brucellosis increases the frequency of HYA/HYA subjects

The availability of stored blood samples provided the possibility to establish the frequency of the HYA/HYA animals present in one herd, before and after 3 years of systematic culling of the animals positive to the agglutination test for brucellosis carried out by the owner of the herd. During this time interval, the frequency of HYA/HYA animals increased from 14% (8/57) to 34% (19/56). This result confirms the role of this haplotype pair in conferring resistance to brucellosis, at least in this herd.

Biological plausibility of the association

One of the functions of the MBL protein is pathogen opsonization and phagocytosis (directly and by activating the antibody-independent pathway of the complement; Takahashi et al. 2005). To probe a possible difference in the biological activity of the protein between resistant (HYA/HYA) and susceptible (LYD/LYD or LYA/LYD) subjects, the B. abortus strain 2308 (10^5 CFU) was incubated in vitro in the presence of heat-inactivated (control) and noninactivated (activity) autologous serum (100 µl 50% diluted) from 15 water buffaloes of each group. For each animal, two blood samples collected at 2-week intervals were tested, each time in triplicate. Following incubation at

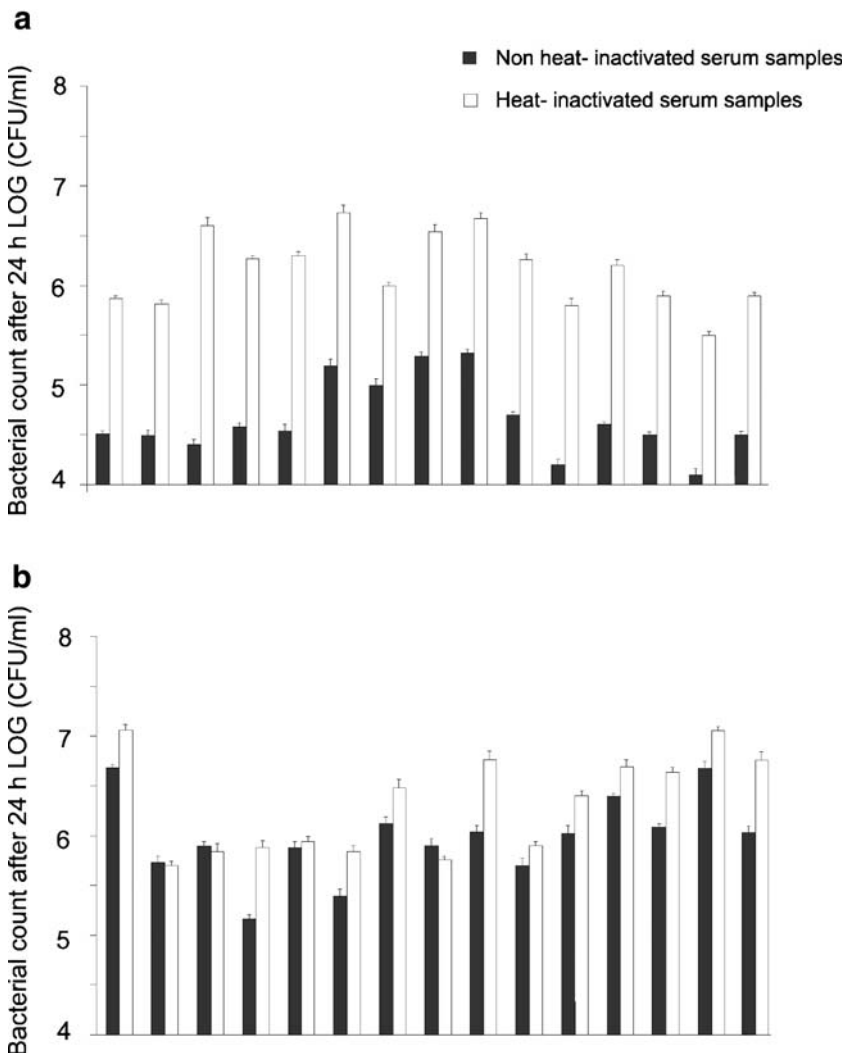

Fig. 2 Antibacterial activity displayed by resistant (HYA/HYA) and susceptible (LYD/LYD) subjects. The difference in CFU/ml between non-heat-inactivated and heat-inactivated (56°C for 30 min) serum samples was highly significant ($P < 0.0035$) in the case of resistant (a), but not of susceptible (b) subjects. Each histogram represents average ± standard deviation from two serum samples, each tested in triplicate

37° for 24 h, the content of each well was plated to count CFU. The serum from HYA/HYA animals displayed a significantly higher antibacterial activity compared with serum from LYD/LYD animals (Fig. 2). Approximately the same results were observed with B. melitensis (data not shown). The evidence that heat inactivation destroys the antibacterial activity of the serum (Fig. 2) suggests that this activity is indeed mediated by MBL, a heat-labile lectin (Anders et al. 1994). Involvement of the MBL in the antibacterial activity was indicated also by the following observations. The antibacterial activity was inhibited by pre-incubation of the serum with 10 mM EDTA, 25 mM mannose or 50 mM N-acetyl-D-glucosamine (Fig. 3), known to specifically inhibit MBL (Anders et al. 1994). Most importantly, the antibacterial activity was inhibited in the presence of mouse anti-human MBL or after passage of the serum through mannan-Sepharose column (Fig. 3). The evidence that genetic analysis and functional (serum antibacterial activity) analysis yield fully concordant results (Figs. 2 and 3) lends biological significance to the protective role of the HYA/HYA genotype against B. abortus infection and the predisposing role of the LYD/LYD genotype.

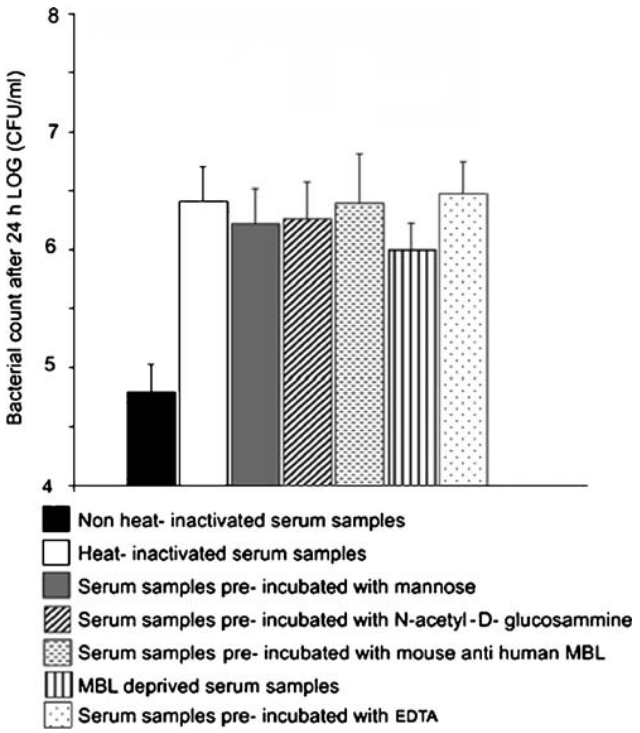

Fig. 3 Neutralization of serum antibacterial activity from resistant (HYA/HYA) subjects by specific inhibition of MBL. The differences in CFU/ml between treated and untreated serum samples were highly significant ($P<0.0001$). Treatment of serum samples included: heat inactivation at 56°C for 30 min, addition of 25 mM mannose, 50 mM N-acetyl-D-glucosamine, 10 mM EDTA, mouse anti-human MBL (25 µl diluted 5×10^{-2}) or MBL depletion (by passage of serum samples over mannan-Sepharose column). Histograms represent the average ± standard deviation from 15 HYA/HYA individual serum samples

MBL haplotypes and milk yield

No difference was found in milk yield (the most important production trait for water buffalo breeders) between resistant and susceptible cows ($t_{0.05}$ 0.10; degrees of freedom, 22; $P=0.92$).

Discussion

In the water buffalo, the distribution of genotypes by disease status showed a pattern of increased prevalence of HYA/HYA subjects among controls ($P<10^{-7}$; Table 4). By contrast, the LYD/LYD ($P=10^{-7}$; Table 5) subjects displayed a pattern of increased prevalence among cases. The evidence that resistant (HYA/HYA) water buffaloes, exposed to the pathogen, remain seronegative (by the agglutination, complement fixation and flow cytometry tests) suggests that this MBL haplotype pair prevents B. abortus infection by acting rapidly, before the immune system of the host detects the presence of the pathogen.

Studies of the human MBL gene have clarified the chemical relationship between single point mutations occurring in this gene and the function of the controlled protein. In particular, it has been shown that single base substitutions caused by mutant alleles interfere with the correct folding of the three basic subunits of the MBL molecule. If this protein contains one defective chain, it becomes prone to degradation or unable to activate the complement (Garred et al. 2003). On the basis of this evidence, we propose that, as observed in humans, resistant water buffaloes have fully functional MBL protein capable of combating B. abortus infection effectively; susceptible animals, on the contrary, have partially inactive MBL protein, unable to carry out rapid opsonization of the pathogen.

Brucellosis is imposing strong selection on the water buffalo MBL locus, at least in the population examined in the present study. Why then do haplotypes conferring susceptibility to B. abortus persist in the population? One explanation is that harmful haplotypes are maintained by compensating advantages (Carrington and O'Brien 2003; Rotter and Diamond 1987). There is evidence that low human MBL levels decrease complement activation and protect the host from excessive tissue damage (Takahashi et al. 2005; Garred et al. 2006). These studies provide support to speculate that mutant MBL alleles may be maintained in the water buffalo population because of their role in modulating inflammation.

Case-control studies have a long record of false-positive results (Ioannidis et al. 2001; Lohmuller et al. 2003).

Confirmation of the association between the HYA/HYA haplotype pairs and resistance to B. abortus using an independent group of animals seemed therefore very important. A second independent study confirmed the initial findings (Tables 6 and 7). The fact that the results were confirmed by a relatively small-sized (and consequently low-powered) study is particularly convincing of the strength of the association.

