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Seminars of the Department of Infectious, Parasitic and Immune-mediated Diseases (2010)

Edited by Patrizia Martucci

Dipartimento di Malattie Infettive Parassitarie e Immunomediate

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The Department of Infectious, Parasitic and Immune-mediated Diseases of the Istituto Superiore di Sanità (the National Institute of Health in Italy) organizes, fortnightly, departmental seminars. This document reports the seminars of 2010 about the research activities carried out in the field of infectious diseases in the Department, and it represents a form of dissemination and promotion outside the ISS. In addition, it testifies the skills, competence and commitment of the staff that made this exciting initiative possible. A special acknowledgment is due to the younger researchers, who have the merit of having presented the contributions of this volume. Should their reports effectively express the complexity of the research approaches, even to non professionals, a double result would be achieved: to train young scientists in properly performing their work, and communicating it.
Key words: Infectious disease; Mechanism of infection; Diagnosis and therapy; Health protection

Istituto Superiore di Sanità
Seminari del Dipartimento di Malattie Infettive Parassitarie e Immunomediate (2010).
A cura di Patrizia Martucci
2012, 78 p. Rapporti ISTISAN 12/10 (in inglese)

Il Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate dell'Istituto Superiore di Sanità (ISS) organizza, quindicinalmente, da ormai diversi anni seminari dipartimentali. Questo volume riporta le relazioni riguardanti l'anno 2010 su alcune attività di ricerca svolte nel settore delle malattie infettive nel Dipartimento, ma soprattutto rappresenta una forma di divulgazione e promozione delle attività anche al di fuori dell'ISS. Inoltre, testimonia la competenza, la professionalità e l'impegno del personale che ha reso possibile questa interessante iniziativa. Un particolare riconoscimento è espresso ai ricercatori più giovani che hanno il merito di aver presentato i contributi del volume. Se le loro relazioni riusciranno ad esprimere la complessità degli approcci di ricerca e al tempo saranno comprensibili ai non addetti ai lavori, si sarà ottenuto un doppio risultato: formare ricercatori in grado di svolgere adeguatamente il proprio lavoro e di comunicarlo.
Parole chiave: Malattie infettive; Meccanismi d'infezione; Diagnosi e terapia; Epidemiologia; Tutela della salute

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INTRODUCTION

The Department of Infectious, Parasitic and Immune-mediated Diseases of the Istituto Superiore di Sanità (ISS, the National Institute of Health), in order to enhance collaboration and exchange of information among its divisions, organizes every fortnight, since many years now, Departmental Seminars.

The seminars aim to present research outcomes, issues related to institutional duties and literature analysis on topics of interest in the fields of the Department's research activities. These meetings give a spur on discussion and training for the several fellows, PhDs and students hosted by the Department. It is indeed important for young investigators to learn how to present the results of their research.

Since 2011, in order to promote the Departmental activities both to the ISS and also to the scientific community, it has been decided to collect, on a voluntary basis, the reports of seminars held during 2010 within this ISTISAN Report. Hopefully, this initiative will be repeated in the next years, with the consent of all the speakers involved.

Like any collection of grey literature, this volume offers a quick glance on the research activities carried out in the field of infectious diseases and it also represents a form of dissemination and promotion of the department's activities.

These contributions will testify the skills, expertise and commitment of the staff present in the department, which made this very fruitful initiative possible.

Finally, a special mention has to be given to the younger employees who have the merit of having shown the majority of reports held in 2010, and consequently of the contributions in this volume.

Should their reports express the complexity of research approaches not only to experts, but also to the layman, a double result would then be achieved: researchers trained to do their job and to communicate it in the best way.

PHYLOGENETIC ANALYSIS OF THE PROTEIN NSs OF TOSCANA VIRUS

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Toscana virus (TOSV) belongs to the large group of the Arthropod-Borne Viruses transmitted by bites of haematophagous insects (mosquitoes, ticks, phlebotomus), the taxonomic classification of whom includes different viral families (*Bunyaviridae*, *Flaviviridae*, *Togaviridae*, *Reoviridae*). Until now, more than 500 arboviruses are known and 100 out of them cause human cases, with a clinical spectrum ranges from asymptomatic infection to rare severe disease.

According to the current International Committee on Taxonomy of Viruses (ICTV) classification, TOSV belongs to the *Bunyaviridae* family, to the genus *Phlebovirus* and to the Sandfly virus serotype Naples serocomplex.

TOSV is embedded in a lipid envelope that contains the viral glicoproteins G_N ang G_C and its genome is composed of three negative single-strand RNA assembled with the nucleocapsid protein N and with the polymerase L.

TOSV was originally isolated in 1971 from the a specimen of *Phlebotomus perniciosus* collected in Grosseto province (Central Italy) (Verani *et al*., 1982). Since its first isolation, several studies have been conducted, in Italy and other Mediterranean countries, in order to investigate its possible animal reservoirs, the spread of the infections and its geographical distribution, and its pathogenycity.

Since the 1983, TOSV has been associated to neuropathological diseases (Verani, 1984). Its main clinical manifestation is neurologic: it is responsible for aseptic meningitis, meningoencephalitis and encephalitis associated with fever, myalgia and headache. However, asymptomatic infection have also been described as result of serological studies (Nicoletti, 1991; Braito *et al*., 1997).

TOSV is present in the European countries of the Mediterranean area: Italy, France, Spain, Portugal, Cyprus (Braito *et al*., 1998; Valassina *et al*., 2000). In Italy TOSV cases have been reported from Tuscany, Marche, Umbria, Latium, Sardinia and Campania Region, and from all those rural areas which guarantee the insect vectors persistence. These vectors belong to the *Phlebotomus perniciosus* and *P. perfiliewi* genera which live at an altitude of 200-600 metres and whose main activity is related to the summer period. Consequently, Toscana infections show a seasonal trend with a peak in August because of the life cycle of the vectors, then the geographical distribution is related to the distribution of the vector.

The diagnostic procedures for the TOSV infection are the isolation method and the detection of viral RNA in the cerebrospinal fluid; specific antibodies can be also detected by serological techniques.

TOSV, as briefly mentioned, has a RNA segmented genome comprised of three units designated large (L), medium (M) and small (S). The L segment encodes the viral polymerase, the M segment encodes the G_N–G_C glycoprotein and a non-structural protein NSm, and the S segment uses an ambisense strategy to express the nucleoprotein N and the non-structural protein NSs.

Several studies have been conducted on the molecular variability of TOSV strains. Based on the analysis of PCR (Polymerase Chain Reaction) products from the L segment, 2 lineages of

TOSV circulate in the Mediterranean basin (Sanbonmatsu-Gàmez *et al*., 2005). Nucleotide sequences of TOSV isolates in Iberian area and in France are significantly different from the Italian prototype strain ISS Phl.3. Based on the analysis of G_N glycoprotein (M segment), 4 lineages of TOSV have been proposed in Italy (Venturi *et al*., 2007). These results have been confirmed based on the analysis of PCR products of the N gene (S segment) (Valassina *et al*., 1998).

These studies suggest a possible link between the grade of variability of each segment and the role of the proteins encoded. L segment seems to be the most conserved segment (it encodes the viral polymerase), the M segment shows high variability (it encodes the glycoprotein G_N and G_C which are the target of host immunitary response), S segment seems to be in the middle (it encodes the nucleoprotein N and the non-structural protein NSs).

Our aim was to investigate the variability of the gene NSs (S segment) of TOSV strains isolated from different hosts and collected in different localities from 1980 to 1995. Nucleotidic and aminoacidic sequences obtained were used to realize a phylogenetic analysis.

The study included 25 samples previously identified as TOSV by neutralization test and complement-fixation test (Verani *et al*., 1988). 18 of them were "clinical strains" isolated from patients hospitalized with neurological disease, and 7 of them were "environmental strains" isolated from pools of phlebotomus and one from the brain of a bat.

We realized specific RT-PCR for the gene NSs (1067 nt) and analized the sequenced PCR products. The nucleotidic and amino acid sequences for NSs protein were corrected and aligned by the Clustal_W program of BioEdit. The sequences of our samples were aligned with representative sequences in a multiple aligment (the Italian ISS Phl.3 and 2 sequences from Iberian strains). Moreover, we calculate the homology rates for the sequences compared to the ISS Phl.3 ones.

Nucleotidic and aminoacidic sequences of 17 of the 18 clinical strains show high identities with ISS Phl.3 (96.4% and 96.6%), while one of the 18 clinical strains (Pt 3_1983 P) show high identity with the Iberian strain (95.3% and 97.2%). This is because Pt 3_1983 P sample come from a clinical case of a tourist who had been in Portugal Pt 3_1983 P. The environmental strains show sensible identities with the ISS Phl.3 (97.9% and 98.2%). This suggests the idea that there is a different evolutionary press acting on the virus over the insect vectors.

To establish genetic relationships and the evolutionary rate according to NSs, nucleotide and amino acidic sequences phylogenetic trees were constructed by using the neighbor-joining, maximum parsimony and maximum likelihood methods by using PAUP software (Swofford, 1993). The clustering of different lineages was strongly supported by bootstrap resampling in order to determine the robustness of the topologies (Figure 1).

Then Bayesian methodology was used by BEAST: this computer platform exploits statistic inference to evaluate the common ancestor, by using the collecting dates of our samples. SFSVN (Sandfly Fever Naples Viruses) NSs sequences were included as an outgroup, to determine the coalescent node for TOSV sequences (Figure 2).

Phylogenetic trees obtained by these two methods showed similar topology. They showed two major lineages coming from a common ancestry (about 500 years ago), one of whom originated the Italian lineage. Currently, there are 3 lineages in Italy: they have been co-evolved in the last 2 ages leading to the present Italian variability frame.

In conclusion, our data for NSs protein show a strong evolutionary negative press aging by the insect vectors, suggesting a possible role of NSs protein in the manteinance of the relationship between the virus and its vector. Furthermore, they show 3 lineages cocirculating in Italy which seem to be co-evoluted and randomly spread on Central Italy.

Figure 1. Phylogenetic relationships of TOSV strains based on the NSs amino acidic sequences constructed by using UPGMA. Arms length is proportional to the amino acidic sosbtitutions

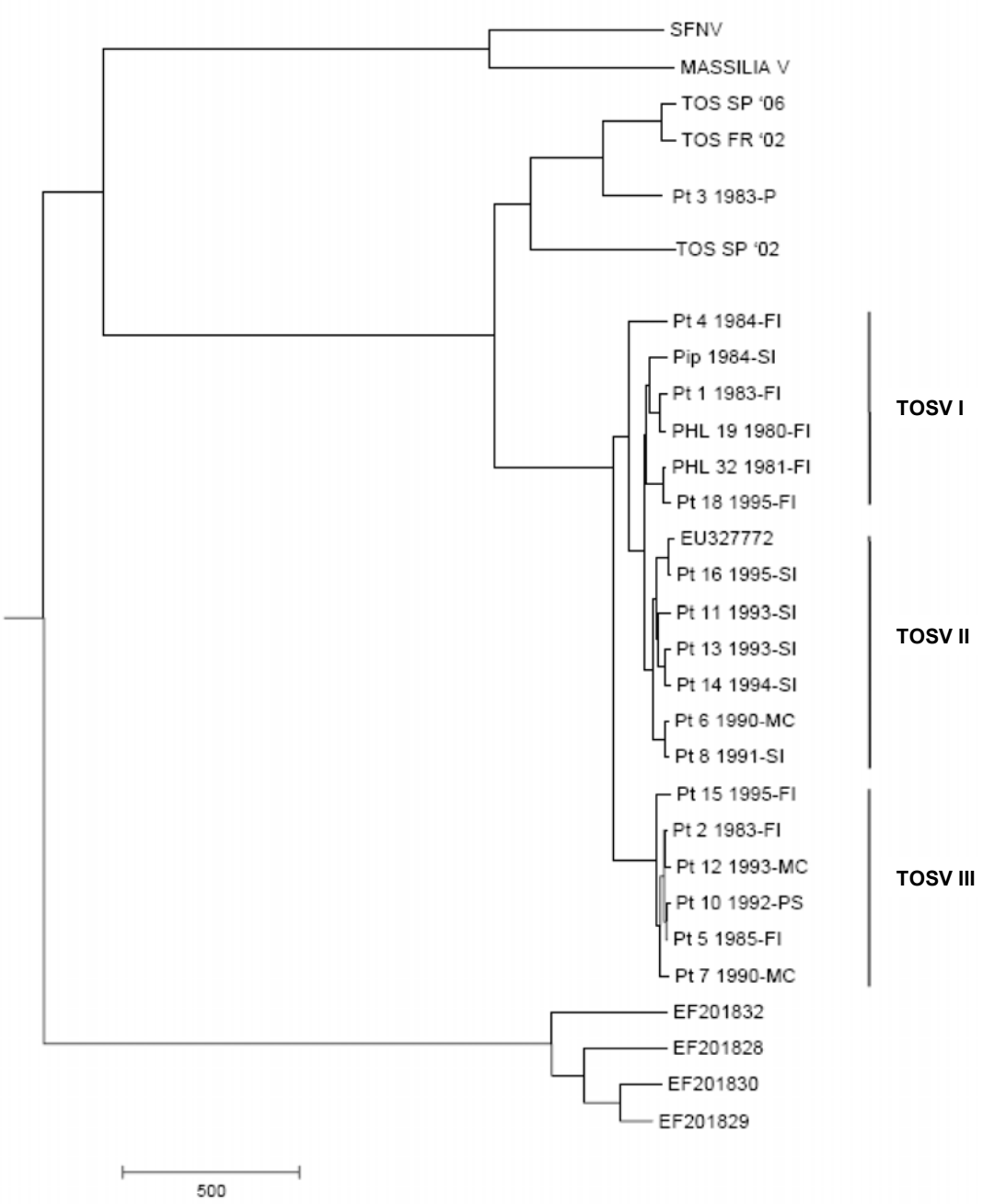

Figure 2. Phylogenetic tree constructed by using Bayesian method. SFSVN was set as the outgroup to root the tree

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PROBIOTIC BACTERIA FOR PREVENTION AND THERAPY OF ALLERGIC DISEASES: STUDIES IN MOUSE MODELS OF SENSITIZATION TO INHALANT AND FOOD ALLERGENS

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Introduction

Allergy can be considered as an inappropriate immune response to otherwise harmless antigens (inhalant and food "allergens"), and is characterised by a disruption of the Th1/Th2 balance towards a pronounced Th2 profile. Th2 lymphocytes play a pivotal role in the development and maintenance of the allergic response by producing IL-4, IL-13, IL-5, IL-9. Their activation leads to the induction of allergen-specific IgE synthesis and recruitment of eosinophils, two factors that mediate most of the clinical symptoms of allergy (1). Non-allergic individuals respond to allergens by developing either a Th1-type or T regulatory (Treg)-type immune response (2-3). Allergic sensitization of the airways with indoor and outdoor aeroallergens is associated with the development of asthma and allergic rhinitis and affects almost 15-30% of the European population (4). Food allergy can be defined as an abnormal immunologic reactivity to food proteins, characterised by expansion and activation of Th2 cells resulting from inappropriate control of these cells by regulatory T cells operating by various mechanisms, associated with a breach in oral tolerance (5-6). IgE-mediated food allergy has been estimated to affect 1-2% of the adult population, and up to 5-7% of children (7).

Epidemiologic studies demonstrate a significant increase in the prevalence of allergic diseases in industrialized countries during the last decades (8). This increase has been associated with environmental factors associated with the Western lifestyle, including high socioeconomic standards and decreasing contacts with microbes and microbial products during early infancy, as stated by the "Hygiene Hypothesis" in its initial form (9) and subsequent revisions (10-11).

Although allergic symptoms can be controlled by pharmacological treatment, specific immunotherapy (SIT) represents until now the only causal therapy toward type I allergy. However, classical SIT is still associated to some drawbacks, such as allergic side reactions in some patients and long duration of the treatment with repeated injections (12). Moreover, the usefulness of SIT to treat food allergy is still controversial, so that no effective treatment is available for food allergy and its primary management still consists of strictly avoiding relevant allergens (13). Consequently, there is great interest in exploring novel therapeutic approaches based on less invasive routes, such as the mucosal route, and new immunomodulatory agents able to restore a favourable Th1/Th2/Treg balance.

There is evidence from epidemiological studies that the development of allergy in genetically predisposed subjects is associated with a different proportion of the bacterial phyla belonging to the gut microbiota in comparison with normal subjects (14). From this observation, the rationale for using "normal commensal bacteria" in the attempt to restore in the gut conditions as close as possible to the healthy subjects is logically derived, and has been proposed for the prevention and therapy of allergic diseases (15-16).

Probiotics, which are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (17), are claimed to beneficially affect the immune system in several physiological and pathological conditions, including inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), obesity, type I diabetes, and allergies. Several clinical studies have been designed to explore the effective role of probiotics in the prevention and therapy of allergic diseases, mostly focused on infancy food allergies and atopic dermatitis. In summary, the results of these studies, although promising, are not conclusive yet, and it is generally accepted that there are currently insufficient data to recommend probiotics as a part of standard therapy in any allergic conditions (18-19). Larger, controlled studies with well defined probiotic bacteria and perhaps mixtures of different strains are needed to determine the actual role of these products, and to elucidate the immunological mechanisms involved.

On these bases, the aim of the present research is to investigate the immunomodulatory capacity of a mixture of different probiotic strains on specific immune responses and anaphylactic reaction in mouse models of allergic sensitization to food and inhalant allergens.

Methods

Probiotic preparation

The probiotic preparation VSL#3 (VSL#3 Pharmaceuticals, Fort Lauderdale, FL, USA), which contains eight different bacterial strains (four Lactobacilli, three Bifidobacteria, and one *Streptococcus thermophilus*) was used throughout the study. We used both the live VSL#3 mixture for oral administration and a sonicated preparation for intranasal treatment.

Inhalant allergy model: prophylactic protocol

A mouse model of sensitization to the major allergen of *Parietaria judaica* pollen (recombinant Par j 1, rPar j 1) has been developed and characterized in our laboratory (20). Different groups of Balb/c mice were administered intranasally the sonicate preparation of VSL#3 or PBS as control for eight days. The sonicate preparation was chosen to avoid an inappropriate colonization of nasal mucosa by live bacteria. Then, mice were intraperitoneally immunized with rPar j 1 in alum. Serum total and specific IgE antibodies and specific IgG subclasses were measured. Fractions of lung tissue were collected for the evaluation of local cytokine gene expression (21).