False-positive results often derive from the very low prior probability that the association between the genetic variant and the disease being studied is real (Wacholder et al. 2004). We tried therefore to verify experimentally the biological plausibility of the correlation between MBL haplotypes and resistance to B. abortus infection. The serum from resistant water buffaloes displayed in vitro much higher bactericidal activity compared to the serum from susceptible ones (Fig. 2). Thus, the study, to the statistical significance of the epidemiological data and reproducibility of the results, adds also the biological relevance of the gene studied. Use of haplotypes with a strong causal relationship to the trait being studied is the characteristic common to a long series of successful (repeatedly confirmed) case-control studies (Risch 2000). In addition, MBL haplotypes comprise polymorphic sites in the coding and the promoter regions, the latter involved in initiating transcription and regulating gene expression. MBL haplotypes therefore are a priori likely to exert a greater effect on the trait, compared to single nucleotides, elevating the probability of a meaning association between the MBL gene and brucellosis in water buffalo.

In conclusion, the association reported here appears sufficiently reliable. This conclusion is based on several characteristics of the study: reproducibility, biological relevance of the candidate gene, statistical power (see "Sample size calculation" section), statistical significance (placing the study in the first class of the BADGE system), careful control of confounding effects (see "Study design" section).

We would like to mention possible caveats of the present study. First, the MBL protein is known to interact with numerous pathogens (Eisen and Minchinton 2003; Koch et al. 2001; Zhang et al. 2005). We therefore cannot formally exclude that the increased frequency of HYA/HYA subjects described here might have been driven in part by other pathogens. Second, in the present study, the HYA/HYA subjects were all invariably resistant to B. abortus infection. However, we would like to point out that we are not excluding that genetically resistant and B. abortus susceptible animals might sporadically be encountered in the future. In humans, the hemoglobin S gene and the α^{+}-thalassemia gene protect from malaria when inherited alone, but the protection is lost when the two genes are inherited together (Williams et al. 2002). In general, innate immunity genes with a one-to-one correspondence between genotype and phenotype certainly exist, but the genes influenced in their expression by other genes perhaps represent the majority. Immune response genes require very tight control, and the delicate balance between pathogen control and tissue damage in fact is often the result of complex gene interactions (Dyment et al. 2005; Gregersen et al. 2006). We must also not lose sight of nongenetic influences. HLA-mediated protection against HIV is not effective when infection occurs through breastfeeding (Carrington and O'Brien 2003). Analogously, the immunity provided by the MBL haplotype pair HYA/HYA might be insufficient to prevent infection if the host is exposed to the pathogen through an unconventional route.

In conclusion, the results reported here illustrate how the option to control animal diseases by genetic selection is realistic. With regard to water buffalo brucellosis, the positive selection of resistant subjects and the concurrent negative selection of the susceptible ones could rapidly increase the level of herd immunity. The relatively high initial frequency of the resistant animals would further facilitate this process.

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ORIGINAL ARTICLE

Heterogeneous shedding of Brucella abortus in milk and its effect on the control of animal brucellosis

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Abstract

Aims: To ascertain whether in Brucella abortus-infected water buffalo herds, the number of newly infected animals could be reduced by culling superspreaders (the animals secreting ‡10^4 CFU per ml of milk).

Methods and Results: The number of B. abortus present in the milk (CFU per ml) from 500 water buffaloes was measured by the culture. Each animal was tested three times, at one month intervals. The presence or the absence of B. abortus in each milk sample was confirmed by PCR. A majority of infected animals shed the pathogen at a low level (£10^3 CFU ml^{-1}). However, a few infected individuals (superspreaders) shed large numbers of B. abortus (‡10^4 CFU ml^{-1}). Quantitative PCR of B. abortus positive milk samples gave comparable results to culture. Culling of the superspreaders was sufficient to arrest the spread of infection.

Significance and Impact of the Study: The approach described here can reduce significantly the cost of controlling brucellosis. Culture and quantitative PCR tests identify superspreaders and, compared with the serological tests in use to detect brucellosis, provide also a more accurate estimate of the disease incidence.

Introduction

The incidence of human brucellosis is especially high in the Middle East, central Asia and several countries in the Mediterranean (Spain, Greece, southern Italy) and Latin America (Argentina, Mexico, Peru) (Pappas et al. 2006). The World Health Organization estimates the number of new cases of human brucellosis at more than 500 000 per year (Queipo-Ortuno et al. 1997). Moreover, cases of Brucella infection of prosthetic implants and devices in humans have increased substantially in the past decade (Dhand and Ross 2007). In the south of Italy, brucellosis is endemic in the water buffalo (Bubalus bubalis) (Anon 2007). This condition represents a risk of disease spread among humans (Boschiroli et al. 2001; Anon 2007).

The recent severe acute respiratory syndrome (SARS) pandemic was characterized by the phenomenon of 'superspreading', whereby a few patients infected a large number of people, while a majority of the patients infected a few people or none at all (Lipsitch et al. 2003). Subsequent studies demonstrated that the presence of superspreaders was not a peculiarity of SARS, but rather a characteristic of most infectious diseases, whether vector-borne (malaria), sexually transmitted (AIDS) or transmitted by direct nonsexual contact (SARS, smallpox or measles) (Galvani and May 2005; Lloyd-Smith et al. 2005). Convincing evidence has been provided that identification and isolation of superspreaders can help bring the epidemic under control (Woolhouse et al. 1997; Galvani and May 2005; Lloyd-Smith et al. 2005; Matthews et al. 2006). This study shows that culling of the relatively few superspreaders present in brucella-infected water buffalo herds can reduce disease transmission.

Materials and methods

Study design

The heterogeneous shedding of brucellae in milk was investigated in 500 water buffaloes from four herds (125 animals from each herd). Herds were selected on the basis of their location [all in the province of Caserta, southern Italy, an area where brucellosis is endemic (Anon 2007)], their size (300–400 lactating cows per herd) and comparable management (diet and bedding). The owners agreed that no new introduction or removal of animals into the herd would occur 2 months prior to the study or during the study. Within each herd, selection of the animals aimed to exclude confounding effects due to innate resistance to B. abortus, vaccination, sex or age. Therefore, unvaccinated lactating cows of 4–10 years of age were tested at the Nramp1 (natural resistance-associated macrophage protein 1) and MBL (mannose-binding lectin) loci, as described (Borriello et al. 2006; Capparelli et al. 2007, 2008). In each herd, the 125 individuals first identified as genetically nonresistant to B. abortus infection (without the NRAMP1-BB genotype or the MBL-HYA/HYA haplotype pair) were included in the study. Shedding of brucellae in milk can be intermittent. Therefore, the 500 individuals were tested for the presence of brucellae in the milk three times – at 1-month intervals – by the culture test and by PCR. During the first visit to the farms, along with milk samples, blood samples were also collected, which were tested for the presence of anti-brucella antibodies by agglutination, complement fixation and flow cytometry. The number of bacteria secreted in milk samples was also measured, by the culture test and by quantitative PCR (qPCR). Both techniques allowed classifying the animals as low shedders (secreting £10^3 CFU ml^{-1}) or high shedders (secreting ‡10^4 CFU ml^{-1}). Finally, to ascertain whether culling high shedders would hold in check the spread of infection, these animals were culled in three of the herds and kept in the remaining one. The control herd was chosen blindly among the herds.

Bacterial culture

Milk samples were collected after washing and drying the teats of the animals. Each sample consisted of approximately 20 ml of milk from each quarter of the mammary gland. Samples were enriched by overnight incubation at 37 C in air supplemented with 10% CO_2 and then centrifuged at 2000 g for 15 min. The cream and deposit were diluted (10^{-1}–10^{-3}) in H_2O and finally seeded (100 **1**l) separately on duplicate agar plates containing Brucella medium base (Oxoid, Milan, Italy) supplemented with antibiotics (polymixin B, bacitracin, nalidixic acid, nistatin, vancomycin) and 5% sterile horse serum. The plates were incubated for 10 days at 37 C in 10% CO_2 atmosphere. To establish the detection limit of the test, a B. abortus 2308 stock suspension was prepared to contain 10^8 CFU ml^{-1} by the direct plate count technique. Dilutions of the stock suspension were spiked (100 **1**l) in triplicate into 20 ml aliquots of a pool of milk samples found absent from B. abortus by the culture and PCR tests. After an equilibration time (to allow incorporation of spikes into the matrix), the samples were seeded on duplicate agar plates, incubated (as described above) and the number of CFU per plate recorded. The detection limit of the culture-based test was 30 CFU ml^{-1} of milk. Each test included a quality control to confirm that the culture medium supported the growth of B. abortus 2308 at the concentration of 30 CFU ml^{-1} of milk (the detection limit of the test). In the event of contamination (which occurred in about 5% of the samples), a new milk sample was collected from the same animal and tested again.

Detection of Brucella abortus by PCR

DNA was extracted from individual milk samples as described (Leal-Klevezas et al. 1995). The concentration and purity of DNA were determined spectrophotometrically by reading at A_{260} and A_{280}. The PCR target was a 223 bp genus-specific sequence common to all B. abortus biovars (Queipo-Ortuno et al. 1997). The 25 **1**l reaction mixture contained 1 **1**l DNA template, 5 **1**l GoTaq buffer (Promega), 4 mmol l^{-1} $MgCl_2$ solution, 200 **1**mol l^{-1} PCR nucleotide mix (Promega), 400 nmol l^{-1} reverse (CGCGCTTGCC TTTCAGGTCTG) and forward (TGGCTCGGTTGCCAATATCAA) primers, 1 U GoTaq DNA polymerase (Promega, Milan, Italy). The PCR profile was set as follows: 3 min at 95 C; 15 s at 95 C, 15 s at 60 C, 15 s 72 C for a total of 40 cycles; 7 min at 72 C. The detection limit of the test – 15 CFU ml^{-1} of milk was established by testing the DNA extracted from the same spiked samples described above. Each sample was tested in duplicate and each test included a negative control (containing all the reagents, but lacking template DNA) and a positive control (containing all the reagents and B. abortus DNA at 15 CFU ml^{-1}, in place of template DNA). It was also established that the assay does not amplify the DNAs from field isolates of Salmonella serovar Typhimurium, Salmonella serovar Typhi, Yersinia enterocolitica O9, Escherichia coli O157:H7, Staphylococcus aureus or Streptococcus agalactiae).

Isolate confirmation

Five typical Brucella-like colonies (small, convex, smooth, translucent) were randomly selected from each plate and subcultured for PCR confirmation.

Detection of brucellae by quantitative PCR

The brucellae present in milk samples were therefore quantified by SYBR Green PCR Master Mix (Invitrogen, Milan, Italy). DNA was extracted from individual milk samples as already described (see above). Primers were those used for Brucella-specific PCR (see above). Optimal primer concentration was established by preliminary experiments. Each reaction mixture consisted of 2 μl of DNA, 0·3 μl (250 μm l^{-1}) of each primer, 3·2 μl of nuclease-free water and 6·2 μl of SYBR Green PCR Master Mix in a total volume of 12 μl. The reactions were performed in duplicate for each sample using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR protocol included: a preliminary step of 2 min at 50 C; a denaturation step of 2 min at 95 C; 40 cycles of 15 s at 95 C, 30 s at 60 C, 30 s at 72 C. Dissociation curve analysis of amplified DNA was carried out at temperatures between 56 and 95 C. A standard curve was constructed using DNA extracted from spiked milk samples (see above).

Detection of anti-brucella antibodies

The serological status of the animals was detected by the Rose Bengal test (RBT), complement fixation tests (CFT) (Blasco et al. 1994) – the official tests used in the European Union countries (Blasco et al. 1994) – and by flow cytometry. Identification of anti-brucella antibodies by flow cytometry was carried out as follows. Brucella abortus 2308 was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. Preliminary tests demonstrated that paraformaldehyde treatment did not interfere with antibody binding to B. abortus. Bacteria ($5 \cdot 10^6$ CFU) and the serum sample to be tested (100 μl serum diluted one in 100 with PBS or 100 μl blood stored at −20 C also diluted one in 100 with PBS) were mixed in Eppendorf tubes and incubated overnight at +4 C. Tubes were centrifuged (8000 g for 5 min), washed with PBS, quenched with 2% milk powder (Sigma, Milan, Italy; 50 μl per tube; 30 min at room temperature) and incubated (2 h) with fluorescein-labelled mouse anti water buffalo IgG diluted $2 \cdot 10^{-3}$ (50 μl per tube) and analysed on a Coulter flow cytometer (Coulter, Miami, FL, USA).

Statistical analysis

Data from the culling experiment were evaluated by the Fisher's two-tailed exact test. The analysis was carried out using GraphPad software (la Jolla, CA, USA).

Results

Heterogeneous shedding of brucellae in milk

Quantitative detection of brucellae in the milk from the 500 animals in the study was first carried out by the culture test. The presence or absence of Brucella organisms in the 500 individuals was then confirmed by PCR. Animals were tested three times at 1-month intervals. The culture and the PCR tests gave fully concordant results. This finding is not surprising, given the close detection limits of the two assays (30 and 15 CFU ml^{-1} of milk respectively). Of the 500 individuals, 399 (80%) were consistently nonshedders; 73 (14·6%) were consistently low shedders (with brucellae counts £10^3 CFU ml^{-1} of milk); 12 (2·4%) animals were intermittently low shedders (Table 1).

Most of the shedding animals (85 out of 101) (84%) were low shedders, while only a small minority (16 out of 101) (15·8%) were high shedders or superspreaders (with

Table 1 Results from bacterial culture and serological tests in each herd

	Culture test (no.)				Serological test* (no.)					
	Shedders				CFT		RBT		FCT	
Herd	Constant low	Intermittent low	High	NonShedders	+	−	+	−	+	−
A	14	3	4	108	70	55	78	47	70	55
B	20	2	4	100	80	45	84	41	78	47
C	17	4	5	99	87	38	90	35	80	45
D	22	3	3	92	83	42	85	40	74	51
Total	73	12	16	399	320	180	337	163	302	198

*CFT, complement fixation test; RBT, Rose Bengal test; FCT, flow cytometry test.

Table 2 Longitudinal distribution of low shedding (£10^3 CFM ml^{-1} of milk) and high shedding (‡10^4 CFU ml^{-1} of milk) water buffaloes during a 2-month interval

Status of animals	Sample order (No. samples)		
	First	Second	Third
Negative	411	399	403
Low-shedding	73	85	81
High-shedding	16	16	16
Total	500	500	500

brucellae counts ‡10^4 CFU ml^{-1}) (Table 1). The thresholds distinguishing low shedders from high shedders (<10^3 CFU ml^{-1} vs >10^4 CFU ml^{-1}) correspond to the highest and the lowest counts detected among low and high shedders respectively. Over the 2-month period, the number of the low shedder animals changed from 73 to 85 and again to 81 (Table 2). The number of the high shedders instead remained constant (Table 2). The same phenomenon was observed among cows shedding high numbers of E. coli 0157 (Matthews et al. 2006). Milk samples from low shedders displayed considerable variation in concentration of brucellae. Milk samples from high shedders instead displayed persistent shedding of bacteria at high level (‡10^4 CFU ml^{-1}).

Selective culling of high shedding animals

High shedding animals were culled in herds termed B, C and D, while being kept in herd A (Table 3). In the absence of high shedding subjects, during a 3-month observation period, only 4 out of the 291 negative animals became positive to the bacteriological test in herds B, C and D; in the control herd A, 7 out of 108 negative animals became positive. Statistical analysis established that the incidence of newly infected animals observed in the control herd A, compared with the incidence in herds B, C and D, is statistically significant (Fisher's two-tailed exact test: P = 0·0136). Selective culling of the high shedding animals is thus effective in reducing the transmission of the infection. Of the seven newly infected animals in herd A, one was a superspreader (Table 3).

Comparison of the bacteriological and serological tests

To compare the performance of the serological tests with that of the gold standard, milk and blood samples were collected on the first visit to the farms. Milk samples were tested by the culture and PCR tests; blood samples were tested by CFT, RBT and flow cytometry. Sensitivity (% true positives) of the serological tests was quite satisfactory (100%). The individuals positive to the culture and PCR tests were in fact all positive also by each of the three serological tests. However, the specificity of the same tests (% of true negatives) varied from 40% to 48% (Table 4). This last result means that 52–60% of the animals were infected according to one or another serological test, but noninfected according to the culture test (the gold standard) and the PCR test. One must note, however, that the specificity of the serological tests would be slightly higher (from 41% to 50%), if it was calculated on the samples collected during the second visit to the farms (rather than the first), when 12 animals originally diagnosed negative to the culture and PCR tests subsequently became positive to these tests (Table 2).

Identification of high and low shedding animals by quantitative PCR

The culture test requires long incubation time (due to the slow growth of the bacteria), is susceptible to overgrowth of contaminating bacteria (due to lack of satisfactory selective media) and exposes laboratory personnel to

Table 3 Culling experiment: results and characteristics of the herds

Lactating cows (no.)											
					Shedders				Newly emerged shedders		
Herd	No of heads	Average age (years)	Parturitions	Abortions	Low	High	High	Culled	Low	High	P*
A	300	6·4	216	4	17	4	108	0	6	1	0·0136
B	380	6·2	266	2	22	4	100	4	3	0	
C	400	5·9	276	3	21	5	99	5	1	0	
D	400	6·6	284	5	25	3	92	3	0	0	

*This is the P value of Fisher's two-tailed exact test.
Causes of abortions unknown.

Table 4 Performance of complement fixation test (CFT), Rose Bengal test (RBT) and flow cytometric test (FCT), compared to that of the culture test (CBT) as gold standard

	Animals (no.)		
Test*	Positive	Negative	Total
CFT	320	180	500
RBT	337	163	500
FCT	302	198	500
CBT	89	411	500

Specificity (% true negative animals) of CFT, RBT, FCT, compared with that of CBT: 44%, 40% and 48% respectively.

Positive predictive value (the proportion of animals with a positive test result that are correctly diagnosed) of CFT, RBT and FCT, compared to that of CBT: 22%, 21% and 23% respectively.

*Results refer to blood and milk samples collected at the time of the first visit to the farms.

infection. To evaluate the possibility of using qPCR assay for the quantitative detection of B. abortus in natural samples, milk samples from all animals positive at any of the three times were tested by the qPCR and the results compared with those from the culture test. There were no significant differences between the numbers counted by the two methods and classification of the animals into spreaders or superspreaders by qPCR was unambiguous (Fig. 1).