Food allergy model: therapeutic protocol

The therapeutic activity of live VSL#3 mixture was assessed in a mouse model of oral sensitization and anaphylaxis to the food allergen Shrimp Tropomyosin (ST), which is the only major allergen of Crustaceans, recognized by above 80% of Crustacean-allergic subjects (22).

C3H/HeJ mice were intragastrically sensitized with purified ST from the shrimp *Metapenaeus ensis* with cholera toxin (CT) as mucosal adjuvant. ST-specific IgE, IgG1, IgG2a in the serum, and IgA in faecal extracts were evaluated by ELISA. To induce *in vivo* anaphylaxis, mice received an oral challenge with ST. Local and systemic anaphylactic reactions were scored according to symptoms observed and to histamine levels in faecal extracts. At the end of the sensitization period and after the first challenge, different groups of mice were orally treated for four weeks with live VSL#3 preparation to allow gut colonization.

The effects of the probiotic treatment were also evaluated by assessing cytokine and transcription factor gene expression in the jejunum (23).

Results and conclusions

Prophylaxis of inhalant allergy

The prophylactic treatment with VSL#3 induced a significant reduction of serum allergen-specific IgG1, but did not affect levels of total and Par j 1-specific IgE and IgG2a. Gene expression of cytokines and transcription factors has been evaluated in lung tissue, comparing the pattern found in the two experimental groups (treated with VSL#3 or PBS) to that observed in naïve mice. VSL#3 pre-treatment was able to completely prevent the IL-13 local upregulation observed in sensitized mice, and to reduce IL-4 expression to the levels found in naïve mice. IL-10 expression, reduced in sensitized and PBS pre-treated mice, was increased in VSL#3 pre-treated mice VSL#3 treatment did not affect GATA-3 expression. On the other hand, the significant reduction of T-bet expression level observed in Par j 1 sensitized mice was restored up to the level found in naïve mice by the probiotic pre-treatment. The consequent reduction of the ratio between GATA-3 and T-bet expression could be associated to the down-regulation of the Th2 response.

Intranasally delivered probiotic bacteria have the capacity to prevent the development of allergen-specific Th2 responses induced *in vivo* against rPar j 1. Probiotic prophylactic treatment does not induce at the local level a switch to Th1-like responses (IFN-γ and T-bet expression in the VSL#3-treated group are comparable to that found in naïve mice), but could rather induce a skewing towards a regulatory pattern mediated by IL-10 producing regulatory cells.

Therapy of food allergy

The food allergy mouse model developed in our laboratory show some significant features allowing its application for the preclinical evaluation of novel therapeutic approaches, such as the use of an allergen relevant for human sensitization (ST), the oral route of sensitization, and the induction of immune and clinical features of human disease, including anaphylactic response. In this setting, oral therapeutic treatment with live VSL#3 was able to significantly reduce both systemic and local anaphylactic symptoms induced by oral challenge with the sensitizing allergen ST. Oral therapeutic administration of VSL#3 to ST-sensitised mice significantly reduces symptom score and histamine release in the faeces following allergen challenge, as well as specific IgE response in the serum. In the jejunum, IL-4, IL-5 and IL-13 tissue content was significantly reduced, whereas FOXP3 and IL-27 mRNA expression, IL-10, TGF-β and IFN-γ tissue content were up-regulated.

These results, obtained in a mouse model mimicking human anaphylaxis to a food allergen, support the therapeutic potential of the oral administration of a probiotic mixture on an established food allergen sensitization, for which no effective treatments based on the pathogenetic mechanisms are available.

The induction of protective immune responses at the sites of direct allergen exposure linked to counter-regulatory local and systemic immune responses, as it can be achieved by mucosal delivery of safe probiotic bacteria balanced mixtures, might become an effective strategy in the

prevention and therapy of type I allergy. Further studies are needed to fully elucidate at the preclinical level the mechanisms of their immunomodulatory action.

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BIOFILM-PRODUCING GROUP A STREPTOCOCCAL INFECTIONS: MANAGEMENT AND TREATMENT

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Streptococcus pyogenes, or Group A Streptococcus (GAS), is a common colonizer of the upper respiratory tract in humans, that represent the only natural host. The most frequent pathological manifestations of GAS are mild suppurative throat and skin infections, with a worldwide estimate of 727 million cases per year. The cost for the health care system is rather heavy because, even though the infections are normally mild and self limiting, antibiotic treatment is suggested, first to relieve discomfort but also to minimize transmission and reduce complications. In fact, in susceptible hosts, GAS infections may lead to life-threatening complications such as sepsis, necrotizing fasciitis and toxic shock, or debilitating sequelae such as rheumatic fever, glomerulonephritis or tics.

The antibiotic of choice for streptococcal infections still remains penicillin. The ability of penicillin and related antibiotics (e.g., amoxicillin) to kill group A streptococci has not changed in more than 50 years. Up to now, there has never been a report of a group A streptococcus grown from a person resistant to this class of antibiotics. Thus, it appears that, in nature, Group A strep are unable to acquire resistance to penicillin.

Other possible therapeutic choices, particularly in case of allergic reactions to b-lactams, include macrolides, although macrolide resistance has showed an increasing trend in the last decades, with resistance rates which vary considerably in different countries. They range around 10%, but may reach up to almost 30% in some part of Europe, with marked regional variations in resistance rates (Table 1).

Table 1. Incidence of macrolide resistance in different countries

Year of publication	Country	Incidence of macrolide resistance (%)
2000	Belgium	10
2002	Finland	16.5 down to 8.6
2007	Canada	42
2007	Italy	26 down to 18
2008	Portugal	26 down to 13
2008	Denmark	3
2009	USA	3.5-4.5 (down from 9%)
2010	Norway	3.4
2010	France	1990 to 2003 - from 6 to 24

Despite the availability of an antibiotic which should be universally effective, *S.pyogenes* infections may fail to respond to antibiotic therapy leading to persistent throat carriage and recurrent infections. Kuhn and colleagues (2001) and Conley *et al*. (2003) examined a cohort of patients (104 and 99 patients respectively) with GAS throat infections, paired for age, sex and other parameters. In both cases one third of infections failed to respond to penicillin therapy.

Having established that group A strep does not carry the genetic determinants for resistance to penicillin, it is clear that such failures must have different explanations.

Recently, Pichichero and coworkers (2007) reviewed a list of factors possibly implied in such failures. Among these, of importance the carrier state which often depends on recurrent exposure; lack of compliance or a too early start of therapy during the infection, which may result in a deficient immune response. Also poor tonsillar penetration of the antibiotic used for treatment; presence of other microorganisms able to produce penicillinase, thus destroying the antibiotic before it came in contact with GAS, may represent important factors. An elegant study published in Lancet a few years ago (2001), suggested that especially macrolide-resistant GAS may be equipped with molecules favouring cell penetration (bacteria are often found intracellularly especially in peritonsillar abscesses); as penicillin does not penetrate epithelial cells, GAS would be protected. In light of more recent studies, also biofilm should be added to the list (Baldassarri *et al*., 2006). It is known that biofilm may certainly contribute to phenotypic resistance to anti-infective agents. Also what has been known as "tolerance" until now may in fact be an aspect of the resistance conferred by biofilm. In stationary-like phase (such as in biofilm), in which cell wall synthesis is minimal, penicillin may be ineffective (Eagle effect), also because several PBPs of GAS are lost when the bacterium enters the stationary phase *in vitro*.

The first indications that also *S.pyogenes,* besides other streptococcal species such as *S.salivarius* or *S.bovis*, was able to produce biofilm came from histological observations of structured communities present in human or animal model lesions (Akyiama *et al*., 2003; Neely *et al*., 2002; Hidalgo-Grass *et al*., 2004)

Biofilm formed *in vitro* is possibly less organized compared to the classical one, such as that produced by *Staphylococcus epidermidis*. Curiously, it appears that GAS start forming biofilm at the extremities of the bacterial chains, a character suggesting a "specialization" of terminal cells that has been already observed for GAS in cell attachment (Molinari *et al*., 2000).

As for the mechanism possibly involved in biofilm formation and regulation (Figure 1), it has been suggested that GAS starts as biofilm, colonizing the mucosa of the upper respiratory tract, then external stimuli may operate on a transcriptional regulator such as srv (which regulates several virulence factors), which in turn affect production/expression of the cysteine protease speB which degrades protein and DNA, which are integral part of the biofilm, releasing cells for colonization of distant sites (Doern *et al*., 2009; Sumby *et al*., 2006; Walker *et al*., 2007).

Alternatively damage by speB is perceived as signal inducing mutation in covS, another regulator, which would repress speB, induce sdaI (a dnase) and again leading to degradation of protein and DNA and cell releasing.

A number of molecules have been suggested to be in relation with biofilm production: such as M protein, or analogous of the M protein (Courtney *et al*. 2009), the product of the gene *has*A (hyaluronic acid capsule) which is not required for biofilm formation in static system, but it may be needed for aggregation and biofilm maturation (evaluated in flow conditions).

As is well known, in general biofilm-embedded cells are more resistant to anti-infective agents compared to planktonic cells of the same culture.

Conley and colleagues (2003) reported that while no strains of 50 from pharingits were resistant to penicillin, only one to the combination of penicillin and rifampin and 7 to rifampin alone, a large percentage of the 30 strains for which the minimal biofilm eradication concentration was determined where non susceptible to the antibiotic tested, including penicillin.

Besides being "insensitive" to penicillin treatment, we observed that penicillin at subMIC concentration may stimulate an increment in biofilm formation (Figure 2).

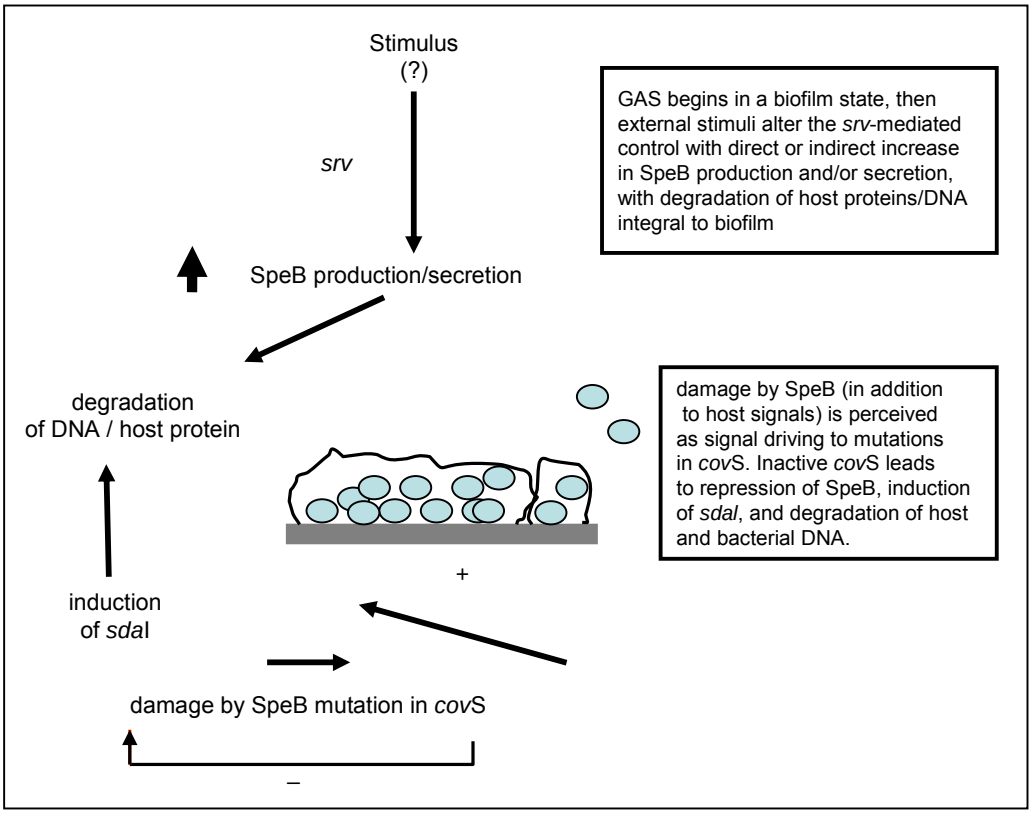

Figure 1. Possible mechanisms involved in biofilm formation by GAS

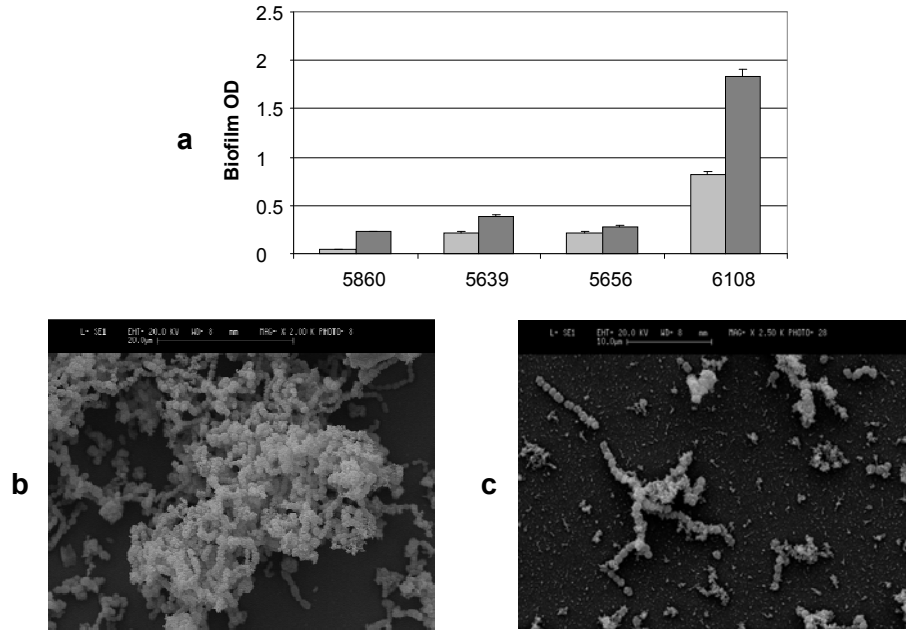

Figure 2. Biofilm OD of four GAS isolates after growth in plain medium (light grey bars) or in the presence of penicillin at ½ of the MIC (dark grey bars) (a).
Scanning electron microscopy of cells grown in presence of penicillin (b) or in plain medium (c)

Analogous effect could be observed for erythromycin, tested on susceptible strains.

We found that such effect was all the more common depending on the antibiotic resistance pattern of the isolates. In fact, those carrying the *erm* gene, which codes for macrolide resistance through methylation of the target site, where less susceptible to the presence of subMIC penicillin, while those carrying genes coding for efflux pumps, were more easily affected.

In the most recent years compounds other than antibiotics have been taken into consideration for their possible action on biofilm-producing microorganism, in general, and on GAS. Among those cationic peptides (CAMPs) have received some attention, as well as other natural compounds such as plant extracts or coral-associated actynomycetes that have been found to interfere with quorum sensing signals and biofilm formation, without effect on growth rates (Limsuwan & Voravuthikunchai, 2008; Nithyanand *et al*., 2009; Rasooli *et al*., 2008).

Cationic peptides in particular came to our attention, as are major factors for their antibacterial activity on mucosal surfaces. In a small collection of isolates, characterized by different resistance pattern, we evaluated the effect of three different CAMPs. While no difference could be found for indolicidin and polimixin E, i.e. all strains showed the same MIC, and interesting findings was that with nisin. We found that biofilm embedded cells were more susceptible to the action of nisin (Figure 3), a finding in agreement with that the strains with higher MIC (that could grow at higher CAMP concentration) were those producing less biofilm.

A possible cross-resistance to the cationic peptide appeared to be conferred by either the methylation or the efflux pump coding genes, as the majority of the susceptible strains would grow at lower nisin concentration, while it was the other way around for resistant isolates.

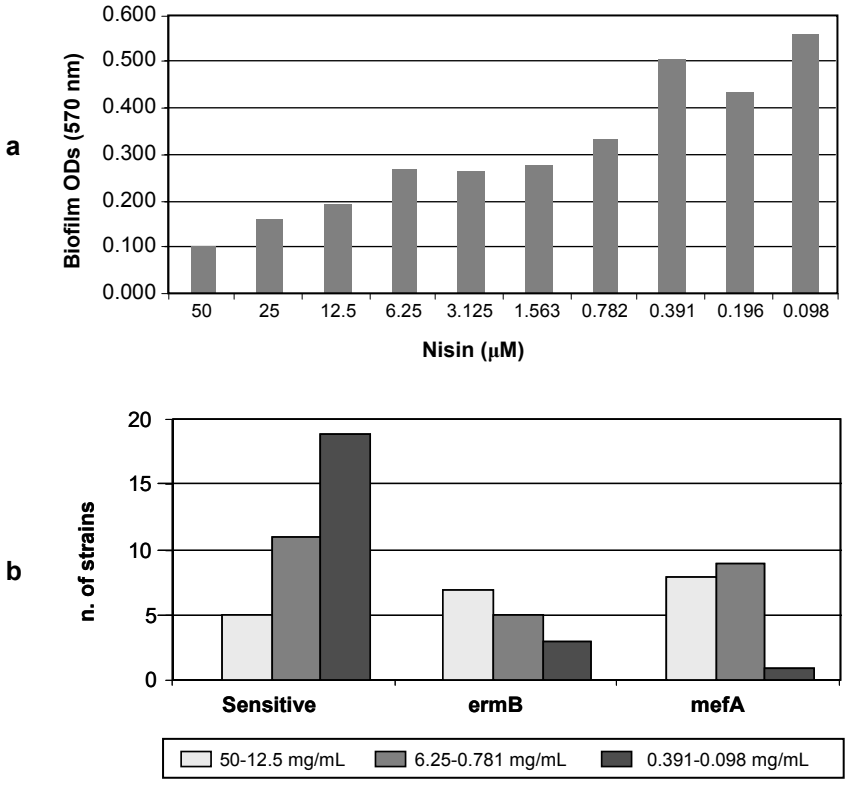

Figure 3. MICs to nisin in a collection of 100 GAS isolates (a), and relationship of MICs to ability to form biofilm (b)

To summarize, clinical practice indicates stabilized procedure for treatment of streptococcal pharyngitis, which are especially important in countries, such as India or Australia, where post streptococcal sequelae such as acute rheumatic fever represent a heavy burden. Thus, long term oral penicillin, erythromycin or clindamycin and vancomycin, are the therapeutic strategies upon which a consensus exists.

However, the best indication to decide the most appropriate therapeutic approach still remains the evaluation of the clinical picture. Also, follow up of the cases should be pursued, for early identification of the carrier state or identification of subjects more prone to recrudescence. Further, knowledge of the local epidemiology as far as resistance rates are concerned is fundamental, to decide appropriate antibiotics alternative to penicillin.

Of fundamental importance remains the pursue of additional information on the mechanism through which antibiotics stimulate biofilm formation and further investigation evaluating substances active on GAS biofilm.

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ANTI β-GLUCAN ANTIBODIES AND ANTIFUNGAL PROTECTION

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Background

Deep-seated infections caused by *Candida*, *Aspergillus* and *Cryptococcus* fungal pathogens are increasingly impacting on public health and carry a high mortality toll, even in the presence of effective antifungal chemotherapy, due to drug toxicity and the emergence of resistant strains.

Particularly daunting are the high mortality rates following nosocomial acquisition of *Candida* bloodstream infection, which approach 40%, especially when considering that this fungal species is now the fourth most common nosocomial bloodstream isolate in the USA and in the European countries. *Candida* species, mostly *C.albicans*, are also responsible of non life-threatening but very distressing infections such as chronic, recurrent vaginitis, which affects nearly 2% women in fertile age and is almost completely refractory to antifungal chemotherapy. Novel therapeutic approaches for a more efficient control of fungal pathogens, are thus intensely investigated. Among these, the development of antifungal vaccines or therapeutic antibodies (Ab) is considered a particularly attractive strategy by most authors in the field.

Several experimental vaccines and Ab preparations have been candidated in recent years as possible, innovative tools for the prophylaxis or theraphy of any of the most widespread fungal diseases (1). Almost all these reagents, however, target fungal molecules present only in one or very few, related fungal species and have a restricted spectrum of protective activity. This may represent a limiting factor in the treatment of patients at risk for fungal diseases, that are tipically predisposed to the attack of different pathogenic fungi, or in the absence of a precise microbiological diagnosis, that is often particularly laborious in fungal infections.

We hypothesized that β-glucan, a cell wall polysaccharide mainly consisting of β1,3 and β1,6-linked repeating units of D-glucose cross-linked together, could represent an ideal, "universal" target for antifungal Ab therapy and vaccination. In fact, this polysaccharide is essential for the growth and the survival of fungal cells, is involved in fungal virulence and is present, with a similar basic molecular structure, in all major pathogenic fungi. Following this concept, we generated a β-glucan (laminarin)-CRM197 glycoconjugate vaccine (Lam-CRM) and an anti-β-glucan, monoclonal Ab (mAb 2G8) and demonstrated that anti-β-glucan Abs raised by Lam-CRM vaccination, as well as administration of mAb 2G8, could induce a high degree of cross protection, in animal models, against different, largely prevalent agents of fungal infections such as *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* (2, 3).

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On the basis of these data, we proposed glucan-based vaccines and anti-β-glucan Abs as particularly interesting candidates for the development of novel, broad-spectrum immunological treatments against fungal diseases. To this aim, however, it was necessary to characterize precisely the Abs and β-glucan antigens that can generate a protective immunity and to elucidate the mechanisms of immune protection, also in consideration of the large variability of protective value among antifungal Abs with similar or even equal antigen specificity. We therefore addressed further studies, using the model fungal pathogen *C.albicans* and different *in vivo* and *in vitro* approaches, to gain insights into the protective β-glucan epitopes, the molecular target(s) of anti-β-glucan Abs in fungal cells and the protection-relevant Ab biological activities.

Protective and non protective anti β-glucan Abs

We generated and described two murine anti-β-glucan monoclonal Abs (mAb) with identical variable regions but different isotypes (mAb 2G8, Ig G2b and mAb 1E12, IgM). Interestingly, despite identity of antigen-binding regions, only the IgG mAb 2G8, but not the IgM mAb 1E12, was able to provide protection against different *C.albicans* infections (Figure 1) (4).

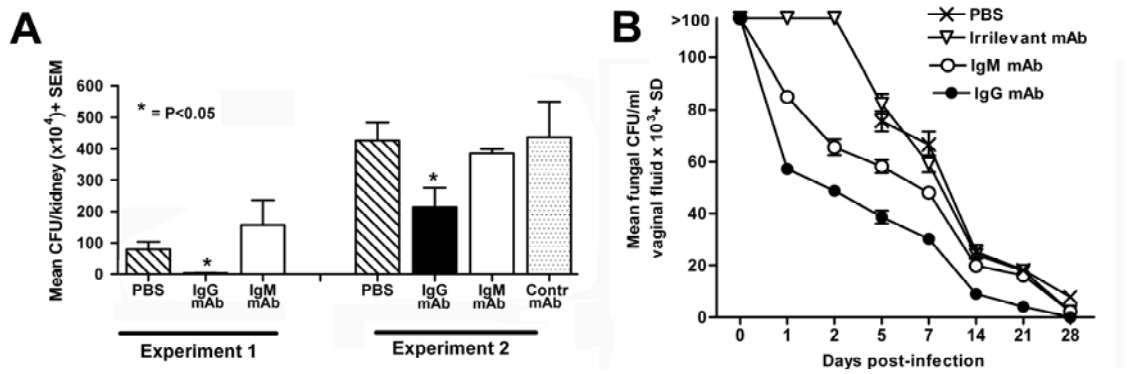


Figure 1. Different anti-*Candida* protective activity of mAbs 2G8 and 1E12

In fact, in a murine model of disseminated candidosis, pretreatment with the IgG mAb (100 µg/mouse, 2 h pre-challenge) was able to reduce significantly the fungal burden (CFU) in the kidney, an activity which is predictive of protection. At difference, pretreatment with the IgM mAb was completely uneffective (Figure 1A).

Similarly, in a rat model of vulvovaginal candidiasis, administration of the IgG, but not of the IgM mAb at 1, 24 and 48 hours after an intravaginal challenge with *C.albicans* resulted in an accelerated fungal clearance from the vagina and a more prompt resolution of the vaginal infection (Figure 1B).

Different ability for antifungal protection by the mAbs was associated to differences in fine β-glucan specificity. In ELISA (Figure 2A) the protective IgG bound strongly laminarin (a glucan with prevalent β1,3 linkages) and only weakly, at high antibody concentration, pustulan (a β1,6 linked-glucan) whereas the non protective IgM bound significantly both laminarin (though less strongly than the Ig G) and pustulan. In addition, microarray analyses performed to

characterize in details the epitopes recognized by the two mAbs showed that the protective IgG mAb bound selectively to laminarin-type (β1,3-linked) oligosaccharides starting from a minimum DP of 4 glucose units and with maximum binding to octaose. In contrast, the IgM mAb bound not only to the β1,3 oligosaccharides but also to those with β1,4 and β1,6 linkages (Figure 2B).

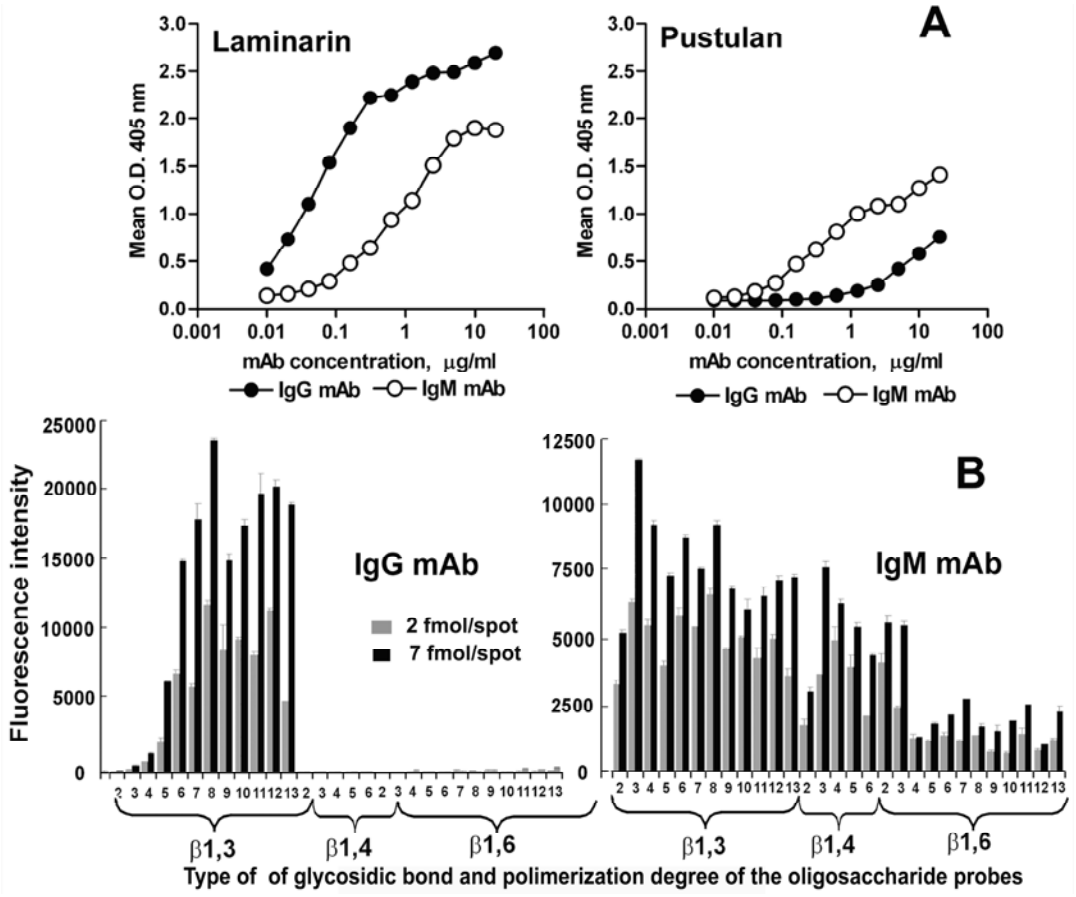


Figure 2. Fine β-glucan specificity of the IgG and the IgM mAbs

To investigate more in depth the possible relationships between recognition of specific β-glucan sequences and protective activity, we performed vaccination experiments with a panel of different CRM197 conjugates constructed with natural or synthetic, β-glucans of distinct molecular structure (Table 1). Reactivity of the Abs induced by the different vaccines toward β1,3 and β-1,6 glucan was evaluated by ELISA (laminarin or pustulan as the antigens) and protective activity was assessed, in vaccinated mice, by the ability to decrease mortality rates and fungal invasion in organs following a systemic challenge with *C.albicans* (5).

These experiments showed that all conjugates, adjuvanted with MF59, were efficacely immunogenic for mice, but elicited different patterns of Ab response in terms of binding specificity to β1,3 or β1,6 linked glucan molecules (Table 1). Moreover, vaccine-induced protection against an experimental, systemic challenge with *C.albicans* was highly variable among the different conjugates, and those eliciting a prevalent or exclusive anti β-1,6 glucan Ab response consistently resulted poorly or not protective (Table 1).

Table 1. Antibody response to different β-glucan-CRM197 conjugates and degree of induced, anti-*Candida* protection

β-glucan	Basic molecular structure	Specificity of induced Abs	Degree of protection
Laminarin	Linear β1,3 glucan with rare β1,6-linked side chains of a single glucose residue	Prevalent anti-β1,3 glucan	High
Curdlan	Linear β1,3 glucan	Anti-β1,3 glucan	High
Pustulan	Linear β1,6 glucan	Anti-β1,6 glucan	None
Synthetic 15 mer	Linear β1,3 oligosaccharide of 15 glucose residues	Anti-β1,3 glucan	High
Synthetic 17 mer	Similar to 15 mer but with two β1,6-linked side chains of a single glucose residue	Mixed anti-β1,3/β1,6 glucan	Low

On the whole, these data indicated that not all anti-β-glucan Abs are equally protective. In particular, the Ab isotype can significantly influence their selectivity and intensity of binding to particular β-glucan sequences and significantly impact their protective properties.

In addition, a restricted specificity for β1,3 glucan sequences appears to be the most favourable for protection, whereas exclusive or prevalent specificity for β1,6 glucan sequences is associated to lack of protective activity.

Worth of note, immunogenicity and protection experiments with the different β-glucan conjugates also indicate that the anti-β-glucan Ab profile induced by vaccination could be directed as desired, at least in terms of β1,3 versus β1,6-glucan specificity, by modifying appropriately the structure of the antigen in the glycoconjugate vaccine, a finding of some relevance for the future development of β-glucan-based, antifungal vaccines.

Antifungal activities of anti-β-glucan Abs

Two major biological activities by which antifungal Abs can provide protection are: i) blocking of fungal adherence to host epithelia, thus preventing tissue invasion; ii) opsonization of fungal cells and consequent enhancement of the antifungal activities of host phagocytes.

Thus, we tested *in vitro* the protective mAb 2G8 for any effects on the adherence of *C.albicans* hyphae to human epithelial cells. In the presence of the mAb we found significantly fewer adherent hyphae in co-cultures of *C.albicans*-Hep-2 cells, as compared to control co-cultures with an irrelevant mAb (Figure 3A). In these experiments, the non protective Ig M mAb was devoid of any effect, suggesting that inhibition of adherence to host epithelia might be relevant for anti-β-glucan Ab-mediated protection *in vivo*, as reported for several other protective antifungal Abs (4).

Next, we addressed the issue of whether the binding of anti-β-glucan Abs on *C.albicans* surface could facilitate the killing of fungal cells by human polymorphonuclear neutrophils, that are crucial defensive effectors in fungal diseases. To this purpose, we exploited two recombinant, mouse-human chimeric Abs derived from mAb 2G8, recently generated and expressed in *Nicotiana benthamiana* plants, in the format of complete IgG or scFv-Fc (6). These recombinant Abs fully retained the β-glucan binding specificity of the cognate murine mAb against *C.albicans* and were able to confer significant antifungal protection in animal models of systemic or vulvovaginal *C.albicans* infection (6). As shown in Figure 3B, the binding of the

recombinant Abs, in both formats, to *C. albicans* cells resulted in a much more efficient fungal killing by neutrophils as compared to the irrelevant Ab-treated *Candida* cells, and this effect was similar to that observed with a human serum containing high levels of opsonizing anti-*Candida* mannoprotein Abs.

Both the 2G8 mAb and the purified IgG fraction from sera of Lam-CRM-immunized mice also proved able to inhibit directly fungal growth, without the intervention of any host cell, an unusual property that characterizes a few other antifungal Abs. This was evaluated, as shown in Fig. 3C and D, in growth inhibition assays where *Candida* cells were treated with the Abs for 18 h and then enumerated by a standard CFU count. Here again, the non protective IgM mAb 1E12 showed only a very weak activity as compared to the 2G8 IgG at the same concentration (Figure 3 C).

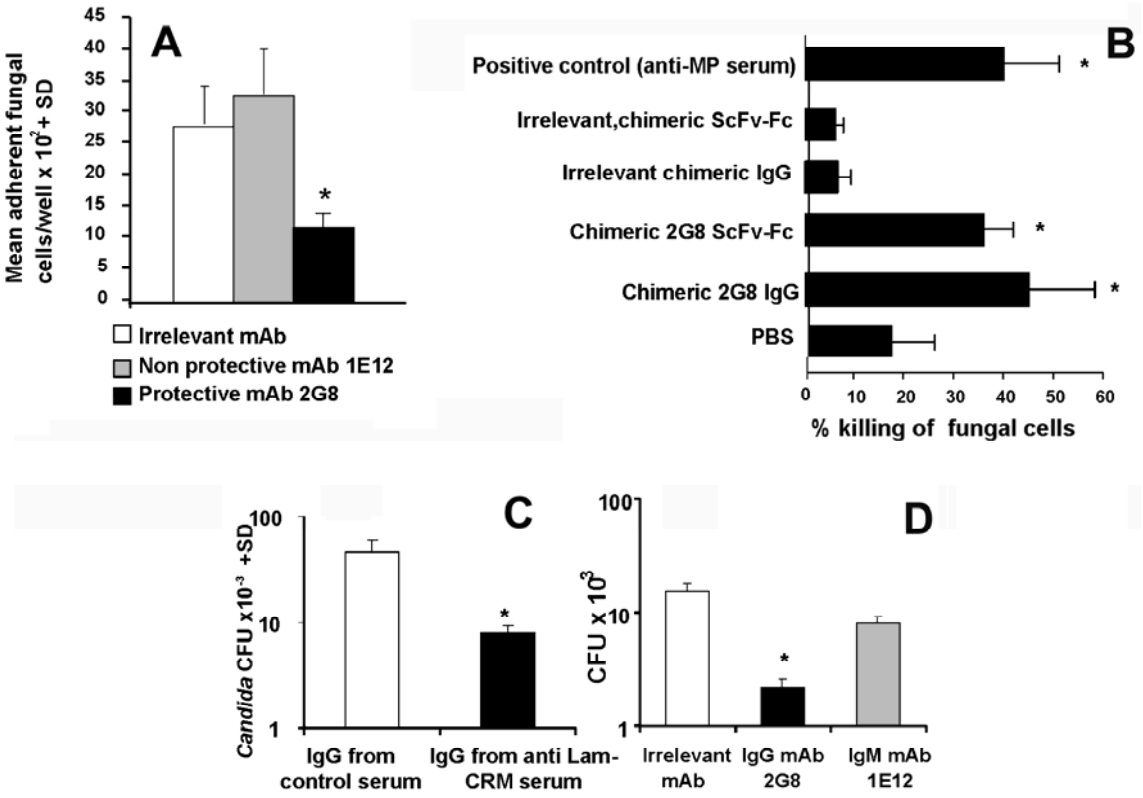


Figure 3. Anti-*Candida* activities of the anti-β-glucan Abs

Overall, these experiments demonstrated that the anti-β-glucan Abs exert different antifungal activities that can account for their protective activity *in vivo*. Besides opsonisation, that is one of the main mechanisms whereby the host can eliminate or control *Candida* through activation of phagocytes, the Abs appear to inhibit directly some critical virulence properties of the fungus, such as hyphal growth and adherence *in vitro*. These latter antifungal activities, that do not require the cooperation of host immune cells, might be particularly suitable for protection of patients with numerical or functional defects of immune cells, that are those mostly at risk for invasive fungal infections.