Discussion

As is well known, B. abortus can be shed in the milk of infected animals intermittingly, in cattle (Sreevatsan et al. 2000) and other species (Blasco et al. 1994). This study supports this result and, in addition, shows that in the water buffalo, while the majority of the animals shed B. abortus at low levels (£10^3 CFU ml^{-1}), a small number of individuals shed B. abortus persistently and at high levels (‡10^4 CFU ml^{-1}) (Table 1). What determines the shedding level of B. abortus in the single animal is not yet known. Age, genetics, diet and management are factors possibly influencing high shedding of E. coli O157 in cattle (Matthews et al. 2006). As in this study the animals

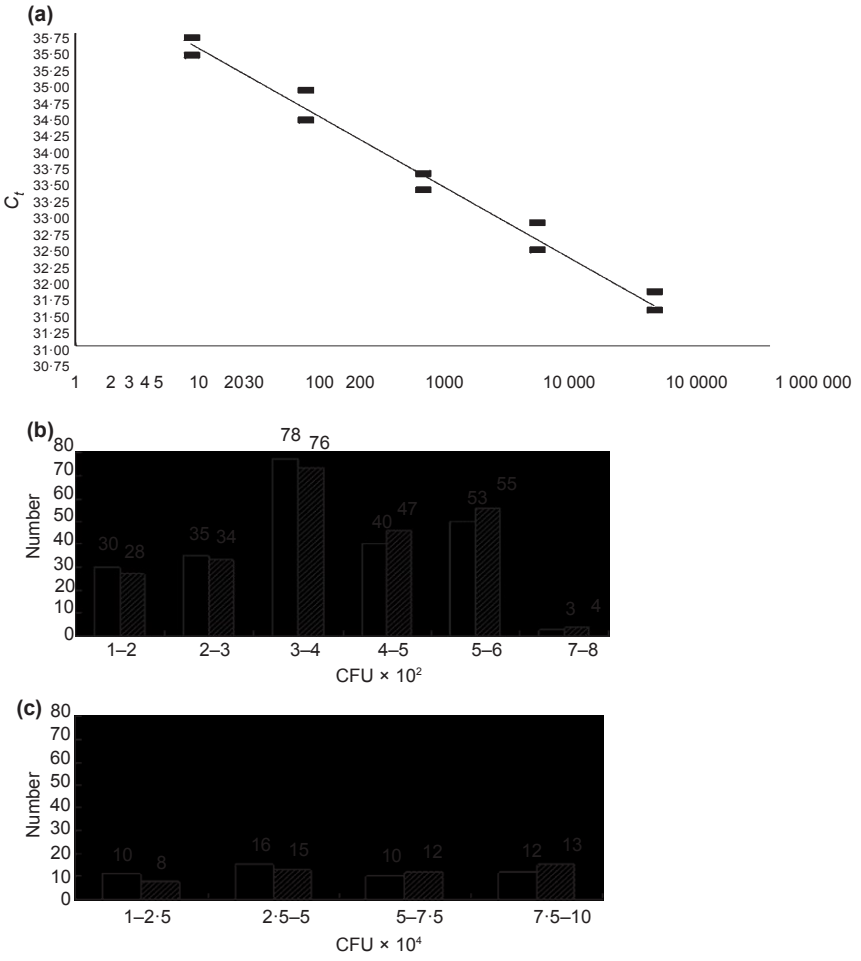

Figure 1 Detection of shedders and supershedders by qPCR. (a) Serial ten-fold dilutions of template DNA extracted from water buffalo milk spiked with B. abortus and tested in duplicate (r^2: 0!926). (b) Comparison of qPCR (□) and culture tests (▨) of shedders and (c) supershedders. C_t is the threshold cycle.

were kept in a relatively constant environment (diet, housing, farm hands and pen mates), it is plausible that the observed heterogeneity in B. abortus shedding might reflect in part genetic differences among the animals. This would open the possibility of using preventive culling of superspreaders to control of animal brucellosis.

Longitudinal follow up of the 500 animals in this study, showed that culling of super shedders is sufficient to arrest the transmission of the disease (P = 0·0136; Table 3). Practical issues (in particular, the cost of culling high shedding animals) limited the size of this study. The data presented here are therefore preliminary and need to be confirmed by studying a larger number of animals over a longer observation period. Still, they build on numerous studies demonstrating the generality of individual variation in infectiousness and its relevance to efficient control of diseases (Woolhouse et al. 1997; Galvani and May 2005; Lloyd-Smith et al. 2005; Matthews et al. 2006). In particular, the results presented in this study recall very closely the shedding characteristics of E. coli O157 in cattle (Omisakin et al. 2003; Robinson et al. 2004; Matthews et al. 2006), where risk assessment simulation has demonstrated that the animals shedding E. coli O157 at a high level, although a minority, are responsible for maintaining the pool of E. coli O157 on the farms (Cassin et al. 1998) and the majority of human infections (Strachan et al. 2002).

Identification of supershedders is often difficult (Galvani and May 2005). Brucellosis is a fortunate exception as supershedders can be easily identified by the culture test. This test has long been known as the gold standard for the diagnosis of brucellosis (Blasco et al. 1994; Dobson and Meagher 1996). However, the culture test has limitations (mentioned in the previous section). To obviate these limitations, we developed (Fig. 1) an SYBR Green 1 real-time PCR (qPCR). This assay, which has already provided fast, sensitive and quantitative detection of several pathogens (Zhou et al. 2007; Ohtsuki et al. 2008), is expected to encourage other people to experiment with selective culling as a new approach to the control of animal brucellosis, an approach with the potential of effectively reducing the cost needed to control this disease. In addition, although our data refer specifically to water buffalo brucellosis in the south of Italy, they are highly likely to be relevant to other geographical areas and to the same disease in other hosts.

Our data show that culture and qPCR tests, compared with serological tests, are more specific. As diagnosis of animal brucellosis at present is based mainly on serological tests, our results need some discussion. According to a recent review, false positive serological reactions represent a major problem that veterinary authorities must govern (Godfroid et al. 2005). False positive reactions result from the presence in test sera of antibodies against organisms cross-reacting with B. abortus (Godfroid et al. 2002; Gall and Nielsen 2004) or from exposure, but not infection of the animal to the pathogen. Exposure does not necessarily imply that the animals are infected (Blasco et al. 1994; Dobson and Meagher 1996; Sreevatsan et al. 2000; Godfroid et al. 2002). There is in fact evidence that animals can clear the infection (Harmon et al. 1989; Price et al. 1990; Sreevatsan et al. 2000; Godfroid et al. 2002). It could be argued that our data contain a large number of chronically infected animals, among which false culture-negative individuals occur frequently (Cheville et al. 1998; Sreevatsan et al. 2000). This possibility, however, is not realistic because the herds included in the present study are monitored periodically by the veterinary service. More plausible is the alternative explanation that the low specificity of the serological tests observed in this study reflects the high number of false-positive reactions. The concordance between results from cultural and PCR tests reported here and the results from numerous studies (Blasco et al. 1994; Dobson and Meagher 1996; Sreevatsan et al. 2000) supports this contention. Notably, the rate of seropositive animals reported here, 2·0 to 2·5 times those positive to the cultural test (Table 4), is reasonably close (3·0 times) to that reported studying brucellosis in the bison (Dobson and Meagher 1996). In conclusion, serological methods currently used for the diagnosis of animal brucellosis are nonspecific; the culture and qPCR tests instead allow identification and culling of superspreaders (the animals apparently responsible for most of B. abortus transmission) and provide also a more realistic estimate of the incidence of brucellosis.

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Bacteriophage Therapy of *Salmonella enterica*: A Fresh Appraisal of Bacteriophage Therapy

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Background. The most serious criticisms leveled at bacteriophage therapy are as follows: phages induce neutralizing antibodies, phages are active only when administered shortly after bacterial infection, and phage-resistant bacteria emerge rapidly in the course of therapy.

Methods. Phages lytic for several *Salmonella enterica* serovars were isolated by means of standard protocols from feces of patients with gastroenteritis. Growth of *S. enterica* serovar Paratyphi B (Salp572$^{\phi S}$) in the presence of phage ϕ1 (selected from among 8 phages for its larger host range) provided a phage ϕ1–resistant bacterial strain (Salp572$^{\phi R}$). The properties of the Salp572$^{\phi S}$ and Salp572$^{\phi R}$ strains and of phage ϕ1 were studied in a mouse model of experimental infection.

Results. Phages induced nonneutralizing antibodies and were active 2 weeks after experimental infection of mice; phage-resistant bacteria were avirulent and short lived in vivo. More importantly, phage-resistant bacteria were excellent vaccines, protecting against lethal doses of heterologous *S. enterica* serovars.

Conclusions. Phage therapy effectiveness has not yet been properly assessed.

Salmonella enterica lives in the gastrointestinal (GI) tracts of mammals, birds, and reptiles and can survive for a long time in water or soil or within foods. Most human cases of salmonellosis are caused by ingestion of food (primarily animal food but also fruits and vegetables) contaminated with animal feces. Clinical manifestations of human salmonellosis range from subclinical gastroenteritis to severe bacteremia, meningitis, and other forms of extraintestinal infections [1]. The more than 2,300 known serovars of *S. enterica* [2] in fact display great differences in virulence [3]. Because of the widespread presence of antibiotic resistance among *S. enterica* isolates [1], fluoroquinolone and third-generation cephalosporins are the only antibiotics available for the treatment of clinically severe forms of *S. enterica* infection [1], hence the great need for antimicrobial alternatives to antibiotics. This study explores the potential of bacteriophage therapy against *S. enterica* serovars.

Originally, the phage specificity for a single bacterial species or serovar [4] was exploited for classifying *S. enterica* bacteria [5]. More recently, phages have been used for the control of *S. enterica* contamination in poultry [6–8], sprout seeds [9], and fresh-cut fruit [10]. In 2008, the US Food Safety and Inspection Service approved a *Salmonella*-specific phage preparation to reduce the contamination level of live poultry before processing [11].

Production of neutralizing antibodies [12, 13], rapid emergence of phage-resistant bacterial strains [12–15], and efficacy of phages only when administered shortly after bacterial infection [16] are the most frequent criticisms of the clinical use of phages. This study describes a phage lytic to *S. enterica* Paratyphi B, which is without the above limitations. This study also describes a phage-resistant strain of *S. enterica* that confers cross-protection against lethal doses of heterologous serovars.

METHODS

Bacteria. The study included 18 *S. enterica* fecal isolates derived from patients with gastroenteritis. Isolates

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belonged to the serovars Paratyphi B (001–005), Typhimurium (001–006), Dublin (001–004), or Virchow (001–003). Specimens were streaked on XLT-4 agar base supplemented with 28% tergitol 4-sodium tetradecyl sulphate solution (Oxoid). Serovars were confirmed by polymerase chain reaction (PCR) assay of the genes *abe, prt, fliC,* and *ctxm9* [17, 18]. For in vivo experiments, bacteria were grown in LB medium (Difco, Becton Dickinson) at 37 C and harvested while in exponential growth phase (optical density at 600 nm [$OD_{600\ nm}$], 0.6–0.8).