Molecular targets of protective anti β-glucan Abs

Until recently, β1,3-glucan was considered a merely structural fungal component, buried in the rigid, inner cell wall layers. Instead, it is now clear that this componentis also present at cell surface (see the pattern of immunogold staining of *C.albicans* cells with mAb 2G8 in Figure 4 A), in close association with cell wall proteins, most of which are covalently linked to β-glucan in the cell wall structure (Figure 4B) (7). It is also known that β-glucan is abudantly released during fungal growth, so that its detection in patient serum is valued as a diagnostic marker of invasive fungal infection, in association with several cell surface glycoproteins that are likewise released into the external milieau. We thus speculated that the anti β-glucan protective Abs could mostly target the glucan bound to some cell wall proteins of the outer cell wall, rather than the chitin-linked β-glucan of the inner cell wall skeleton.

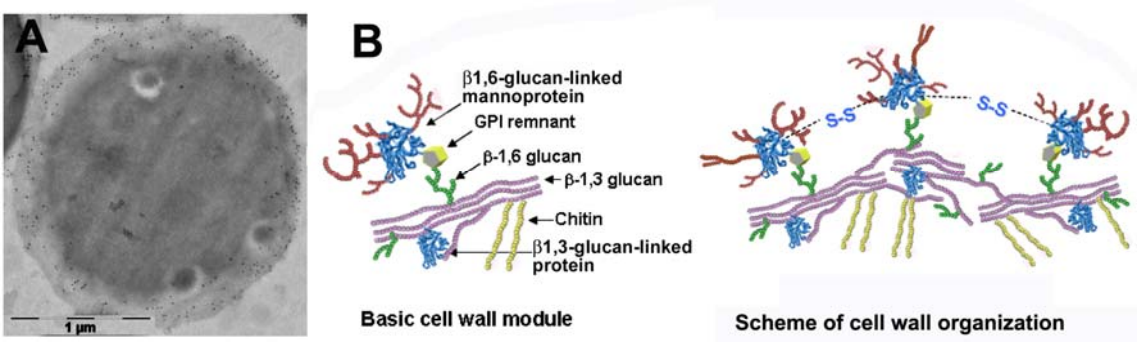
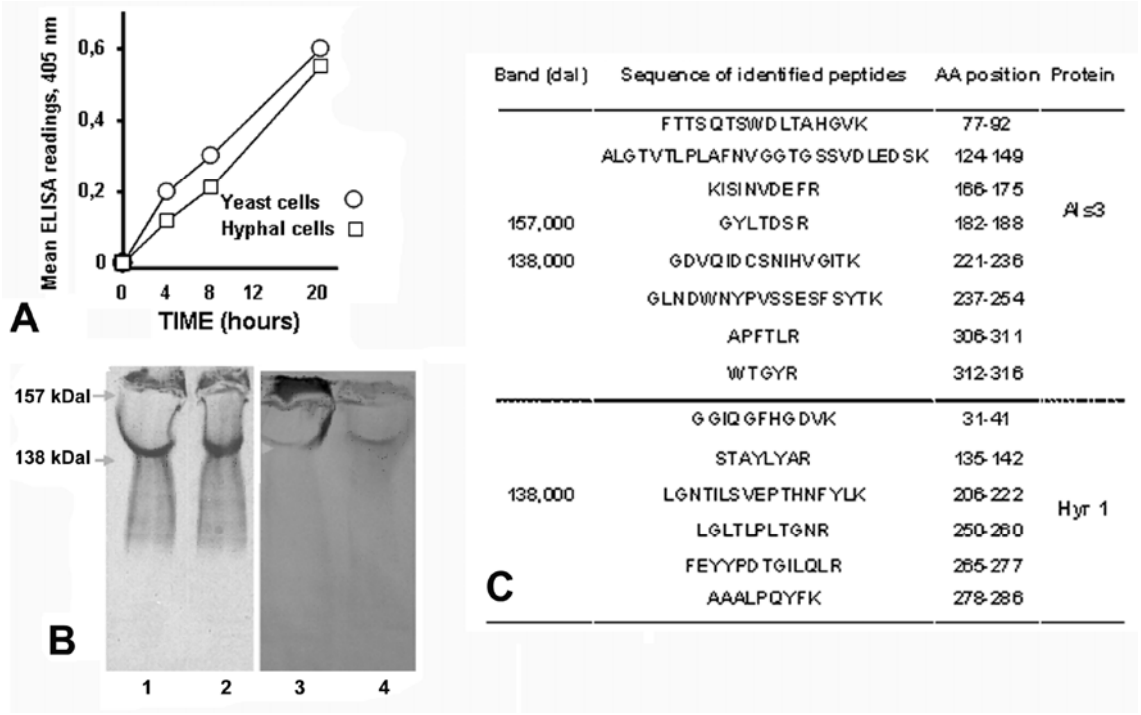


Figure 4. β-glucan in cell wall structure of *C.albicans* and expression of mAb 2G8-reactive β-glucan epitopes in fungal cell

To test this hypothesis, we first analysed by ELISA *C.albicans* secretion, and found that a significant amount of β-glucan material reactive with the protective mAb 2G8 was progressively released during growth by both yeasts and hyphae (Figure 5A) (4). Then, we analyzed this secreted material by SDS-PAGE and Western blot to identify possible, discrete protein components bearing mAb-reactive β-glucan motifs. This analysis revealed, whitin a polydisperse, heterogeneous smear, two fairly distinguishable mAb 2G8-reactive bands, with an approximate molecular weight of 157 and 138 KDal (Figure 5B. Lane 1 and 2: secreted material from yeast cells and hyphae, respectively). Two corresponding bands were also detected among cell wall proteins extracted by SDS or β1,3 glucanase from isolated cell wall preparations (Figure 5B, lane 3 and 4, respectively), suggesting that the secreted proteins bound by mAb 2G8 originated from fungal cell wall. To identify the 138 and 157 KDal bands in fungal secretion, these were excised from the gels, subjected to controlled proteolysis with trypsin and analyzed by mass spectrometry, yielding several peptide mass signals. A MASCOT search was carried out against the fungal protein sequences in the NCBInr database, that clearly identified the Als3 protein in both 157 and 138 KDal bands and the Hyr protein in the 138 KDal band (Figure 5C). These are two well known GPI-anchored cell wall proteins that exert critical roles in *C.albicans* virulence. In particular, Als3 is a major fungal adhesin, also involved in biofilm formation, whereas Hyr1 is activated upon contact with host phagocytes and is critical for fungal transition from the yeast form to the more invasive hyphal form (8-11).



Band (dal)	Sequence of identified peptides	AA position	Protein
	FTTSQTSWDLTAHGVK	77-92	Als3
	ALGTVTLPLAFNVGGTGSSVDLEDSK	124-149	
	KISINVDEFR	166-175	
157,000	GYLTDSR	182-188	
138,000	GDVQIDCSNIHVGITK	221-236	
	GLNDWNYPVSSESFSYTK	237-254	
	APFTLR	306-311	
	WTGYR	312-316	
	GGIQGFHGDVK	31-41	Hyr 1
	STAYLYAR	135-142	
138,000	LGNTILSVEPTHNFYLK	206-222	
	LGLTLPLTGNR	250-260	
	FEYYPDTGILQLR	265-277	
	AAALPQYFK	278-286	

C

Figure 5. MAb 2G8-reactive proteins in *C.albicans* secretion

Both Als3 and Hyr 1 are known to be actively secreted by *C.albicans*. Their strong binding by the mAb 2G8 would indicate that they are secreted with attached β-glucan, in particular β1,3-glucan, moieties and suggests that these proteins might be possible targets of the protective action of the anti-β-glucan Abs. To this regard, recent data demonstrated that the Hyr1 protein increases fungal resistance to neutrophil antimicrobial activities and that Hyr1 neutralization by anti-Hyr1 Abs promotes *C.albicans* killing by neutrophils (9). In addition, it has been reported that some anti-Als3 Abs can block *C.albicans* adhesion to human epithelial and endothelial cells and/or inhibit fungal growth *in vitro* (8-11). Therefore, it is possible that the anti-β-glucan Abs, at least in part, exert their antifungal effects through their interaction with the Als3 and Hyr1 proteins. It cannot be excluded, however, that the protective Abs interact with, and inhibits the function of other, unidentified glucan constituents exerting a role in fungal virulence or other critical biological proprieties *in vivo*.

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MULTIDRUG RESISTANCE IN *SALMONELLA*: MOLECULAR CHARACTERIZATION OF A NEW EMERGING CLONE OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM

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Salmonellosis is still one of the most widespread foodborne bacterial illnesses in humans with clinical manifestation ranging from an asymptomatic state to a very severe disease (Galanis et al., 2006). Based on the presence or absence of somatic (O), capsular (Vi), and flagellar (H) antigens (Yan *et al.*, 2003) Salmonella enterica is divided in more than 2400 serovars or serotypes which include typhoidal *Salmonella* (*S.* Typhi, *S.* Paratyphi C and *S.* Dublin) and non-typhoidal *Salmonella* (NTS).

All NTS are etiological agent of zoonoses, diseases and/or infections that can naturally transmit between vertebrate animals and man, mainly by consumption of food of animal origin.

Salmonellosis is one of the infectious diseases monitored across the Europe by the European Center for Diseases Prevention and Control (ECDC) in the framework of Foodborne and Waterborne diseases network (http://www.ecdc.europa.eu/en/Pages/home.aspx) and *Salmonella* infections are the 2nd most commonly reported human zoonoses in Europe, with 108,614 confirmed human cases reported in 2009 (EFSA & ECDC, 2011).

In Italy *Salmonella* infections are subject of mandatory notification (class II of the infectious diseases) to National Health Service (NHS), which registers about 10000 cases of salmonellosis but, for which information on serotypes is often lacking. This information is acquired by a laboratory-based Salmonella surveillance (Enter-net Italia http://www.iss.it/ente/), coordinated by the Istituto Superiore di Sanità, which collects human, environmental, and food isolates through a network of 1000 medical and 10 environmental protection reference laboratories (Dionisi *et al.*, 2011).

Further characterization and subtyping of strains collected through the network are performed using standardized protocols and include antimicrobial resistance tests, phagetyping and molecular typing by Pulsed Field Gel Electrophoresis (PFGE). This last technique represents the basis of a molecular surveillance network for food-borne infections in Europe, the Pulse-Net Europe.

Analysis of Enter-net Italia database shows that *S.* Typhimurium (STM) and its monophasic variant *S.* 4,[5],12:i:– (Echeita *et al.*, 2001), represent more than 50% of all salmonella serotypes over the last three years (Dionisi *et al.*, 2011). In addition to their wide diffusion, both STM and its monophasic variant show a high proportion of multidrug resistant strains (MDR,resistant to 4 or more drugs) (Dionisi *et al.*, 2011). Most of the MDR strains are represented by a clonal lineage associated to phagetype DT104 simultaneously resistant to 5 antimicrobial agents, ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (R-type ACSSuT) emerged in UK in the 80s but subsequently this clone became widely distributed in at least four continents (Threlfall *et al.*, 2002).

STM R-type ACSSuT clone is diffused also in Italy however, since 2000, strains of STM and its monophasic variant with a tetra-resistant pattern ASSuT, with or without additional

resistances, but lacking resistance to chloramphenicol, have emerged either among human and food animals isolates (Busani *et al.*, 2004; Graziani *et al.*, 2008; Dionisi *et al.*, 2009).

The main objective of the present study has been the characterization of both STM and S. 4,[5],12:i:– strains with R-type ASSuT, using molecular typing technique in order to evaluate their clonal origin and the relationships with the ACSSuT strains. In addition, by the use of the Pulse-Net database, the study aimed to evaluate if ASSuT strains were present in other European countries in order to set up an international collection of strains for further characterization by the identification of resistance genes, the investigation of their localization, and determination of the resistance region.

A total of 553 STM and *S.* 4,[5],12:i:– strains showing the ASSuT or ACSSuT patterns, with or without additional resistances, has been typed by PFGE (Figure 1).

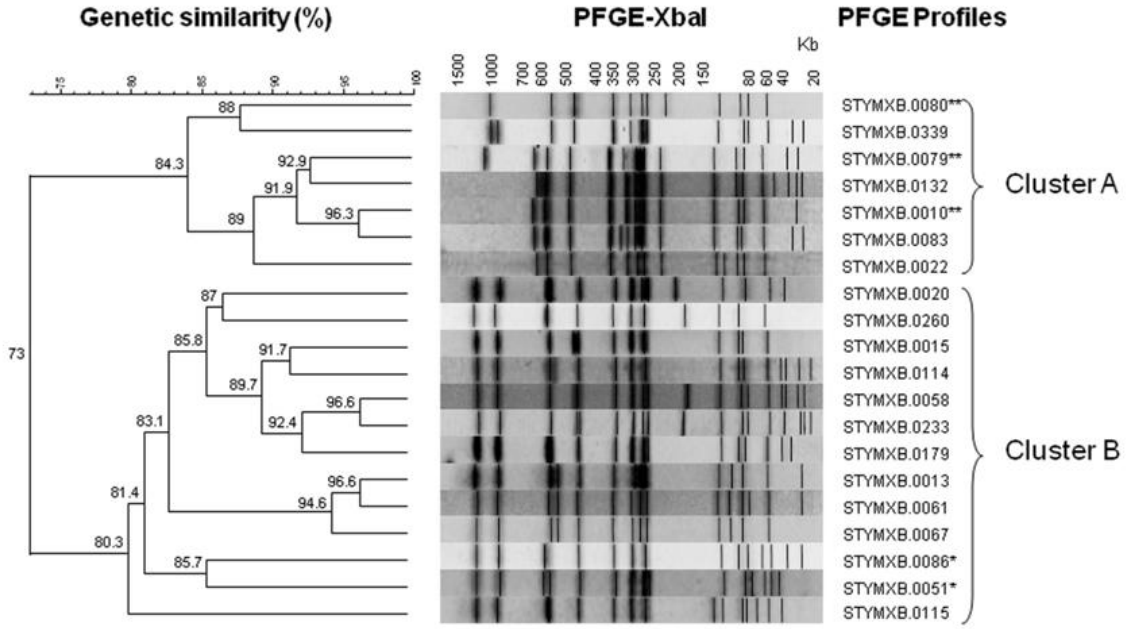

Figure 1. Cluster analysis generated by BioNumerics software of 20 PFGE patterns of ASSuT and ACSSuT *S.* Typhimurium and *S.* 4,[5],12:i:– strains
(From: Foodborne Pathog Dis 2009;6:711-7)

Overall, two large clusters with a 73% of genetic homology were identified. Cluster A included 275 out of the 283 (97.2%) ASSuT strains, while cluster B, included 169 out of the 179 (94.4%) ACSSuT strains, regardless of STM or *S.* 4,[5],12:i:– serovar. In particular the main PFGE profile in ASSuT strains was STYMXB.0079, which accounted for 53.2% of the STM strains and 73% of the monophasic strains. Instead 67.1% of ACSSuT strains belong to PFGE profiles STYMXB.0061 and STYMXB.0067.

By using the Pulse-Net Europe database (Lukinmaa *et al.*, 2006), we were able to identify ASSuT strains, both STM and *S.* 4,[5],12:i:–, isolated in other European countries with the same PFGE patterns as the Italian strains, suggesting that the ASSuT clone is circulating in different European countries.

A total of 64 STM and *S.* 4,[5],12:i:– strains, isolated in Italy, Denmark and United Kingdom, with the ASSuT resistance pattern, were selected in order to identify, by PCR, their resistance genes (bla_{SHV}, bla_{OXA}, bla_{TEM}, bla_{PSE}, *strA-strB*, *aadA2, sul1, sul2, tet*(A), *tet*(B), *tet*(C), and *tet*(G)). Strains were also tested for the presence of class 1 integron.

All 64 ASSuT strains, were positive for bla_{TEM-1}, *strA-strB, sul2* and *tet*(B) and negative for all the other genes tested and for class 1 integron(s).

Conjugation and transformation experiments performed on two strains of R-type ASSuT, belonging to 2 different PFGE profiles, STYMXB.0079 (the most prevalent) and STYMXB.0339, failed, thus demonstrating the inability of horizontal transfer of resistance genes.

Chromosomal localization of resistance genes was demostrated by PFGE using the I-*Ceu* I, a restriction enzyme that recognize only chromosomal DNA, followed by southern blot hybridization using specific probes for *tet*(B), *strA-strB* and 16S r-DNA. The characterization of the chromosomal resistance region was performed by construction of a genomic library of an ASSuT strain. Two clones, positive for the presence of *tet*B and *strAB* gene, identified by hybridization, were subsequently subcloned and sequenced. The consensus map obtained is shown in Figure 2.