Phage isolation. Feces were diluted in saline, centrifuged (8 10^3 *g* for 10 min), filtered through a 0.22-mm membrane, and screened for the presence of phages by a spot test. Supernatants with positive spot test results were tested again by a plaque-forming assay. Individual plaques were expanded in LB broth (2 mL) containing the susceptible host (10^6 colony-forming units [CFUs]). Adsorption rate, latent period, and phage burst tests were performed as described elsewhere [19]. Phage purification was performed as described elsewhere [20].

Mice. Experiments were performed on female BALB/c mice. Phages (10^7 plaque-forming units [PFUs] in 100 mL of saline) were inoculated intravenously. The main target organs of the *S. enterica* isolates used in this study (liver and GI tract) were dissected and weighed. One gram of each sample was homogenized in 1 mL of saline and serially diluted in saline. CFUs were evaluated by plating each dilution on a lawn of *S. enterica*.

S. enterica *transformation.* The green fluorescent protein (GFP) gene was cloned into the HindIII-PstI sites of the pUC19 plasmid and then introduced into the Salp572^{flS} strain by electroporation as described elsewhere [21]. In vivo experiments demonstrated that transformation did not statistically significantly influence the virulence of the Salp572^{flS} strain.

Phage-neutralization test. Phage f1 (10^5 PFUs suspended in 100 mL of saline) was incubated with rat anti-f1 antiserum (diluted 10 ^{2} and 5 10 ^{2} in saline) for 3 h at room temperature. Bacteria (10^5 CFUs in 1 mL of LB broth) were added to the antibody-coated phage particles, and the mixture was incubated for 3 h at 37 C. CFUs and PFUs were then counted.

Real-time reverse-transcription PCR (RT-PCR). Reactions were performed in 20 mL in triplicate with the following thermal profile: 95 C for 10 min and 45 cycles of 15 s at 95 C and 45 s at 60 C; 1 min at 60 C; and 15 s at 95 C plus 0.3 C at each step of the melting curve. The relative quantification of gene expression was calculated using the relative standard method, with the glyceraldehyde-3-phosphate dehydrogenase gene (*gapA* for virulence genes and *gapdh* for cytokine genes) used as an endogenous control. The sequences of PCR primers used (0.2 mmol/L each) are reported in Table 1.

Other methods. Restriction endonucleases analysis–pulsed-field gel electrophoresis of wild and mutant *Salmonella* strains was performed as described elsewhere [22, 23]. Bacteria were prepared for electron microscopy examination as described elsewhere [24]. Mice infected with GFP-labeled bacteria were analyzed using the Leica Macrofluo instrument (Wetzlar) equipped with the Leica application suite software (version 3.1.0). The O-antigen was detected by the slide agglutination test (using antiserum from Staten Serum Institut [Copenhagen, Denmark]). Survival rates of mice were analyzed using the Fisher exact test. Bacterial counts and cytokines levels were analyzed using the Student *t* test.

Table 1.
Sequences of the Polymerase Chain Reaction Primers

Primer	Sequence 5′-3′
Abe forward	GGCTTCCGGCTTTATTGG
Abe reverse	TCTCTTATCTGTTCGCCTGTTG
Prt forward	ATGGGAGCGTTTGGGTTC
Prt reverse	CGCCTCTCCACTACCAACTTC
FliC forward	AACGAAATCAACAACAACCTGC
FliC reverse	TAGCCATCTTTACCAGTTCCC
Ctxm9 forward	GTGACAAAGAGAGTGCAACGG
Ctmx9 reverse	ATGATTCTCGCCGCTGAAGCC
Gapdh forward	TTCACCACCATGGAGAAGGC
Gapdh reverse	GGCATGGACTGTGGTCATGA
IL-6 forward	AAAGAGTTGTGCAATGGCAATT
IL-6 reverse	CAGTTTGGTAGCATCCATCAT
TNF-a forward	TCTCAGCCTCTTCTCATTCCT
TNF-a reverse	GTCTGGGCCATAGAACTGATG
IL-4 forward	AATGTACCAGGAGCCATATCCAC
IL-4 reverse	TCACTCTCTGTGGTGTTCTTCGT
IFN-g forward	AGCGGCTGACTGAACTCAGATTGTAG
IFN-g reverse	GTCACAGTTTTCAGCTGTATAGGG
ccmE forward	GTCGTAGAGACTGAAGTTCACTGA
ccmE reverse	ACCTGCGACGTAAAAACC
sthE forward	CTTATTCTGCTGTTCGCGTATTC
sthE reverse	GCGCTACCGGGTTCATATAAAAA
cheY forward	GCATGTTCCAGTCGGAGATAATA
cheY reverse	ATGGCGGATAAAGAGCTTAAATTTT
motA forward	ATCGGTACAGTTTTCTGGTGGTT
motA reverse	TAGAGCAACGCCAGCAAATCCA
ygbA forward	ACTCAATCAGGCTATCGAACATG
ygbA reverse	AGAAAAACAAACTGCGAAAAGGAAT
ttk forward	TCTGCTTCATCTCCTCGCGTTTA
ttk reverse	GTATCGCTCGTGAAAAGGTAAAC
gapA forward	TTTGGCCGTATCGGTCGCATT
gapA reverse	AGCGGTAACACGGATTTTTTTAC

RESULTS

Phage isolation and in vitro characterization. Two samples of feces were diluted with sterile phosphate-buffered saline and filtered through a 0.22-mm filter. Serial dilutions of the filtrates were then tested for the presence of phages on LB agar plates against a panel of 18 *S. enterica* isolates. Of the 8 phages isolated,

f1 was chosen for further studies in view of its higher in vitro lytic activity (Figure 1*A*) and larger host range (Table 2). Phage f1 was further characterized with regard to the absorption rate (1.6 10^8 mL/min), latent period (30 min), burst size (10^2 PFUs), and genome size (Figure 1*B*). Incubation of f1 with an excess of mouse anti-f1 antibodies did not interfere with phage's capacity to lyse susceptible bacteria. This result demonstrates that the antibodies elicited by f1 are nonneutralizing.

Phage f1 activity in vivo. Two groups of mice (10 mice per group) were injected intravenously with a lethal dose (10^7 CFUs per mouse) of a phage f1–susceptible isolate of *S. enterica* serovar Paratyphi B (for brevity referred to as Salp572^{f1S}). One group of infected mice was used as the untreated control, whereas the second group was injected intravenously with phage f1 (10^7 PFUs per mouse) immediately after infection. The animals of the control group all died within 48 h after infection. The mice treated with the phage all survived. Cumulative results from 3 independent experiments showed that although 100% (30 of 30) of the mice of the control group died, none (0 of 30) of the phage-treated mice died. The clinical advantage of phage therapy was also evident on quantifying the bacterial load in the blood and in the 2 organs most heavily infected with Salp572^{f1S} (liver and GI tract). Control mice displayed a high bacterial load (mean bacterial load standard deviation [SD], 4 10^6 0.20 10^6 CFU/mL in the blood and 6 10^6 0.3 10^6 CFU/gr in the GI tract), whereas no bacteria were isolated from the blood, GI tracts, or livers of phage-treated mice. In addition, phage f1 statistically significantly reduced inflammation in *Salmonella*-infected mice (Figure 1*C*).

Efficacy of phage f1 delayed treatment. The above experiment demonstrated that f1 is active in vivo, at least when administered immediately after infection. Typically, bacterial infections are caused by a small initial inoculum. Therefore, to be clinically useful, phage therapy must be effective when started weeks after infection. To see whether f1 displayed this potential, mice were infected with a sublethal dose of Salp572^{f1S} (10^5 CFUs per mouse) and treated with phage f1 (10^7 PFUs per mouse) 2 weeks later. In this experiment, the parameter to measure the efficacy of the therapy was the ability of phage f1 to sterilize the animals (rather than the ability to rescue the animals from a lethal bacterial dose, as in the above-described experiment). Phage f1, administered 2 weeks after infection, was entirely effective in sterilizing the animals, as shown by microscopic analysis (Figure 2) and confirmed by bacteriological tests (data not shown).

Host density threshold. Mice were infected with a sublethal dose of Salp572^{f1S} bacteria (10^6 CFUs per mouse) and 2 d later treated with phage f1 (10^7 PFUs per mouse). Circulating bacteria and phages were measured at 2-d intervals for 14 d. Phage replication began only when the concentration of the Salp572^{f1S} bacteria in the blood was 10^4 CFU/mL (Figure 3*A*). The same threshold was also required for phage replication in the GI tract and liver (Figures 3*B* and 3*C*). Thus, phages, in common with other microbial predators [25], display a density threshold for reproduction. This notion, as discussed below, is important for a correct clinical use of phage therapy.

Phage f1 resistance produces loss of virulence. In vivo and in vitro experiments have demonstrated that acquiring resistance to a phage or to an antibiotic imposes a fitness cost [26–29]. This study exploited acquisition of resistance to phage f1 as a means to curb the virulence of the wild Salp572^{f1S} strain and to use the avirulent phage-resistant derivative strain (Salp572^{f1R}) to protect mice from infection. The phage-resistant strain Salp572^{f1R} was isolated from the phage-susceptible strain Salp572^{f1S} in the presence of phage f1. The common origin of the parental and derivative strains is documented by their similar genomic profiles (Figure 1*D*). Salp572^{f1R} was fully resistant to phage f1 (Figure 4*A*). Prolonged liquid subculture for several months in the absence of the phage, storage at 80 C, and passages in vivo did not alter the resistance of Salp572^{f1R} to phage f1. On LB agar, Salp572^{f1R}, compared with the parental strain, formed smaller colonies (data not shown); Salp572^{f1R} also displayed reduced size and a translucent and thickened cell wall (Figures 4*B* and 4*C*), loss of the O-antigen, reduced growth rate during the exponential growth phase (after incubation for 3 h, broth cultures of the wild-type Salp572^{f1S} strain and of the mutant Salp572^{f1R} strain had an $OD_{600\ nm}$ of 0.6 and 0.2, respectively), and increased doubling time (mean doubling time of Salp572^{f1S} SD, 30 3.5 min; mean doubling time of Salp572^{f1R} SD, 45 2.8 min). Acquisition of phage resistance also altered the transcriptional profile of Salp572^{f1R}. The 6 virulence genes that were analyzed were all statistically significantly underexpressed in Salp572^{f1R} (Figure 5*A*). Interestingly, in common with several *S. enterica* strains [30–32], Salp572^{f1R} gained phage resistance by the loss of the O-antigen.

In vivo characterization of the Salp572^{f1R} strain. Two groups of mice (10 mice per group) were infected with Salp572^{f1S} and Salp572^{f1R} (10^7 CFUs per mouse), respectively. The animals infected with Salp572^{f1R} were totally sterile 7 d after treatment. The animals infected with Salp572^{f1S} all died within 48 h. The experiment was repeated with 2 more f1-resistant mutants of *S. enterica* serovar Typhimurium and 2 f1-resistant mutants of *S. enterica* serovar Dublin. Again, bacteria were cleared within 4–7 d. Salp572^{f1R} caused no signs of physical stress (ruffled fur or fever) in the treated animals, repressed the transcription of the tumor necrosis factor (TNF) a and interferon (IFN) g genes, and induced the transcription of the interleukin (IL) 4 and IL-6 genes, as determined by RT-PCR analysis 24 and 48 h after treatment (Figure 5*B*). Salp572^{f1S}, in contrast, activated the TNF-a and IFN-g genes and repressed the IL-4 and IL-6 genes (Figure 5*B*). The above results dem-

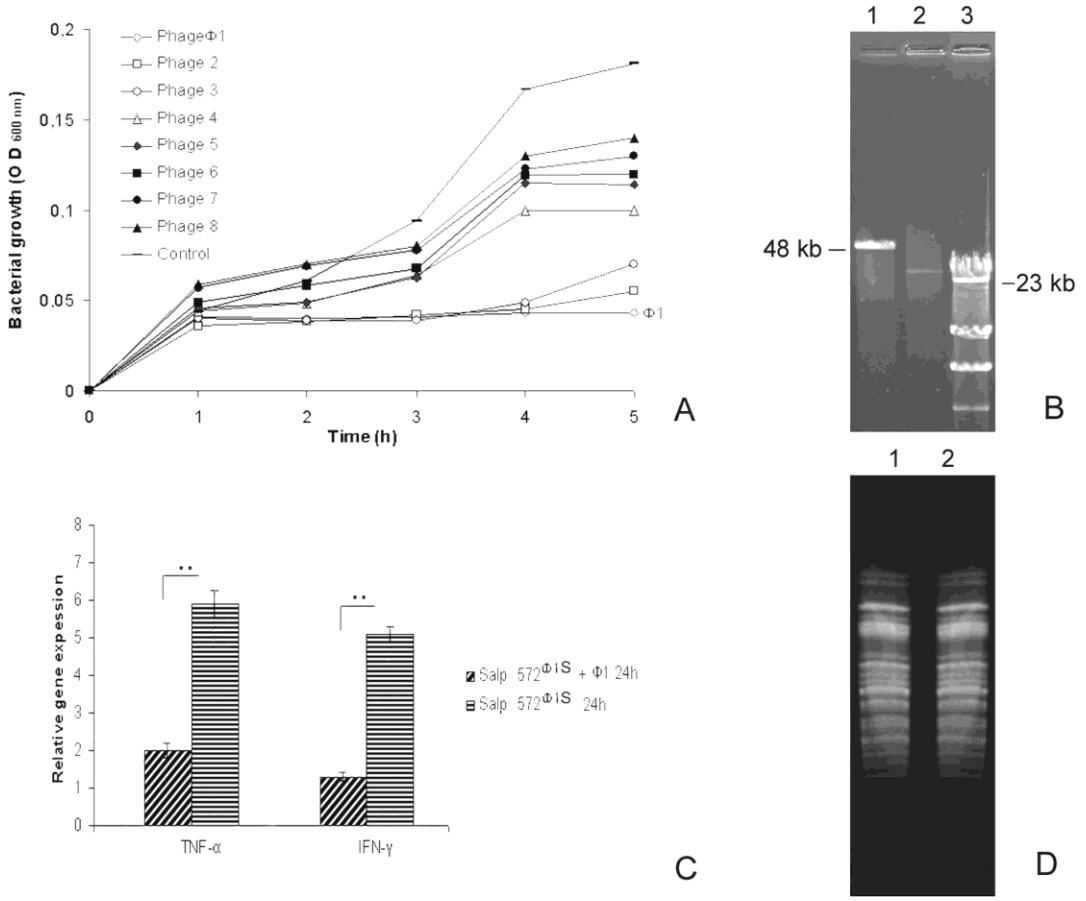

Figure 1. Characteristics of bacteriophage **f**1 and of the phage **f**1-susceptible Salp572^{f1S} and phage **f**1–resistant Salp^{f1R} bacterial strains. *A,* Lytic activity of the 8 phages isolated in the course of the study. The experiments described in the study were performed with the use of phage **f**1. "Control" refers to the bacterial strain Salp572^{f1S} grown in the absence of phage **f**1. *B,* Genome size of phage **f**1. Pulsed-field gel electrophoresis (PFGE) was run at 10 C with a pulse ramping between 1 and 10 s for 16 h using Lambda DNA (Promega) as marker. *C,* Anti-inflammatory activity of phage **f**1. Twenty-four hours after treatment with phage **f**1, Salp572^{f1S}-infected mice displayed statistically significantly reduced levels of proinflammatory cytokines, compared with mice infected with Salp572^{f1S} but not treated with phage **f**1. *D,* PFGE profiles of the Salp572^{f1S} (*left*) and Salp572^{f1R} (*right*) strains. The identical PFGE patterns reflect the common origin of the 2 strains. Restriction fragments were resolved in a single run at a constant voltage of 6 V cm^2 and an orientation angle of 120 between electric fields for 20 h with a pulse ramping between 1 and 15 s. IFN-g, interferon g; $OD_{600\ nm}$, optical density at 600 nm; TNF-**a**, tumor necrosis factor **a**.

onstrate that Salp572^{f1R} is rapidly cleared in vivo and induces a balanced anti-inflammatory response.

Immunization with Salp572^{f1R} protects against lethal Salp572^{f1S} infection. One group of 20 mice was immunized intravenously with Salp572^{f1R} (10^7 CFUs per mouse) and 2 weeks later infected with Salp572^{f1S} (10^7 CFUs per mouse). The control group (10 mice) was infected directly with a lethal dose of Salp572^{f1S} (10^7 CFUs per mouse). Survival of the Salp572^{f1R}-treated mice was monitored for 4 weeks after experimental infection. The Salp572^{f1R} vaccine strain protected 20 (100%) of 20 mice from a lethal dose of Salp572^{f1S}, whereas all 10 of 10 control mice died within 48 h (P ! .001). Blood, GI tracts, and livers of the Salp572^{f1R}-vaccinated animals were sterile within 7 d after experimental infection, whereas the same organs and blood from the control mice at the time of death displayed high bacterial loads (mean bacterial load SD, 4.0 10^6 0.18 10^6 CFU/mL in the blood and 7.0 10^6 0.34 10^6 CFU/gr in the GI tract). The same schedule was followed to repeat the experiment with a vaccine of heat-killed Salp572^{f1R}. This time no difference was observed in survival time between the control group and the group treated with heat-killed Salp572^{f1R}. This result suggests that protective immunity is probably elicited by a gene (or multiple genes) that are induced during infection. The experiment was repeated once more with a vaccine of live Salp572^{f1R} and *S. enterica* serovar Dublin, *S. enterica* serovar Typhimurium, or *S. enterica* serovar Virchow as challenging bacteria (10^7 CFUs per mouse). The mice of the control groups (5 mice per group) all died

Table 2. Properties of the 8 Isolated Bacteriophages

Salmonella enterica serovar	Bacteriophage f1	f2	f3	f4	f5	f6	f7	f8
Paratyphi B, 001		+	+	+	+	+	+	+
Paratyphi B, 002								+
Paratyphi B, 003		+		+				+
Paratyphi B, 004	+		+	+	+			+
Paratyphi B, 005		+		+	+	+		+
Typhimurium, 001		+		+		+	+	+
Typhimurium, 002	+		+	+	+			+
Typhimurium, 003		+	+	+	+	+	+	+
Typhimurium, 004	+		+	+	+	+	+	+
Typhimurium, 005								+
Typhimurium, 006		+				+		+
Dublin, 001								+
Dublin, 002								+
Dublin, 003								+
Dublin, 004								+
Virchow,								001
Virchow, 002	+							
Vrchow, 003								

within 2–3 d; at the end of the experiment (5 weeks after infection), all the mice immunized with Salp572^{f1R} (10 mice per group) were alive. These data show that the Salp572^{f1R} vaccine is effective only when alive and, in this state, is active against different *S. enterica* serovars. Live attenuated *Salmonella* strains can provide short-term, nonspecific protection because of persisting vaccine bacteria [33]. The Salp572^{f1R} strain is cleared within 7 d after administration. Therefore, the protection described in this study cannot be a result of a nonspecific immune response.

Anti–Salp572^{f1R} antibodies protect against Salp572^{f1R} injury. One group of 10 mice was injected intravenously with 10 mL of normal rat serum (control mice). A second group of 10 mice was injected with 10 mL of serum from rats immunized with Salp572^{f1R}. The next day, both groups of animals were challenged with a lethal dose of Salp572^{f1S} (10^7 CFUs per mouse). Survival was monitored for 14 d. The anti-Salp572^{f1R} rat serum provided protection to 10 of 10 mice, whereas all 10 of 10 control mice died within 2 d. At the end of the experiment, the blood, livers, and GI tracts of mice passively immunized with anti-Salp572^{f1R} antibodies were sterile, whereas at time of death, the blood, livers, and GI tracts of the control mice displayed a high bacterial load (mean bacterial load SD, 4 10^6 0.4 10^6 CFU/mL in the blood and 6.5 10^6 0.5 10^6 CFU/gr in the GI tract).