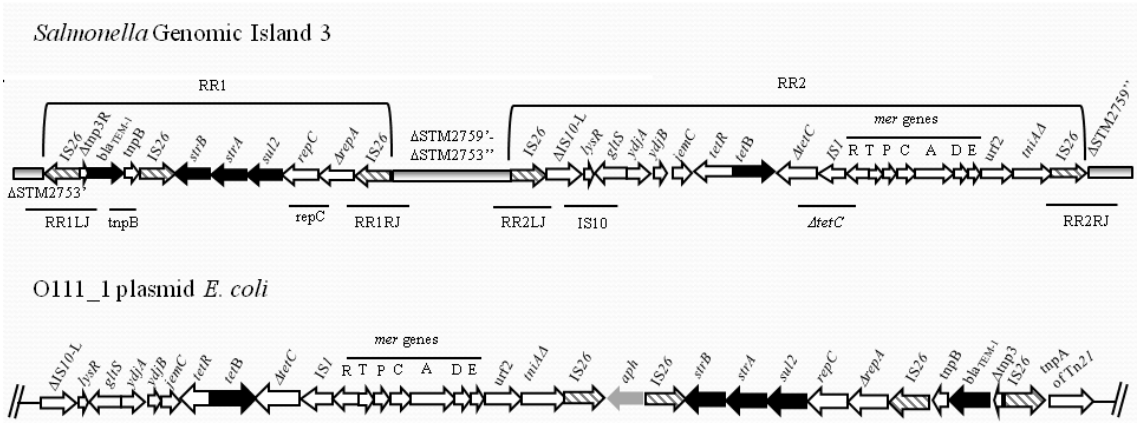

Figure 2. Comparison of the genetic organization of the ASSuT resistance region and O111_1 plasmid (Ac. n° AP010961). Antibiotic resistance genes and IS elements are indicated by black arrows and grey hatched arrows, respectively. Grey boxes indicates chromosomal DNA. Light grey show the fragment lacking in ASSuT resistance region
(From: J Antimicrob Chemother 2012;67(1):111-4)

The ASSuT resistance region genes are localized in two regions: Resistance Region 1 (RR1-8761 bp) and Resistance Region 2 (RR2-14587 bp), inserted in two adjacent loci of the chromosome, which were involved in the polysaccharides transport and metabolism. In particular the RR1, conferring resistance to ampicillin, streptomycin and sulphonamides, was inserted at the STM2753 gene causing a deletion of 190 bp, while the RR2 region, harboring the *tet*(B) gene, was inserted at the gene STM2759, causing a deletion of 6 bp. The chromosomal fragment located between the 2 composite transposons includes the genes from STM2753'' to STM2759' (6543 bp) but in inverted orientation.

The RR1 interrupted the ΔSTM2753' gene and is composed by an IS*26,* a truncated Tn*3* transposon containing the delete resolvase gene (Δ*tnp3R*) and the bla_{TEM-1} gene, followed by a

tnpB and an IS*26* in opposite orientation. Downstream there are the *strA, strB, sul2* resistance genes*, repC* and *ΔrepA* encoding replication protein of RSF1010 plasmid, and at the end another IS*26* element. When compared with multidrug resistance clusters previously described, the RR1 showed 99% sequence identity with a region of the plasmid O111_1 harbored in a *Escherichia coli* strain isolated in Japan in 2001 (Ac no. AP010961). However, in RR1 the resistance gene $bla_{\text{TEM-1}}$ comprised between two IS*26*, is located upstream IS*26*-ΔrepA and inversely oriented.

At the right side of RR1 there is the inverted chromosomal DNA fragment: it starts with ΔSTM2759' and continues till the ΔSTM2753'' gene. This chromosomal fragment is followed by the RR2 which starts with an IS*26* and a defective Tn*10* transposon. This transposon includes a truncated IS*10*-left transposase, *lysR, gltS, ydjA, ydjB* and *jemC* genes with hypothetical functions; *tetR, tetB* genes encoding respectively the tetracycline repressor protein and the tetracycline resistance protein, and Δ*tetC* gene. The Tn*10* transposon has been interrupted by the insertion of an IS*1,* which causes the deletion of *tet*D gene and IS*10*-R. Downstream there is a partial Tn*21* transposon (6025 bp), the mercury resistance operon, followed by *urf2* and *tniAΔ* gene, encoding a hypothetical protein and a putative transposase respectively. The Tn*21* transposon has been interrupted by an IS*26*, and at the right the chromosome continues with ΔSTM2759'' and STM2760 genes (Figure 1).

Also the RR2 show 99% sequence identity with a regions of the O111_1 plasmid. However, in RR2 is present a defective Tn*10*, lacking of the right terminal, as well as in O111_1 plasmid, but truncated also at the left terminal (Figure 1). Finally, our sequence lacks of the *aph* gene, conferring resistance to kanamycin, present in O111_1 plasmid.

This plasmid together with pHCM1, a plasmid found in a *S.* Typhi isolated in Vietnam in 1993 (GenBank AL513383) pAKU_1, an IncH1 plasmid of *S.* Paratyphi A isolated from a Pakistani patient in 2002 (GeneBank Accession no. AM412236) and a 120-kb IncF plasmid pRSB107 isolated from activated-sludge bacteria in a sewage-treatment plant in Germany (accession no. AJ851089) showed a resistance cluster highly similar. In addition the 9-kb sequence, incorporating $bla_{\text{TEM-1}}$, *sul2* and *strA-strB*, flanked by IS*26* elements has been described in other STM strains: on a IncF-like plasmid pU302L (Atlanta; accession no. AY333434) or integrated in the chromosome (Ireland, 1998, accession no. AY524415): all these structures are carried by IS*26* elements as well as in our composite transposons.

A mechanism to assembly this chromosomal resistance region could be mediated by IS*26* but the lacking of direct repeats (DR) flanking the IS*26* elements, as previously reported (Chen *et al.*, 2006; Doublet *et al.*, 2008), allows to exclude a transpositional mechanism. So, even if, there are DNA segment bracketed by two IS*26* elements in direct orientation, the lacking of DR indicates that the various segment of resistance island were probably inserted by multiple illegitimate recombination, rather than by a transpositional mechanism. In addition, the presence of IS*26* elements may have contributed to the intrinsic instability of the resistance regions, resulting in deletions and inversions.

In conclusion all this data demonstrates that the tetra-resistant ASSuT strains of STM and *S.* 4,[5],12:i:–, increasingly isolated in Italy, belong to a same clonal lineage and that the *S.* 4,[5],12:i:– strains circulating in our country mainly derive from this STM clonal lineage. In addition this new multidrug resistance clone is also present in Denmark and United Kingdom.

The antimicrobial resistance pattern conferred by a chromosomal island, with an organization similar to previously reported cluster, deserves concern since it is thought that the resistance will be stably maintained even in the absence of selective pressure.

Finally, this work confirmes once again the necessity and the utility to monitor salmonellosis infection through the surveillance networks, that has permit the identification of this new multidrug resistance clone.

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EMERGING MOSQUITO-BORNE DISEASES IN ITALY: IMPORTANCE OF THE ENTOMOLOGICAL SURVEILLANCE

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The infections biologically transmitted to a vertebrate host by the bite of infected arthropod species are commonly known as Vector-Borne Diseases (VBDs). The main potential vectors are included in the two classes of the Phylum of Arthropods, the class of Arachnids with ticks and the class of Insects, with mosquitoes and sandflies (Figure 1). The pathogens transmitted by blood feeding may be viruses, bacteria, protozoa and filarial worms. Moreover among potential vectors only several species can play a real role of medical importance, depending on the vector competence of the single species, i.e. the intrinsic ability to replicate and transmit the pathogen after an infective blood meal. However each vector species may show different degrees of susceptibility to different pathogenic species or strains. VBDs mainly affect tropical and subtropical countries, where they are still responsible for human diseases. Malaria, leishmania, yellow fever and dengue cause millions cases a year and still represent a very important public health problem. However VBDs, and in particular those transmitted by mosquitoes (MBDs), are becoming a source of concern also in Europe due to their spread and establishment in new areas, outside their natural range (1).

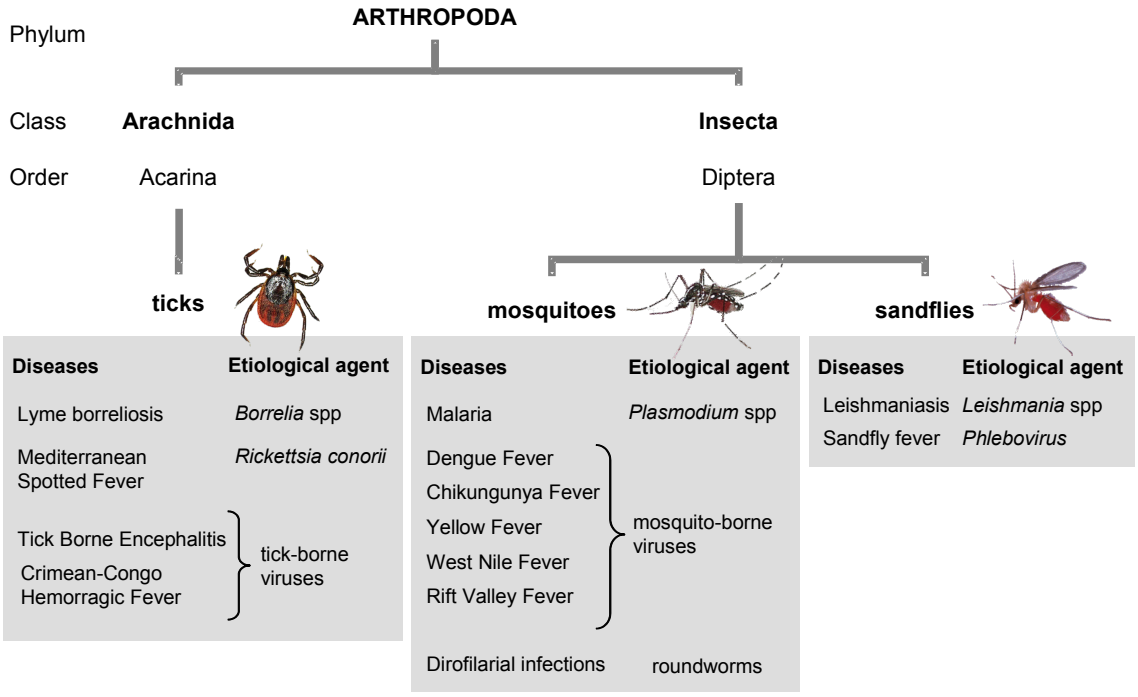

Figure 1. Systematics of the main vectors of VBDs and their respective etiological agents

Climate is a major environmental driver influencing VBD-epidemiology and in combination with other favourable biological, ecological and socioeconomic factors, it can influence the appearance or the reappearance of infectious diseases in a particular area (2, 3). As a result of the globalization, the intensification of travel and trade routes between countries facilitates the movement and spread of pathogens and their vectors farther and faster than ever before (4). Although distance seems to be not a significant barrier to vector movement, climate at the point of entry still represents a fundamental constraint to a new vector establishment. Temperature results as the main critical factor because arthropods, being ectothermic organisms, cannot physiologically regulate themselves and are critically dependent on climate for their survival and development (5). It is estimated that a growth of 1.0–3.5°C of the average global temperatures expected by 2100, will increase the likelihood of many vector-borne diseases (6).

Despite evidence that climatic changes have direct effects on epidemiology of VBDs, many Authors suggest to deepening the ecology and biology of species involved in order to better understand and predict the effects of future changes in the environment (7).

In Italy, after the eradication of malaria achieved in the 1950s, the endemic VBDs, transmitted mainly by ticks and sandflies, resulted of local interest, being relegated to scattered and limited foci (8, 9). The recent Chikungunya and West Nile outbreaks occurring in our country have renewed among health authorities the concern of rising in prevalence and spreading of them or other arboviruses to new areas (10, 11). In our country the mosquito species of actual medical interest are about more than ten and among them, the main mosquito vectors of pathogens are:

Anopheles labranchiae

The main malaria vector, *Anopheles labranchiae,* belongs to the *An. maculipennis* complex that includes cryptic species impossible to distinguish by adult morphological characters, being identifying on egg morphology and molecular approaches (Figure 2).

Figure 2. *Anopheles labranchiae*

In Italy, malaria was officially eradicated only in 1970 and even if *An. labranchiae* populations were dramatically reduced in their densities after a five-year national campaign launched in 1947, the species slowly re-colonized part of the endemic areas, such as Maremma. This region is a narrow coastal plain shared between Tuscany and North Latium regions where hyperendemic levels of the disease were maintained up to 1950's by *An. labranchiae*. In 1997, an autochthonous-introduced *Plasmodium vivax* case, reasonably transmitted by indigenous *An.*

labranchiae, occurred in a rural locality of Grosseto province, demonstrating that Maremma is still a suitable area for malaria reintroduction (12). At present malaria cases in Italy are all of importation, with the exception of some cases due to accidental events (i.e. blood transfusions, accidental introduction of infected anophelines by baggage) (13).

Promoted by the EDEN EU-project (FP6), an our recent research was mainly aimed to evaluate the malariogenic potential of Maremma area, by the assessment of the three parameters that determined it: the receptivity (presence, density, and biologic characteristics of the vector in the study area); the infectivity (degree of susceptibility of mosquitoes to different *Plasmodium* species) and the vulnerability (number of gametocyte carriers during the period in which malaria transmission is possible). Moreover the theoretical vectorial capacity (VC) of *An. labranchiae* was also assessed. VC represents the vector efficiency to transmit the pathogen and, in turn, is influenced by three factors: anthropophily, longevity, and density of the vector (14). The results of the entomological surveys and the development of a map of *An. labranchiae* distribution show a high receptivity of Maremma (15). Relevant densities of the species are strongly related to the presence of the main breeding site of the species, the rice-fields, and to the livestock density in the area, according to anthropic activities that may allow the colonization and the massive reproduction of the species, by modifying the natural environment. The VC of *An. labranchiae* results very high, especially in those areas where the high density of vector depends on rice-field occurrence. A known infectability of *An. labranchiae* from Maremma for exotic *P. vivax* strains has already been confirmed (12) and attempts of artificial infections show a very low susceptibility of this mosquito vector to afro-tropical strain of *P. falciparum*, at least in laboratory conditions (16). The study area also confirms a very low vulnerability due to the scarce presence of gametocyte carriers potentially circulating during the favourable transmission season (13). Therefore in Maremma the malariogenic potential appears to be low, as in other areas of the country where *An. labranchiae* still persist in relevant densities; so our findings exclude a return to an endemic situation, also due to the improved cultural and socioeconomic conditions (15). Nevertheless the occurrence of sporadic, isolated *P. vivax* cases appears improbable but not impossible, as it had already occurred in this area in 1997 (12).

Aedes albopictus

Aedes albopictus, commonly known as the Tiger mosquito, is a mosquito native to the tropical and subtropical regions of South-East Asia and represents the most invasive mosquito in the world (Figure 3).

Figure 3. *Aedes albopictus*

This species has an aggressive human-biting behaviour, carried out preferably outdoors and during daytime and shows an ability to vector many viruses (17). In its native range it is proved vector of Chikungunya (CHIK) and dengue (DEN) viruses, ranking second only to *Aedes aegypti* as a DEN vector, while it exhibits a higher vector competence for CHIK (18).

After its introduction into Italy (19), Tiger mosquito quickly spread throughout the northern and central regions of the country, causing considerable concern among public health authorities (20, 21). A national reference centre for surveillance and control at Istituto Superiore di Sanità (ISS) was established in order to coordinate the surveillance activities, monitoring the spread of the species and to produce guidelines for effective control interventions (22). At present, scattered foci of *Ae. albopictus* are reported in all regions with the exception of Valle d'Aosta and in over 3000 municipalities belonging to 93 provinces (23). After no long time *Ae. albopictus* was considered a merely source of nuisance, the problem of its occurrence being shifted from a health concern to an environmental issue, and monitoring and control were demanded to municipal local governments (23). Nevertheless in the summer of 2007, the mosquito confirmed its competence as an arbovirus vector with the first outbreak of CHIK fever in Europe, occurring in the province of Ravenna, Emilia Romagna Region, and causing about 250 confirmed cases (10, 24). The early epidemiological investigation detected the index case coming from Kerala, a region of India, where CHIK is endemic and the entomological survey identified *Ae. albopictus* as the potential vector. Subsequently the etiological agent was isolated in two mosquito pools by RT-PCR, confirming the high vectorial competence of the species. Phylogenetical analysis of Italian viral strains showed a high similarity with strains from the earlier outbreak on Indian Ocean islands.

In Italy, in these last years *Ae. albopictus* has also been found infected with Usutu (USU) virus (25), and proved to transmit the two dog worms *Dirofilaria immitis* and *Dirofilaria repens* (26, 27).

Culex pipiens

West Nile (WN) virus is widely distributed throughout Africa, Europe, Middle East, Asia, Oceania (with subtype Kunjin) and more recently has spread to the New World, where it was first detected in New York City (28). The natural transmission cycle involves birds (the natural reservoir hosts) and mosquitoes, mainly from the *Culex* genus (29). Humans, horses and other mammals can occasionally be infected when a mosquito species acts as both the enzootic and the bridging vector. These mammals are sensitive to the virus but are considered as dead-end hosts of the virus because they do not develop sufficiently high titres in their blood to infect mosquitoes (30). In Europe, WN disease was reported as a major outbreak in humans for the first time in 1996 from Romania with more than 500 clinical cases and a case-fatality rate approaching 10%, mainly in the elderly (31, 32). Since then, there have been some sporadic human cases from various EU countries and increasing reports of WN infection in horses and birds, as well as detection in mosquitoes (33). In Europe, WN virus circulation is confined to two basic types of cycles and ecosystems: rural cycle (usually involving migratory and wetland birds and ornithophilic mosquitoes) and urban cycle (with synanthropic or domestic birds and mosquitoes feeding on both birds and humans) (32). The virus has been isolated from more than 75 mosquito species from eleven different genera, but in Europe the mosquitoes that have been determined to have high vector competence for WN are *Culex pipiens* complex, *Culex. modestus*, and *Coquillettidia richiardii*, while *Ae. albopictus* demonstrates only moderate vector competence (32, 33). The *Cx. pipiens* complex comprises two distinct forms, named *pipiens* and

molestus, that are morphologically indistinguishable, but exhibit significant behavioural and physiological differences, occupying different niches (Figure 4).