DISCUSSION

The purpose of this study was to review the therapeutic potential of phages. First, we showed that phages are highly effective when administered 2 weeks after the experimental infection; second, we showed that bacteria that gain phage resistance are often avirulent (or scarcely virulent) and do not survive in vivo for long. These results alone invite an objective assessment of phage prospects as antimicrobials. The study also shows that phage-resistant bacteria, with their reduced virulence, can be excellent vaccines. Selection for phage resistance as a straightforward approach to the production of vaccines is a new and significant contribution of phages to the control of bacterial diseases. It is also the most important result of this study.

Phage f1, given concurrently with a lethal dose of Salp572^{f1S}, rescued 100% of the animals. More importantly, the same phage, administered 2 weeks after the experimental infection with a sublethal dose of Salp572^{f1S}, sterilized 100% of the animals (Figure 2). The infection model that used a small initial bacterial inoculum shows clearly the true potential of phages and brings phage therapy closer to the preclinical testing phase. Sterilization was attained in all of the target organs (liver and GI tract) and in the blood without bacterial rebound. The same results were also observed in another study, which involved a different phage (M^{Sa}) and a different pathogen (*Staphylococcus aureus*) [34]. The conclusion that phages do not have a temporal limit on their ability to kill bacteria therefore seems of general significance. In none of the experiments did phage f1 cause adverse effects that could be attributed to the rapid lysis of bacteria, nor did the phage therapy induce neutralizing antibodies, despite claims that it might [12, 13]. The same results were also observed in another study with phage M^{Sa} [34].

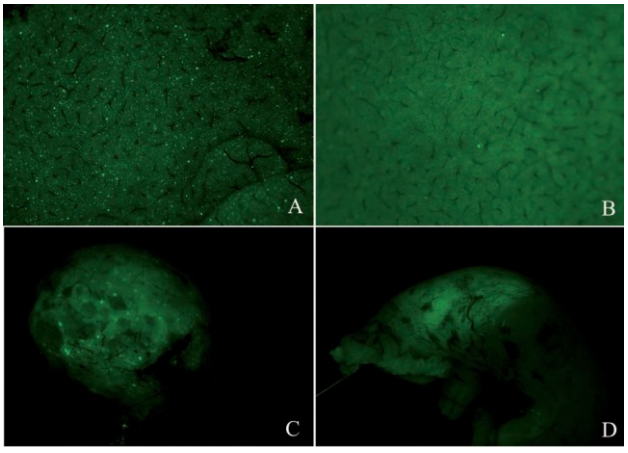

Figure 2. Efficacy of delayed bacteriophage therapy. Mice were infected with a sublethal (10^6 CFUs per mouse) dose of the Salp572^{f1S} strain that was transfected with the pUC19 plasmid expressing the green fluorescent protein (Salp572$^{f1S\text{-}GFP}$). Two weeks later, mice were treated with phage f1 (10^7 PFUs per mouse) or left untreated. Liver tissue samples of untreated (*A*) and treated (*B*) mice and stomach tissue samples of untreated (*C*) and treated (*D*) mice are shown; samples from treated mice were analyzed 24 h after phage administration.

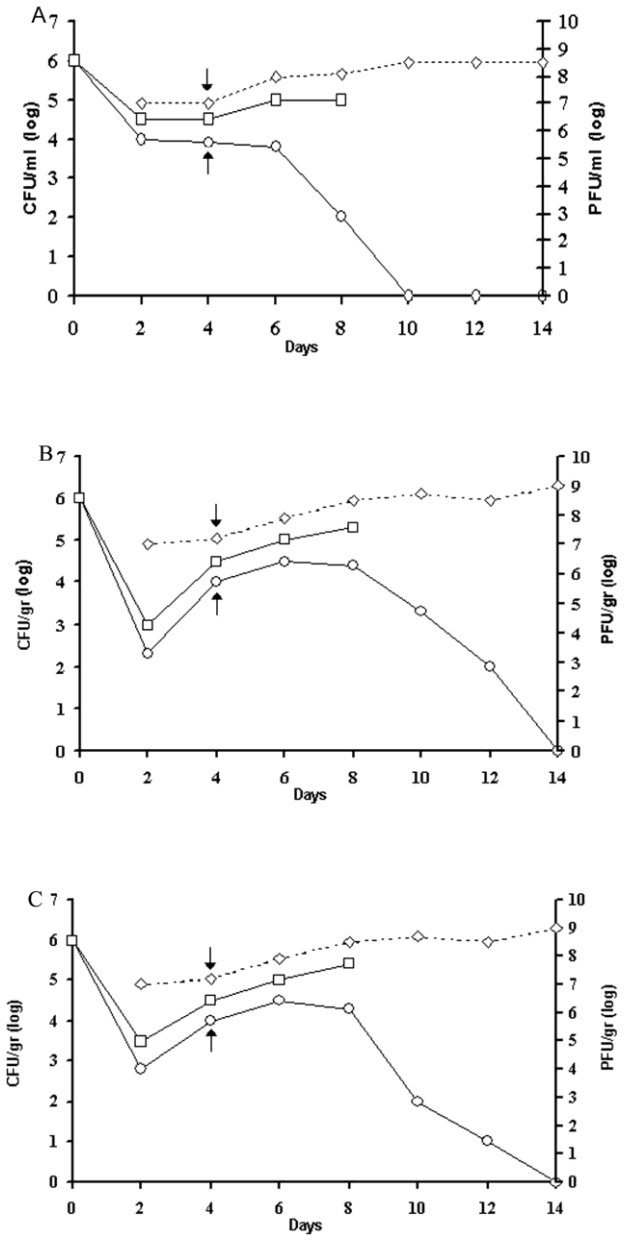

Figure 3. Evidence that bacteriophage f1 replication requires a bacterial density threshold, showing the concentration of bacteria in the blood (*A*), gastrointestinal (GI) tract (*B*), and liver (*C*). One group of 20 mice (*squares*) was infected with Salp572^{f1S} (10^6 colony-forming units [CFUs] per mouse). At 2-d intervals, 2 mice were killed to monitor Salp572^{f1S} replication in vivo, in the absence of phage f1. The mice of this group all died within 8 d. A second group of 20 mice was infected with Salp572^{f1S} (10^6 CFUs per mouse) and 2 d later treated with phage f1 (10^7 plaque-forming units [PFUs] per mouse). At 2-d intervals, 2 mice were killed to monitor phage replication (*diamonds*) and bacterial lysis (*circles*). The results from this group of mice show that phage replication starts when bacteria reach the threshold density of ~10^4 CFU/mL (in the blood) or 10^4 CFU/gr (in the GI tract or liver). Arrows show the time when phage replication starts and the bacterial density when phage replication starts.

Mice inoculated with Salp572^{f1R} bacteria returned to being sterile within 7 d. To exclude the possibility that this finding was peculiar to Salp572^{f1R} bacteria, 2 more phage f1–resistant mutants isolated from *S. enterica* Paratyphi B and 2 phage f1–resistant mutants isolated from *S. enterica* Dublin were inoculated intravenously into mice. Again, within 4–7 d, the mice were sterile. Reduced survival in vivo has also been reported for phage-resistant strains of *Vibrio cholerae* [29, 35], enteropathogenic *Escherichia coli* [36], and *Campylobacter jejuni* [28]. Although the above data do not downplay the possibility that resistant bacteria might occasionally emerge in the course of phage therapy, they do collectively indicate that resistant strains in vivo are generally cleared by innate immunity (at least more often than has been suspected so far). It has recently been reported that phage-resistant bacteria in vitro display little or no fitness cost, in comparison with the original susceptible bacteria [14]. The same phenomenon has also been observed in the course of the present study. However, the point that needs to be stressed is that what is observed in vitro does not necessarily reflect what happens in vivo (in the presence of the immune system).

The finding of an inverse association between the presence of virulent phages and susceptible bacteria (Figure 3) is of significance for a sounder use of phage therapy. It explains why, contrary to what occurs with antibiotics, early phage administration (when bacterial density is below the threshold) might be counterproductive. This finding also helps in deciding when to use a single phage dose and when multiple doses are necessary. If the target bacterial population is replicating rapidly, then 1 phage dose is sufficient (Figure 3); if bacteria replicate slowly—as in the abscesses—then multiple doses are definitely more effective than 1 dose [34]. The dynamics of the interactions between phages and bacteria might also help us to understand the forces regulating the cyclical nature of salmonellosis and serovar changes [1, 2, 37]. We can hypothesize that, as in the case of cholera [38, 39], if lysogenic and nonlysogenic bacterial strains of *S. enterica* were present in the same environment, then the lysogenic strains would liberate phages that attack nonlysogenic strains, which would cause cyclical bacterial strain replacement.

The Centers for Disease Control and Prevention estimate the annual number of cases of salmonellosis in the United States at ~1.4 million [37]. The emergence of antibiotic-resistant strains of *S. enterica* [1] makes treatment of these patients more difficult and, at the same time, the need for a vaccine more urgent. In view of the complex array of virulence factors produced by the many *S. enterica* serovars, it is unlikely that a vaccine targeting a single virulence factor (ie, a subunit vaccine) might be satisfactory. In addition, the factor targeted by a subunit vaccine might have limited distribution among clinical isolates or might not be expressed in vivo. However, if the

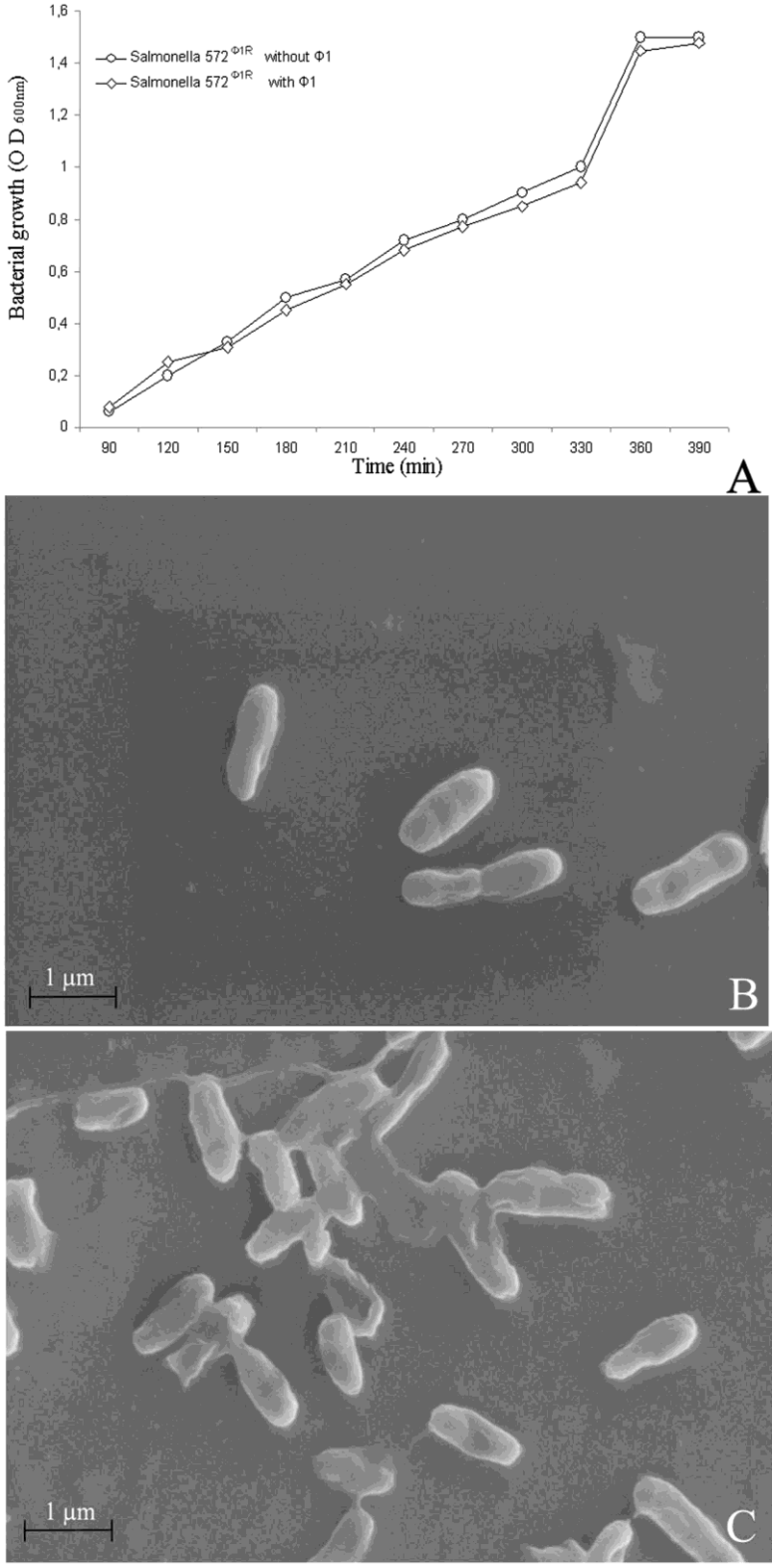

Figure 4. Resistance of the Salp572^{f1R} strain to bacteriophage f1. *A,* Evidence that the presence of phage f1 did not interfere with in vitro multiplication of the Salp572^{f1R} strain. Electron micrographs of the f1-susceptible (Salp572^{f1S}) (*B*) and f1-resistant (Salp572^{f1R}) (*C*) strains in the presence of phage f1. Phage f1 lysed only the Salp572^{f1S} strain. In addition, Salp572^{f1R} bacteria displayed reduced cell size and thickened cell walls. $OD_{600\ nm}$, optical density at 600 nm.

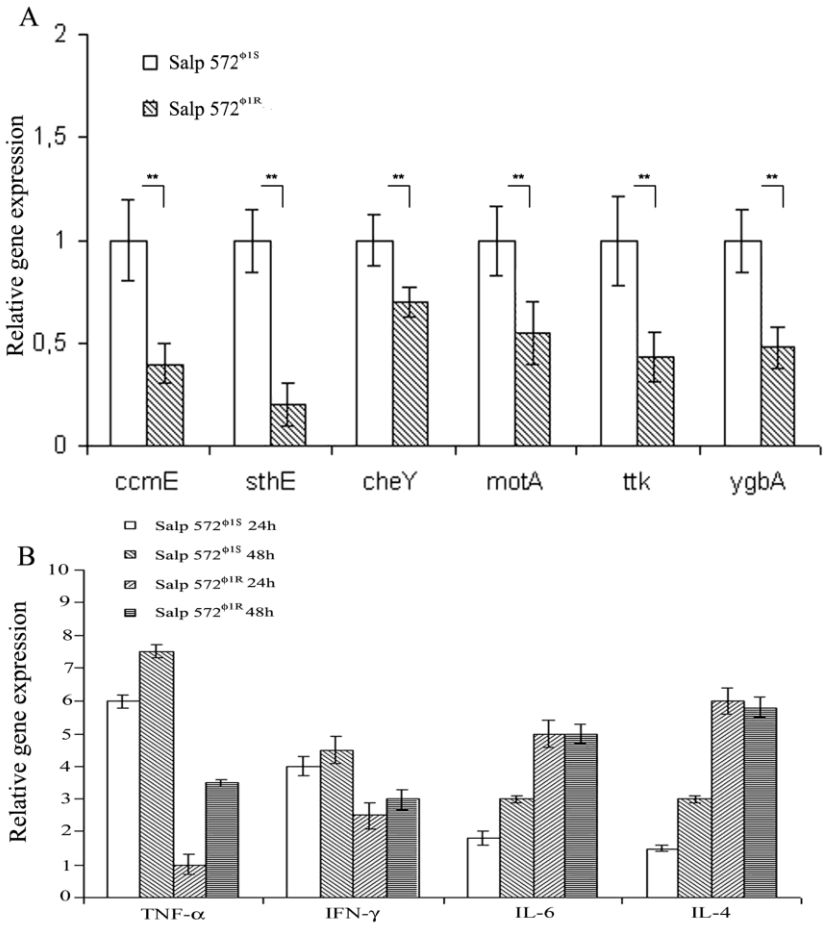

Figure 5. Expression levels (measured by reverse-transcription polymerase chain reaction) of bacterial virulence genes and host cytokine genes. *A,* Expression level of 6 virulence genes (*ccmE*, periplasmic heme-dependent peroxidase; *sthE*, putative major fimbrial subunit; *cheY*, chemotaxis regulator; *motA*, proton conductor component of motor; *ttk*, putative transcriptional regulator; *ygbA*, putative cytoplasmic protein) in the f1-susceptible (Salp572^{f1S}) and f1-resistant (Salp572^{f1R}) strains of *Salmonella enterica* serovar Paratyphi B. The endogenous control was the glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*). *B,* Evidence that the Salp572^{f1R} strain, compared with the Salp572^{f1S} strain, induces in the mouse a more balanced expression of proinflammatory (tumor necrosis factor a [TNF-a] and interferon g [IFN-g]) and anti-inflammatory (interleukin 4 [IL-4] and interleukin 6 [IL-6]) cytokines. Liver cytokine levels were measured 24 and 48 h after infection of mice with 10^7 CFUs per mouse of Salp572^{f1R} or Salp572^{f1S} bacteria. The endogenous control was the eukaryotic glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*).

factor is common among *S. enterica* serovars, then preexisting antibodies in the host might neutralize the vaccine before it can be effective, as was shown in a different context [40]. A multiple-component vaccine, in the form of an attenuated strain of *S. enterica,* therefore seemed to be the appropriate choice, and selection for phage resistance—which often occurs at the expense of virulence [28, 29, 41]—seemed to be the simplest approach to a multiple-component vaccine. The phage-susceptible virulent strain Salp572^{f1S} was grown in vitro in the presence of phage f1. As expected, compared with the parental strain, the phage-resistant strain Salp572^{f1R} was much attenuated in vivo as a result of down-regulation of numerous virulence genes (Figure 5*A*). The Salp572^{f1R} strain was fully protective against different *S. enterica* serovars, provided protection in antibody transfer experiments, and protected mice from inflammation (Figure 5*B*). One potential concern about using the Salp572^{f1R} strain as vaccine is its reversion to phage sensitivity and the accompanying virulent phenotype. However, the Salp572^{f1R} strain showed (in vitro and in vivo) remarkable stability, which presumably reflects the drastic and irreversible changes (Figure 5*A*) that accompanied acquisition of phage resistance.

The data reported here have some limitations that need to be discussed. First, the fitness cost imposed on bacteria to gain phage resistance does not refer to virulence alone but also refers to environmental fitness (the ability to compete with wild-type

strains in nature). This means that one cannot assume with certainty that all phage-resistant strains are attenuated enough to be used as a vaccine. Second, knowing the genome size of phage f1 (30–35 kb) (Figure 1*B*) is not sufficient to conclude that phage f1 is different from the *Salmonella* phages deposited in public databases. However, given the vast phage genetic diversity—such that "no genomically defined phage has been isolated more than once" [42]—the hypothesis that phage f1 is unique seems reasonable. Third, the O-antigen is the attachment site for many gram-negative phages, especially *Salmonella* phages [43, 44]. Salp572^{f1R} bacteria, in common with several *Salmonella* strains, acquired phage resistance by losing the O-antigen [30–32]. However, at present, we cannot exclude the possibility that, in addition to O-antigen loss, other mechanisms also contribute to protect Salp572^{f1R} bacteria against phage f1. In conclusion, despite leaving some issues undefined (the phage f1 sequence and a detailed analysis of the Salp572^{f1R} strain conversion from phage susceptible to phage resistant), the data presented in this study have important implications for the role that phages can have as antimicrobials.

Acknowledgments

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