Figure 4. *Culex pipiens*

In particular *Cx. pipiens pipiens* feeds predominantly on birds (ornithophilic), whereas *Cx pipiens molestus* prefers to feed on mammals, including humans (mammophilic). The taxonomic status, the evolutionary relationships, and especially the role that these forms could play in the WN transmission remain controversial (34). In Italy the first WN outbreak occurred in Tuscany in 1998, involving race-horses only (35). After this event, in 2001 a national plan for the surveillance of WN virus circulation was set up under the coordination of the National Reference Centre for Exotic Diseases of animals (CESME) and with the entomological consultancy of ISS. In 2008 a large WN outbreak was reported with many foci in three North-eastern regions Veneto, Lombardy and Emilia Romagna and with the involvement of humans (9 cases), horses and autochthonous birds (36). Moreover mosquito pools of *Cx. pipiens* were found positive for WN virus. In the following year a new epidemic re-emerged in 2008 area, with an increase of the foci number, but it spread also in additional isolated foci of Central Italy (37). In 2009 besides many horse and bird cases, there were 16 in humans, with two deaths and other detections of positive mosquito pools (38, 39). In the North-eastern area of WN virus circulation was identified the first human case of USU virus, another arbovirus transmitted by mosquitoes and frequently found in co-circulation with WN virus (40, 41). Moreover positive mosquito pools were found for USU virus (25, 42). In 2010 WN outbreaks occurred in the same North-eastern area and in new scattered foci of Central and Southern Italy, with human (3 cases), equine and bird involvement (personal communication). To date there is some evidence of endemicity of WN virus in autochthonous bird species, and consequently of WN disease in Italy (Figure 5) (43).

In conclusion, the re-emergence of malaria cases in some European countries, the arrival and expansion of WN, CHIK and USU viruses, combined with the predicted global warming and the increase of the modern transport, raise questions as to which pathogens and/or vectors will next appear in Europe. The actual risk of a reintroduction of DEN in our continent, and in particular in Italy, is strictly related to the possible spread of *Ae. aegypti*, the main DEN vector. This species occurred in the past in several harbours of the Mediterranean basin and in Greece it was actor of large DEN outbreaks (44).

In Italy, besides the implementation of the national surveillance plan for WN disease, directives from Ministry of Health for the early detection of imported and autochthonous cases of human arbovirosis, as DEN and CHIK fevers, have been released.

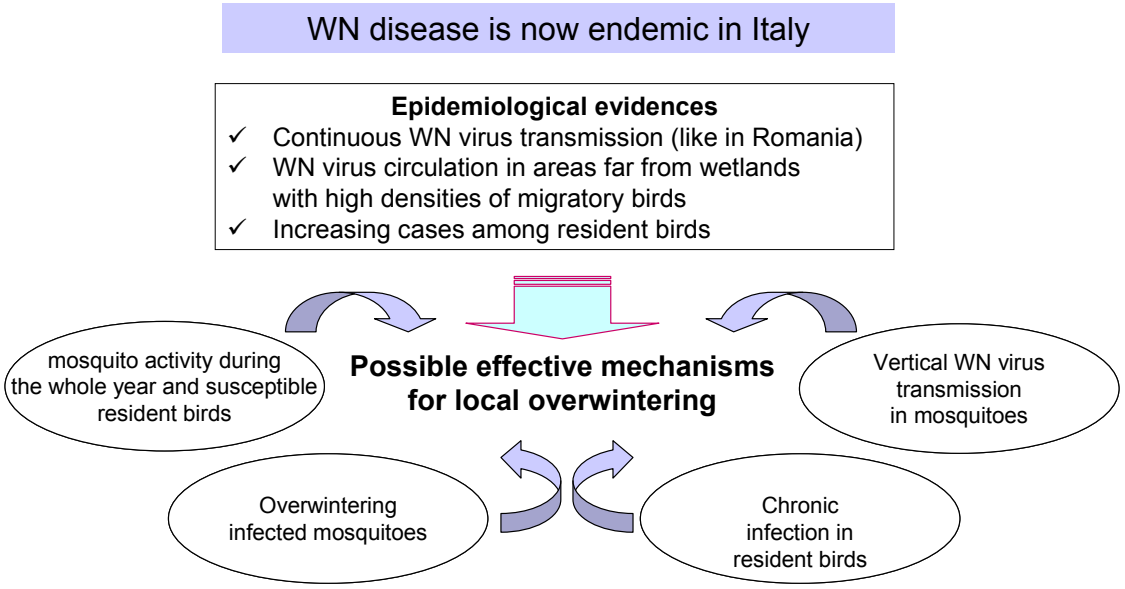

Figure 5. Epidemiological evidences of WN endemicity in Italy and possible mechanisms involved in overwintering of WN virus

At present no plan has been implemented for preventing the introduction, the spread and the possible establishment of exotic vectors. An effective prevention of MBDs and, in general of all VBDs, depends on the development of surveillance integrated control programmes, funded at local or central level, and comprising of entomological monitoring where pathogen occurs or not. It is essential for example to know the mosquito species composition and the relative densities in a certain area and to deepen the mosquito bionomics and ecology in order to evaluate the potential role in the vector-borne transmission and the vectorial competence. Moreover, the localization and mapping of breeding sites for all vector species should be carried out. Mosquito control should be implemented early in the year and focused on larval control using an integrated approach that includes source reduction, water management, chemicals, and biological control methods (29, 45).

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PATHOGENESIS OF CANDIDIASIS: THE ROLE OF THE MP65P, A PUTATIVE β-GLUCANASE ADHESIN, IN THE MORPHOGENESIS AND VIRULENCE OF *CANDIDA ALBICANS*

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Candidiasis are increasing infections, ranging from fatal systemic infections in immunocompromised patients to mucosal infections observed in patients with AIDS.

Candida spp. are the fourth-leading cause of nosocomial infections and in those patients with candidemia the attributable mortality rate is ~50%. Furthermore, oropharyngeal candidiasis (OPC) occurs in approximately 70% of patients with AIDS, ~70% of all women (with or without AIDS) will experience at least one episode of vaginitis caused by *Candida spp*. and ~20% will experience recurrent disease.

The wide spectrum of candidiasis and its recognized clinical importance has stimulated interest to study the components of the host-fungus interaction involved in the pathogenesis of this disease. The mannoproteins are the fungal cell wall components that play a main role in host-parasite relationship. In particular the Mp65p, a mannoprotein of 65 Kilodalton, belonging to the β-glucanase family, is the main target of the immune response against the human opportunistic fungus *Candida albicans* (1, 2).

In order to investigate the role of this protein in the morphogenesis and virulence of the fungus, we have constructed, by ura-blaster protocol, the *MP65* knock-out mutants [*MP65/mp65* heterozygous (het), *mp65/mp65* homozygous (hom or *mp65*Δ) and *mp65/mp65-MP65* revertant strains (rev)]. The correct construction of the *MP65* knock-out mutants was verified by Southern, Northern, Western-blot and immunofluorescence (Figure 1).

The *mp65*Δ mutant, although viable, is inable to form hyphae, *in vitro* and *in vivo*, and to adhere to plastic (Figure 2 and 3). Figure 2 shows colony morphology of *mp65*Δ mutant strain on solid media. The wild-type (wt), *mp65*Δ mutant (hom) and revertant (rev) strains were plated in Spider and modified Lee's medium and incubated at 37°C. Peripheral hyphae morphologies after 3 days of growth of each strain on Lee's and Spider media are shown. The magnification bar corresponds to 0.8 mm. Figure 3 shows adherence properties of *mp65*Δ mutant strain. Each strain was inoculated in modified Lee's medium and incubated at 37°C for 3h in polystyrene wells. After washing, plastic-adherent cells were incubated in solid media for 24h, colonies were counted and the results expressed as percentage of the inoculated cells. Values are mean (+SD) of triplicate independent assays.

Furthemore, the *mp65*Δ mutant is significantly less virulent than the wild-type strain (wt) in a systemic murine infection model, as shown by increased survival, lower CFU counts in the kidney and organ histology (Figure 4). Moreover, in an estrogen-dependent rat vaginal infection model, the early clearance of the fungus is also significantly more accelerated in the *mp65*Δ mutant than in the wild-type strain (3). Figure 4A reports Kaplan-Meyer curves of mouse survival following experimentally induced candidiasis. CD2F1 mice (10 mice per group) were infected via tail vein with $5x10^5$ yeast-form cells of *C. albicans* and animal survival was monitored for 30 days. Figure 4B shows kidney sections of infected mice. Kidney of mice challenged with wt and mutant strains were removed from euthanized mice, 3 days post challenge, fixed, dehydrated and stained with Grocott. The magnification bar corresponds to 0.6 mm and 30 μm for the upper and lower panels respectively.

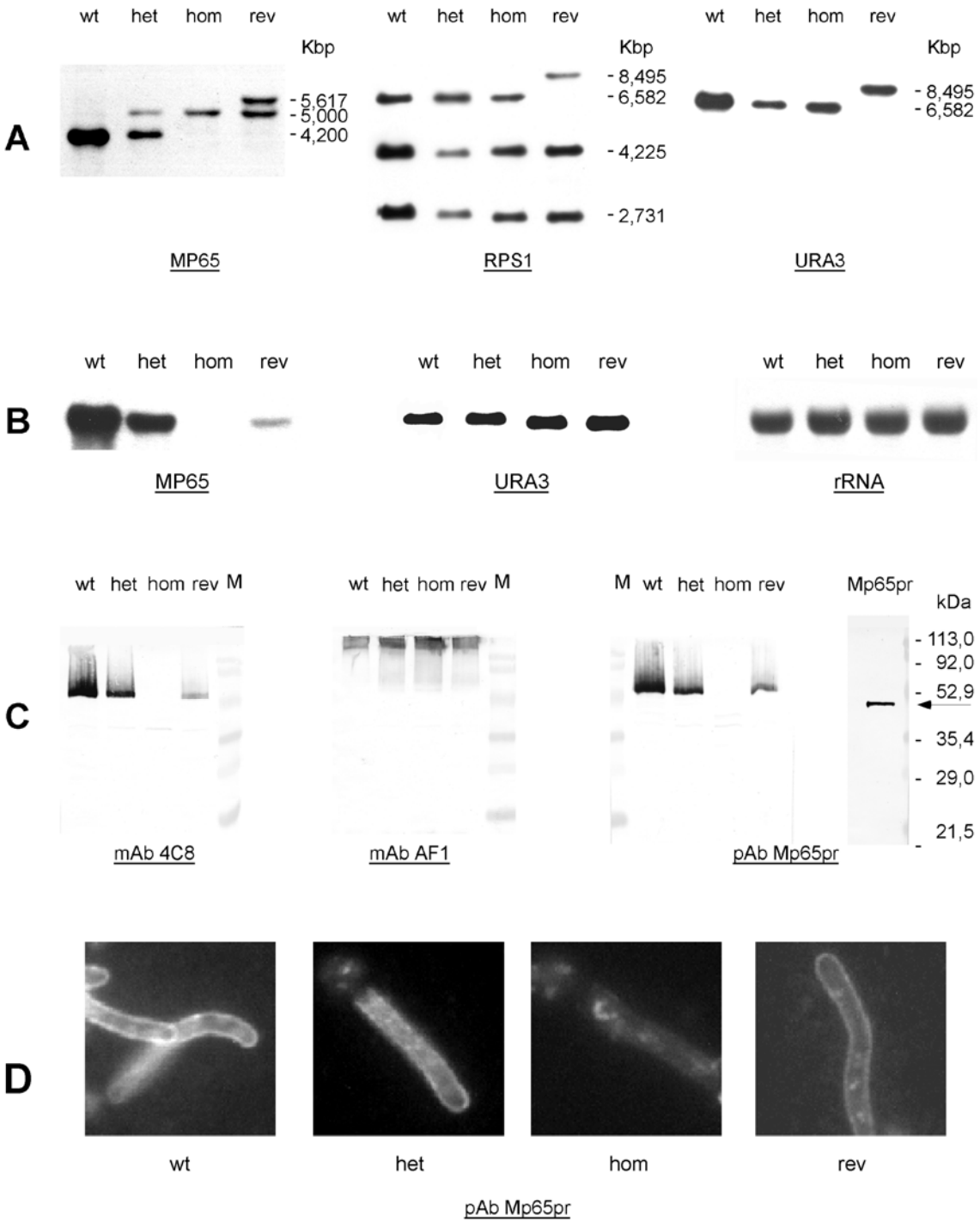

Figure 1. Targeted disruption of the *MP65* gene. Southern (A), Northern (B), Western (C) blot and immunofluorescence analysis (D) of the wild-type (wt), heterozygous (het), homozygous (hom) and revertant (rev) strains
(modified from Sandini* et al., *2007) (3)

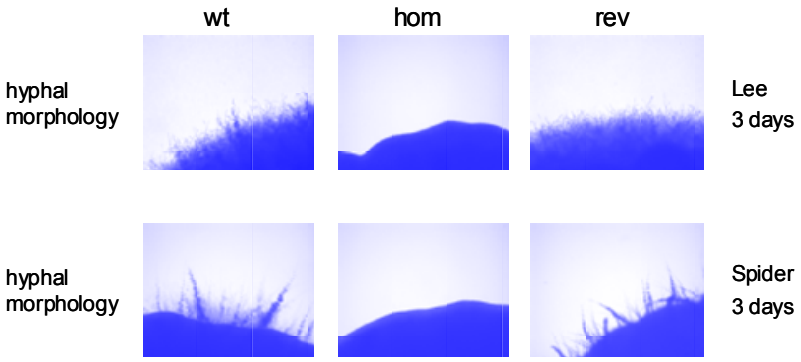

Figure 2. The *MP65* gene is required for hyphal morphogenesis
(modified from Sandini* et al., *2007) (3)

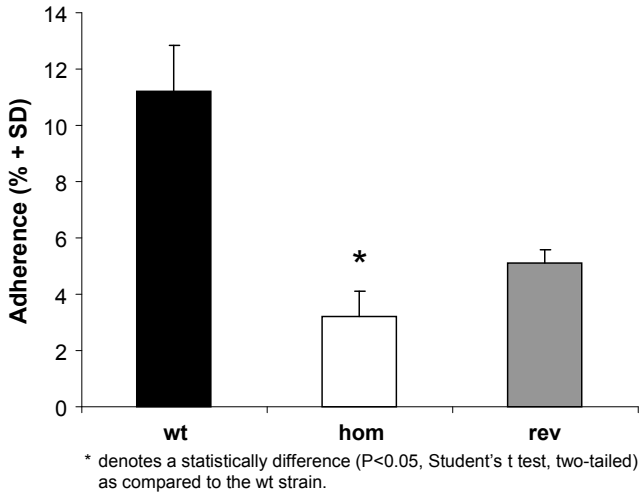

Figure 3. The *MP65* is required for adherence to the plastic
(modified from Sandini* et al., *2007) (3)

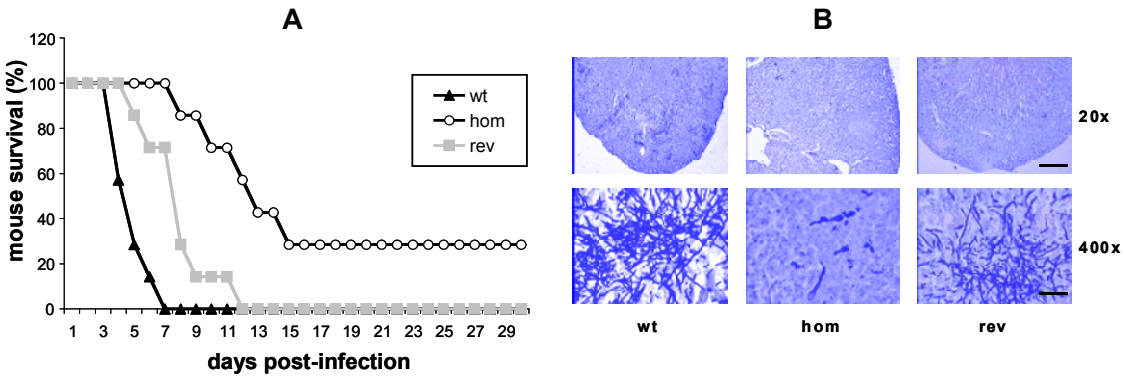

Figure 4. Experimental pathogenicity of *mp65*Δ mutant strain in mouse bloodstream infection: Kaplan-Meyer curves of mouse survival (A) and kidney sections of infected mice (B)
(modified from Sandini* et al., *2007) (3)

We also performed an extensive analysis of the *mp65*Δ mutant to assess the role of this protein in cell wall integrity, adherence to epithelial cells and biofilm formation.

The *mp65*Δ mutant showed a high sensitivity to a range of cell wall-perturbing and degrading agents (Figure 5), especially Congo red, wich induced morpholocical changes such as swelling, clumping and formation of hyphae (Figure 6).

Figure 5 shows the sensitivity of *mp65*Δ mutant to different cell wall-perturbing agents. The wild-type (wt), *mp65*Δ mutant (hom) and revertant (rev) strains were tested by spotting decreasing cell counts on YEPD plates with or without cell wall-perturbing agents. Column 1 corresponds to the cell suspension and columns from 2 to 6 corresond to 1:5 serial dilutions.

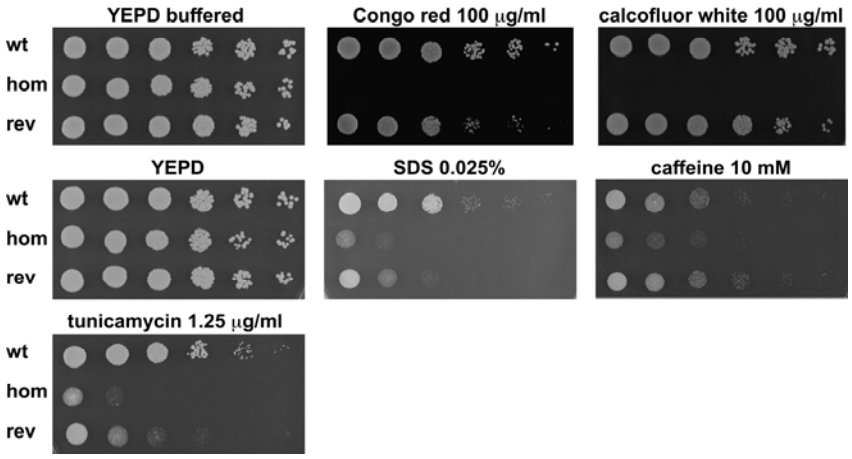

Figure 5. Sensitivity of the *mp65*Δ null mutant to different cell wall-perturbing agents
(from Sandini* et al., *2011) (4)

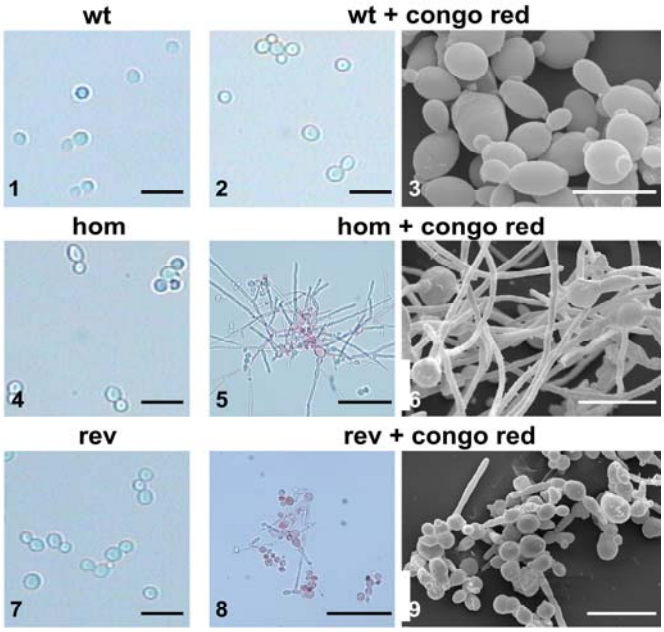

Figure 6. Morphological analysis of the the *mp65*Δ mutant
(from Sandini* et al., *2011) (4)

Figure 6 shows morphological analysis of the *mp65*Δ mutant. The wild-type (wt), *mp65*Δ mutant (hom) and revertant (rev) strains were grown in YEPD for 24h at 28°C with or without Congo red (50 µg/mL) and then observed under a light microscope and SEM. The magnification bar corresponds at 15 µm (panels 1, 2, 4, 6, 7 and 9), 5 µm (panel 3), and 60 µm (panels 5 and 8).

The *mp65*Δ mutant exhibited a constitutive activation of two MAPKs (Mkc1p and Cek1p) and a high level of expression of two stress-related genes (*DDR48* and *SOD5*), associated with modulated expression of β-glucan epitopes, but not gross changes in cell wall polysaccharide composition (Figure 7).

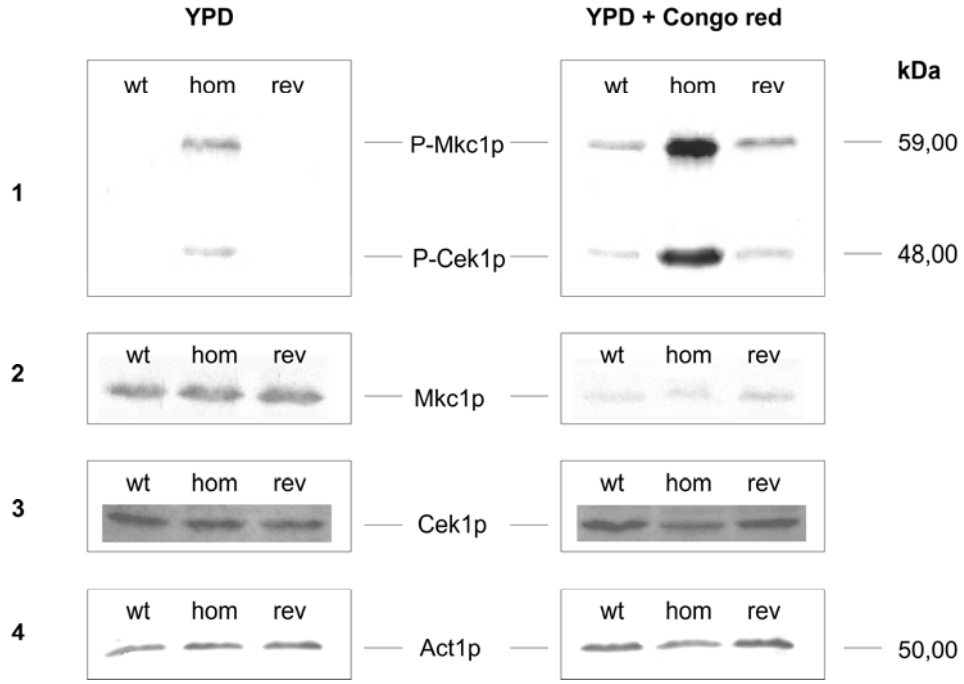

Figure 7. Activation of the cell wall integrity pathway
(from Sandini* et al., *2011) (4)

Figure 7 shows the activation of cell wall integrity pathway through the loss of the *MP65* gene. Activation of the cell wall integrity pathway was determined by Western blot analysis. The wild-type (wt), *mp65*Δ mutant (hom) and revertant (rev) strains were grown in YEPD for 1.5 h at 28°C with or without Congo red (50 µg/mL). Protein extracts (150 µg) were loaded in each lane and analyzed with anti-p44/42 MAPK (panel 1), anti-MAPK (panel 2), anti-Cek1p (panel 3) and anti-Act1p (panel 4) antibodies.

Interestingly, the *mp65*Δ mutant dispayed a marked reduction in adhesion to BEC (Buccal Ephitelial Cells) and Caco-2 cells (Figure 8) and severe defects in biofilm formation when compared to the wild-type (Figure 9).

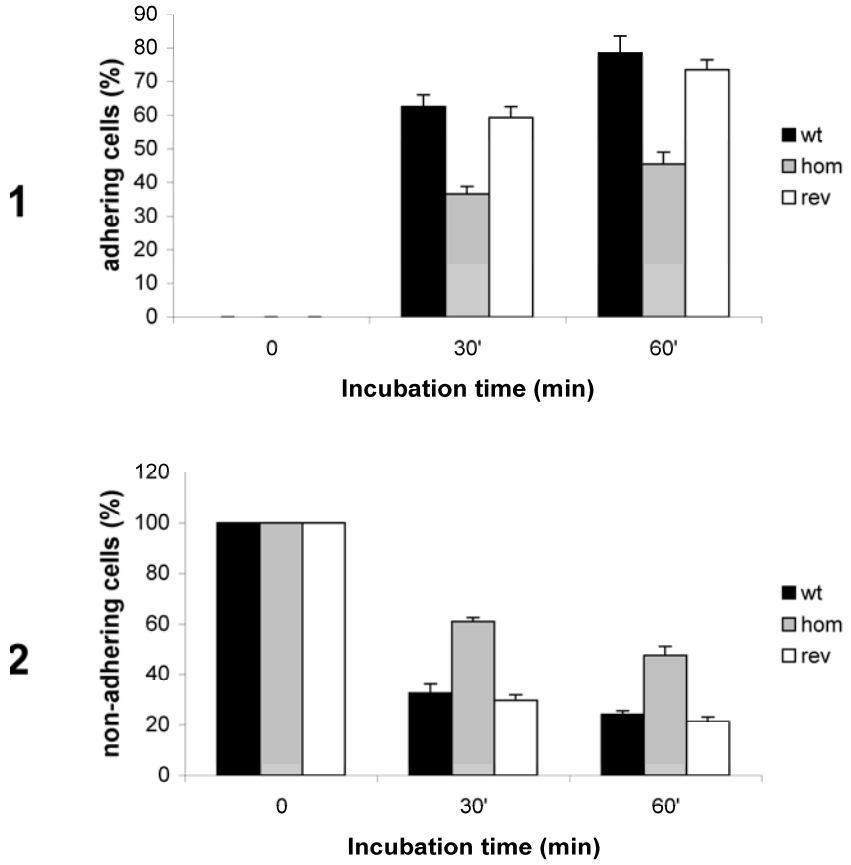

Figure 8. Adhesion of *mp65*Δ mutant to Caco-2 cells
(from Sandini* et al., *2011) (4)

Figure 8 shows the adhesion of the *mp65*Δ mutant to Caco-2 cells. Recovery of *Candida* cells [wild-type (wt: black column), *mp65*Δ mutant (hom: grey column) and revertant (rev: white column) strains] at different time points (30 and 60 min) of incubation with Caco-2 cells. Adherent cells recovered after thorough washing out of the microplate (panel 1). Non-adherent cells recovered from the supernatant (panel 2). The results are the mean of 3 independent experiments. The bars indicate the standard deviations.

Figure 9 shows the biofilm analysis of *mp65*Δ mutant by crystal violet (CV) staining. Equal numbers of cells from the wild-type (wt), *mp65*Δ mutant (hom) and revertant (rev) strains were suspended in 250 µL of RPMI medium and incubated in 24-well plates for 48 h. Non-adherent cells were then removed by washing, and adherent cells were stained with CV. The biofilms were visualized before (panel 1) and after (panel 2) staining and then captured by using either Gel Doc system (Bio-Rad), or using an inverted microscope at 40x magnification (panel 3).

All the above properties were totally or partially recovered in a revertant strain, demonstrating the specificity of gene deletion together with a gene-dosage effect (3, 4).

In conclusion, the knock-out of the *MP65* gene affects biological properties (morphogenesis, pathogenicity, cell wall integrity, adhesion, biofilm formation) that are of potential relevance for candidiasis.

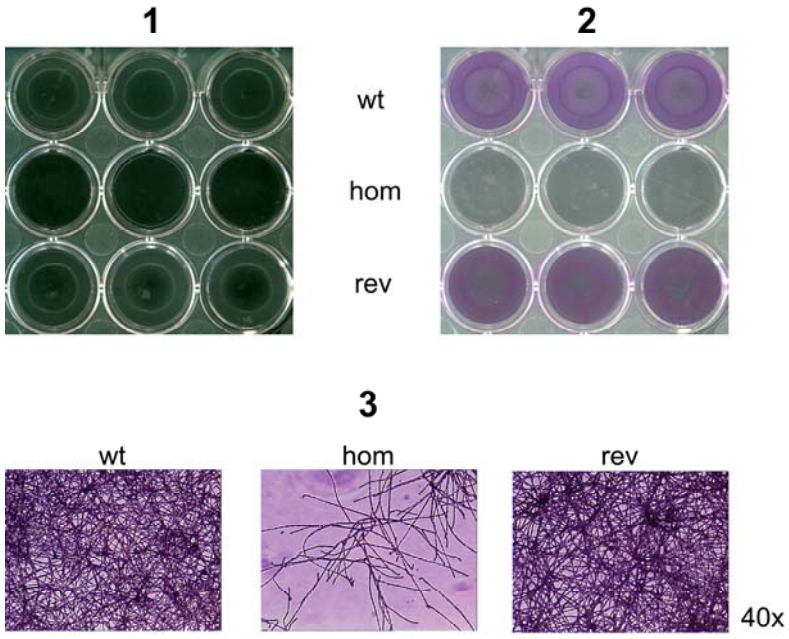

Figure 9. Biofilm analysis of the *mp65*Δ mutant
(from Sandini* et al., *2011) (4)

For all of the discussed reasons, and with the previously reported evidence of Mp65p being a major target of host immune response to *C. albicans* (1), this protein remains an interesting potential target for therapeutic or immunotherapeutic interventions.

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ANTIGEN 85B OF *MYCOBACTERIUM TUBERCULOSIS:* FRIEND OR FOE IN VACCINATION AGAINST TUBERCULOSIS?

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Mycobacterium tuberculosis (*Mtb*) is responsible for more deaths than any other single infectious organism; there are almost 8 million new cases and approximately 2 million deaths annually. Tuberculosis (TB) can be cured by chemotherapy, but the complex and long-lasting treatment, involving at least three drugs for 6 months, means compliance is often incomplete, resulting in a rising incidence of multi-drug and extensively-drug-resistant strains. In addition, the public health impact of *Mtb* has become increasingly severe, partly because of HIV epidemic (1, 2). In this context, vaccines provide the only realistic hope of effective prevetion. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine, developed more than 80 years ago, protects against severe forms of childhood TB, but unfortunately, does not lead to eradication of Mtb and protective activity of the vaccine weakens during adolescence. As a consequence, BCG does not protect against the most prevalent disease form, pulmonary TB, in adults. The causes of failure in developing an effective TB vaccine are still unknown but strictly dependent on the complex and unsettled interactions between *Mtb* and the host immune system. One of major problems is that several immune responses involved in protection are also involved in the pathology underlying the disease (1-3).

In this context, we investigated why an initial protective response specific for the *Mtb* antigen 85B looses its efficacy. Ag85B, a fibronectin-binding protein with mycolyltransferase activity, is the major secretory protein in actively replicating *Mtb*. Ag85B is highly immunogenic, as shown by the easy detection of specific humoral and cell-mediated immune responses both in latently- and actively-infected TB patients. In mice, a large proportion of $CD4^+$ T cells infiltrating the lung after a *Mtb* challenge, recognize Ag85B. For these reasons, Ag85B has been intensely investigated as a promising candidate for subunit TB vaccines. We observed that in mice, a primary vaccination with Ag85B-encoding plasmid DNA (DNA-85B) was protective against *Mtb* infection. Protection was associated with Ag85B-specific $CD4^+$ T cells producing IFN-γ able to control intra-macrophagic *Mtb* growth. Surprisingly, the protection was eliminated when mice immunized with DNA-85B were boosted with adjuvant-free Ag85B protein. Loss of protection was associated with an overwhelming Ag85B-specific $CD4^+$ T cell proliferation and IFN-γ production (Figure 1) (4-6). However, these $CD4^+$ T cells lost the ability to recognize *Mtb*-infected macrophages and to control bacterial intra-macrophagic growth. It seems that the protein-dependent expansion of non-protective $CD4^+$ T cells determined dilution or loss of those protective Ag85B-specific $CD4^+$ T cells induced by DNA-85B vaccination. Therefore, the enhancement of IFN-γ producing $CD4^+$ T cells, that are critical for protection, did not guarantee the development of protective immunity in our TB model but even turned it for the worse. The data suggest that Ag85B protein, during vaccination or secreted by *Mtb* during natural infection could be involved in the instability of protective anti-tuberculosis immune response, and actually concur to disease progression. It is tempting to speculate that this Ag85B protein-mediated immuno-evasion might take place also during *Mtb* infection in humans.

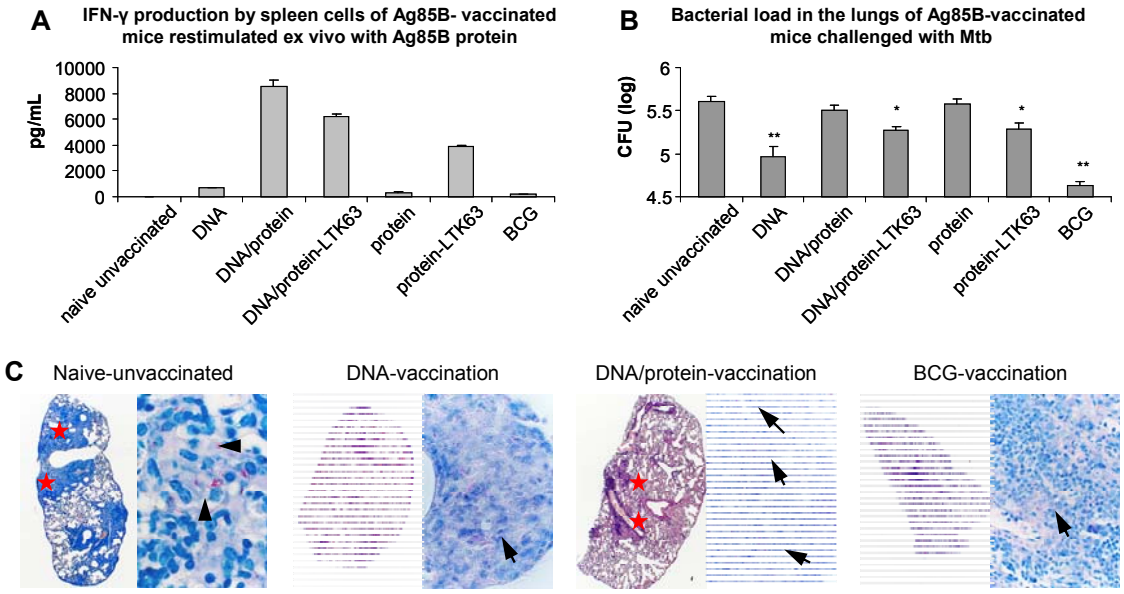

Figure 1. Protection provided by DNA-85B immunization was lost after boosting with adjuvant-free Ag85B protein despite an high Ag85B-specific IFN-γ response

In fact, high levels of circulating Ag85B protein have been found in active TB patients; activation of Ag85B-specifc $CD4^+$ T cells in TB patients is not always associated with a favourable prognosis and secretion of IFN-γ was predominant in patients who displayed bilateral pulmonary lesions and lung cavitation. Therefore, Ag85B protein, normally secreted during *Mtb* infection, could induce, in a positive feedback loop, selection and expansion of non-protective Ag85B protein-specific $CD4^+$ T cells secreting excessive IFN-γ level, thus determining both a progressive decay of the efficacious effector/memory pathogen-specific T cells, and a consolidation of inflammatory lung lesion. A parallel could be made with the well-known "Koch phenomenon" observed when *Mtb* culture filtrate extracts were given to TB patients, causing florid reactions and several deaths in many of the patients with a relatively mild disease.

With the aim to control non-protective Ag85B-specific IFN-γ producing $CD4^+$ T cells during vaccination, we considered the possibility to use an adjuvant to modulate antigen-specific Th1 immunity. For this reason, mice primed with DNA-85 were boosted with Ag85B protein administered with LTK63 adjuvant. LTK63, a non-toxic derivative of heat-labile enterotoxin of *Escherichia coli*, elicits strong humoral and Th1 immunity. However, LTK63 also exerts a negative control on Th1 polarization. Prior intratracheal administration of LTK63 decreased lung inflammation and tissue damage that resulted in an improvement of the animal ability to resolve lung infection by respiratory sincitial virus, influenza virus and *Cryptococcus neoformans*.

We found that Ag85B protein co-administration with the adjuvant LTK63 in DNA-85B primed mice reduced the expansion of Ag85B-specific IFN-γ producing $CD4^+$ T cells and partially restored the protection against *Mtb*-infection (Figure 1) (5). The restrain of Ag85B-specific IFN-γ response allowed the survival of those protective Ag85B-specific $CD4^+$ T cells able to recognize *Mtb*-infected macrophages and induced by the DNA-85B vaccine. The recovery of protection through a down-modulation of antigen-specific IFN-γ response by an adjuvant was a novel finding which could be of relevance in TB vaccination. Moreover, LTK63 drove the commitment of memory antigen-specific T cells towards Th17 response. Considering

that IFN-γ and IL-17 responses are negatively regulated by each other, the induction at the same time of both responses may help to prevent that one response, at the expense of the other, can expand without control causing inflammation-mediated lung damage during *Mtb* infection. The ability to activate several mechanisms to contain uncontrolled expansion of Th1/Th17-mediated inflammatory process may be the reason of the efficacy of LTK63 adjuvant in TB vaccination.

Next, we asked whether it was possible to modulate antigen-specific, IFN-γ producing $CD4^+$ T cells not in the induction phase, as mentioned above describing the ability of LTK63 adjuvant, but after they have been generated.

Moreover, we investigated how to control non protective antigen-specific IFN-γ production without affecting secretion of protective IFN-γ. Namely, we used the previously established TB model of protective DNA-85B and non-protective DNA/free adjuvant Ag85B protein-immunizations. We found that both protective and non-protective Ag85B-immunization generated antigen-specific $CD8^+$ T cells that suppressed Ag85B-specific IFN-γ-secreting $CD4^+$ T cells. Moreover, ligation of the co-stimulatory molecule 4-1BB selectively reduced the high but non-protective $CD4^+$ T cell-mediated IFN-γ response in DNA-primed and protein-boosted mice without affecting the low IFN-γ response associated with protection in mice vaccinated with DNA only (Figure 2) (6). 4-1BB, the murine homologue of human CD137, is a member of the TNF-receptor super-family expressed primarily on antigen-receptor activated T cells. Although 4-1BB co-stimulates primary and secondary responses of both $CD8^+$ and $CD4^+$ T effector cells, signalling via this molecule also results in the activation of regulatory $CD4^+$ or $CD8^+$ T cells. Due to their ability to activate both effector and suppressive T cells, 4-1BB/CD137 ligands are being evaluated for treatment of cancer and auto-immune diseases.

In our model of TB vaccination, we observed that the 4-1BB-mediated IFN-γ inhibition selectively in spleen cells of DNA-primed and protein-boosted mice was linked to the induction of 4-1BB, upon Ag85B stimulation both *in vitro* and *in vivo*, exclusively on suppressive $CD8^+$ T cells of these mice (Figure 2). The 4-1BB-mediated IFN-γ inhibition did not require soluble IL-10, TGF-β, XCL-1 and MIP-1β. In addition, engagement of 4-1BB expressed on $CD4^+$ T cells of protein-boosted mice down-modulated the secretion of IL-10 and MIP-1 β, cytokines often associated with TB susceptibility.

Therefore, the exclusively expression of 4-1BB only on $CD8^+$ T cells found in mice developing a massive non-protective IFN-γ response open novel strategies for intervention in TB pathology and vaccination through T-cell co-stimulatory-based molecular targeting.

To further investigate the mechanisms involved in protective and non-protective immunity elicited by the mycobacterial antigen Ag85B and the cellular and molecular mechanisms that regulate their activation/exhaustion, now we are studying:

1. the peculiar role played by B cells in the optimal release of IFN-γ, MIP-1β and IL-10 (but not IL-17) by Ag85B-specific memory $CD4^+$ T cells;
2. modulation induced by other *Mtb* antigens, in particular the phosphate binding lipoprotein (PstS1) (7) on Ag85B-specific IFN-γ and IL-17 responses during priming or memory T cell effector phase;
3. progressive impairment of Ag85B-specific immunity in spleens of vaccinated mice during *Mtb* infection.

C57BL/6 female mice were immunized twice or four times at 2-week intervals, with DNA-85B, Ag85B protein in the presence or absence of LTK63 adjuvant or combination DNA/protein. As a positive control, a single dose of BCG was injected s.c. Four weeks after the last immunization, some mice were killed to recover spleen. Pooled spleen cells (5 mice/group) of naive unvaccinated mice or vaccinated mice were re-stimulated with Ag85B protein (5 μ g/mL) for 96 h before testing IFN-γ by a commercial ELISA kit (Figure 2A).

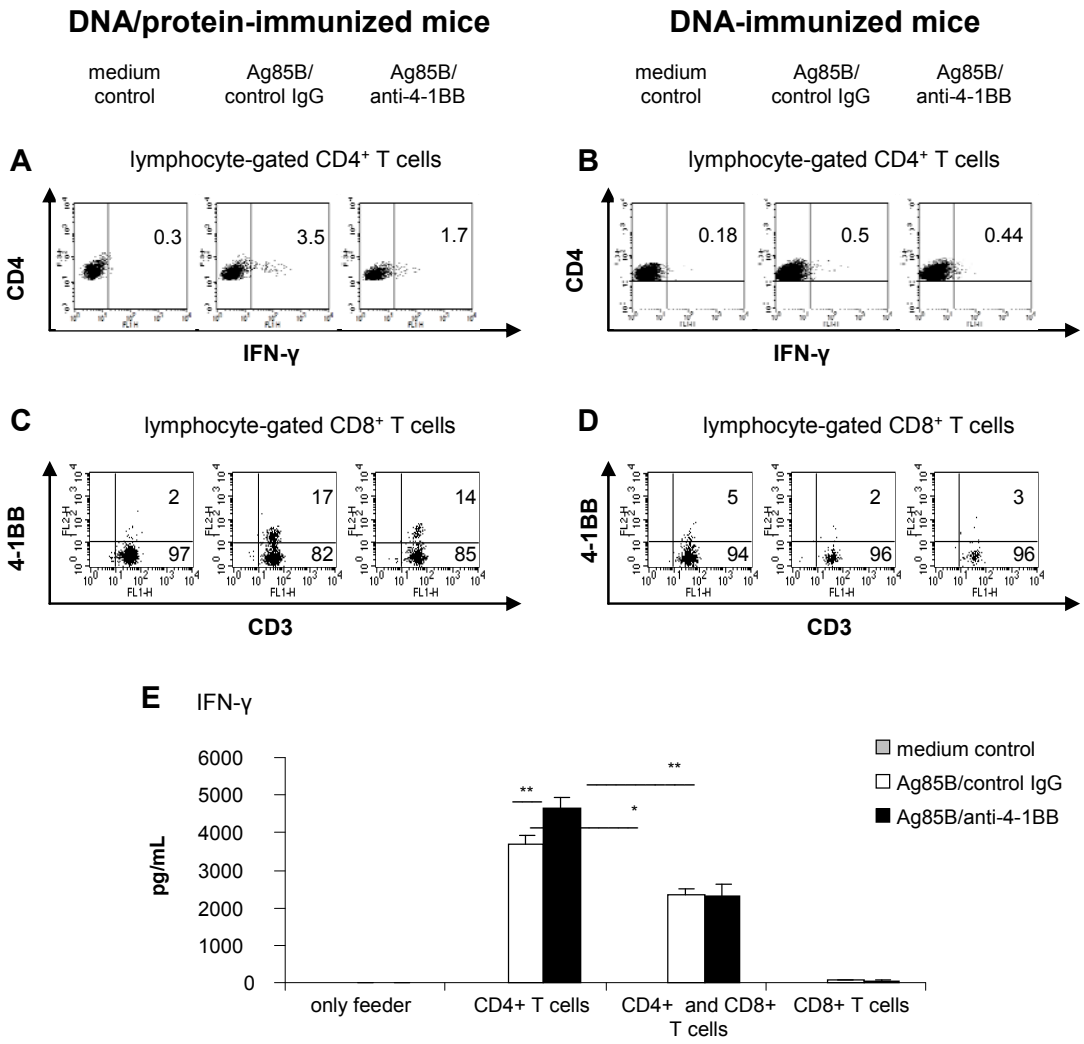

Figure 2. 4-1BB ligation inhibited non-protective IFN- γ production by CD4$^+$ T cells of DNA/protein-immunized mice while the protective IFN-γ response of DNA-immunized mice was not affected: Ag85B stimulation induced 4-1BB expression exclusively on suppressive CD8$^+$ T cells of DNA/protein-immunized mice

No cytokines were detected in unstimulated splenocytes. Data are expressed as mean ± SE of five separate experiments. Other mice were challenged i.v. with 10^5 CFU of *Mtb* H37Rv. Four weeks after infection, the bacteria in the lung were enumerated by CFU assay (Figure 2B). Data are expressed as mean of the five individual mice ± SE and are representative of 1 experiment out of 2. The level of statistical significance for differences between test groups and the control vector were determined by ANOVA test (*, significant). In Figure 3C, haematoxylin-eosin staining of lungs of *Mtb*-infected mice. In naïve unvaccinated and DNA/protein-immunized mice pathological changes and infiltration are more severe (some of them marked with asterisk in the low power magnification) compared to BCG- or DNA-vaccinated mice. The Ziehl-Neelsen staining for acid-fast bacilli (red) (high magnification) also demonstrated that the

infiltration in naïve and DNA/protein-immunized mice contained a lot of acid fast bacilli some of which are depicted by arrows.

In conclusion, the mycobacterial antigen Ag85B induces memory IFN-γ-producing $CD4^+$ T cells associated with protection against *Mtb* infection but the protein endows also a subtle ability to make ineffective a previous protective host immunity. In fact, Ag85B protein turn a protective IFN-γ response induced by DNA-85B vaccine into a non-protective response by expanding a sub-population of Ag85B-specific $CD4^+$ T cells producing high level of IFN-γ unable to control MTB replication, that caused severe inflammation and lung damage. Several cellular and molecular mechanisms can control the expansion of these non-protective Ag85B-specific $CD4^+$ T cells both in the induction (LTK63 adjuvant) or during the effector phase (suppressive $CD8^+$ T cell, the co-stimulatory molecule 4-1BB). These findings are preparatory for novel molecular strategies for intervention in TB pathology and vaccination.

Pooled spleen cells of DNA/protein-immunized mice (panels A and C) or DNA-immunized-mice (Figure 2B and Figure 2D) were re-stimulated with Ag85B protein (5 μg/mL) in the presence of agonistic anti-4-1BB mAb or rat IgG2a control Ab (5 μg/mL each). In panels A and B, spleen cells after 4 days of culture and an overnight incubation with brefeldin A were stained with CD4-PE-Cy5 and then intracellular stained with FITC anti-IFN-γ Ab. Cells were then analyzed by flow cytometry. Dot plot were generated after gating on live $CD4^+$ T lymphocytes and show frequency of IFN-γ-producing cells. In panels C and D cells were labelled with CD8 PE-Cy7, CD3 FITC and 4-1BB PE and analyzed by flow cytometry. Within each panel, the percentage indicates the number of $CD8^+$ T cells expressing the 4-1BB receptor. In Figure 2E, untouched purified $CD4^+$ T cells ($5x10^4$ cells/well) and $CD8^+$ T cells ($3x10^4$ cells/well) obtained by negative magnetic bead separation from spleen cells of DNA/protein-immunized mice were cultured, alone or together on a feeder of $CD3^+$ T cell-depleted spleen cells of naive mice (1.5×10^5 cells/well). Cells were re-stimulated with Ag85B protein (5 μg/mL) in the presence of agonistic anti-4-1BB mAb or rat IgG2a control Ab (5 μg/mL each). Culture supernatants were harvested at 72 h for IFN-γ detection by a specific quantitative sandwich ELISA Kits. Data are presented as mean of 4 independent experiments. Error bars indicate SEM. The level of statistical significance for differences between Ag85B-induced responses (both in the presence or absence of agonist anti-4-1BB mAb) by $CD4^+$ T cells alone and co-cultured with $CD8^+$ T cells were determined by a two-tailed Student's *t*-test (*, $p<0.05$; **, $p<0.01$).

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DEVELOPMENT OF A LOW COST THERAPEUTIC VACCINE FOR THE CURE OF PRECANCEROUS AND CANCEROUS LESIONS CAUSED BY PAPILLOMAVIRUS GENOTYPE 16

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Human Papillomavirus type 16 (HPV16) is associated with the development of benign and malignant lesions of the oral and genital tract (1). HPV16 belongs to the Papillomaviridae family, has an icosahedral capsid of 55 nm formed by 72 capsomers (Figure 1), circular double-stranded DNA genome of about 8000 bp, coding for 8 genes (Figure 2).

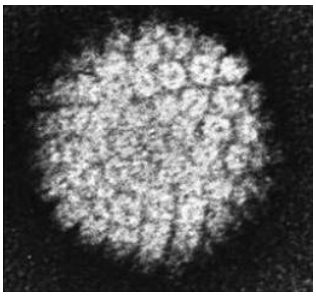

Figure 1. Electron micrograph of a Human Papillomavirus *(NIH-Visuals Online# AV-8610-3067)*

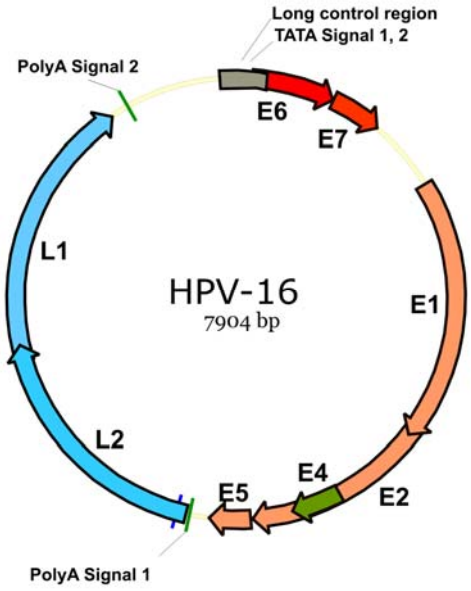

Figure 2. Schematic representation of HPV16 genome divided into three functional regions: the early region (E), encoding six early proteins E1 to E7; the late region (L), encoding the viral capsid proteins (L1 and L2); and a long control region
(from en:wiki, Xmort)

Papillomaviridae are epitheliotropic viruses which have been isolated from all vertebrates. The International Committee on Taxonomy of Viruses (ICTV) classify in 29 genera the 189 papillomavirus (PVs) isolated and sequenced so far. In humans 120 genotypes (HPV) have been well characterized, while 64 papillomavirus (PV) have been isolates from other mammals, 3 PV have been isolates from birds and 2 from reptiles. More than 30 HPV genotypes infect the anogenital mucosa, about 15 of them are defined at oncogenic risk (3). A recent retrospective study on HPV genome detection in 22661 cervical cancer (CC) specimens, collected worldwide, showed that HPV16 is present in 61% of the CC samples. HPV16 with 18, 45, 31, 33, 52, 58 and 35 genotypes are present in 92% of the CC samples (4), confirming previously data (5).

To fight the HPV infections, and consequently the anogenital cancer, prophylactic vaccines have been recently introduced in Italy as well in many European countries, in USA, in Australia and New Zealand. Preventive HPV vaccines aim to prevent HPV infection by inducing neutralizing antibodies response that block HPV particles before their entry into epithelial cells. Two formulations of HPV vaccines have been licensed: the tetravalent Gardasil (Merck-Sanofi Pasteur) based on yeast-produced virus-like particles (VLPs) of four genotypes (HPV16, HPV18, HPV6 and HPV11) in Alum adjuvant; the bivalent Cervarix (GlaxoSmithKline) based on Sf9-produced VLPs of the two oncogenic genotypes HPV16 and HPV18 in AS04 adjuvant. In Italy the two vaccines are purchased by the Italian Health Regional authorities through public tenders (Figure 3).

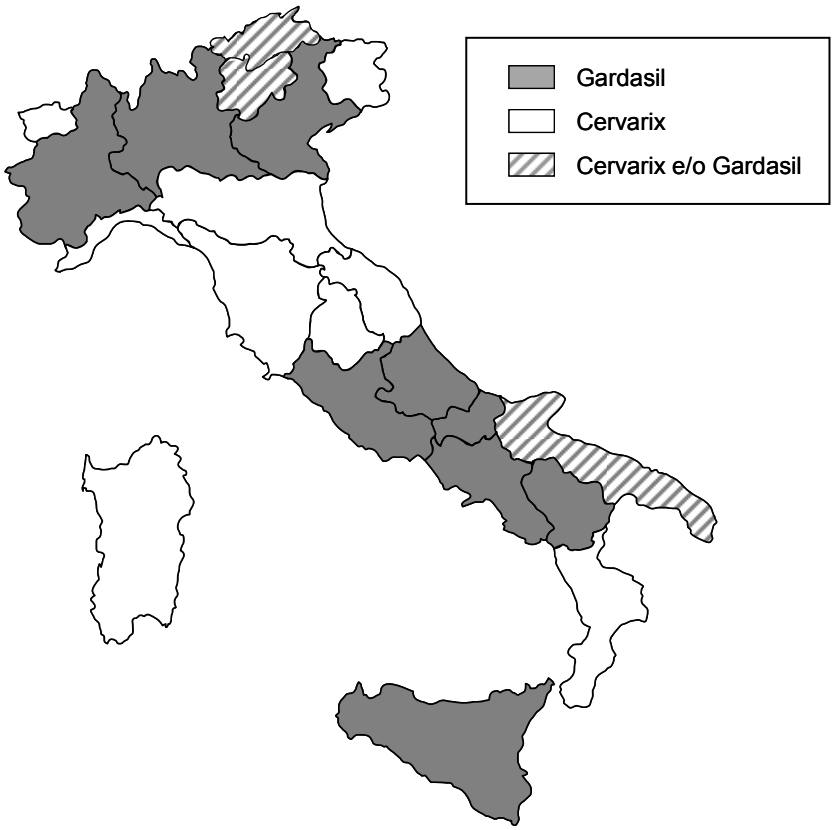

Figure 3. Distribution in Italy of the two prophylactic HPV vaccines in 2010

The HPV prophylactic vaccines have been licensed for use in children, adolescents and women of 9-25 years old. At moment, several trials are ongoing to enlarge this population. These trials are funded by the companies producing the vaccines and have the aim to use the vaccines in men, in persons belonging to groups at risk such as HIV-1 seropositive people, in chronic and autoimmune disease affected people. Trials using HPV vaccines in combination with other vaccines (HBV, polio IPV, DPT) are also ongoing. In the other hand, Public Institutions and Private Foundations founded also trials to study 1) the vaccine safety on groups at risk, 2) long-term immunity after vaccination, 3) different vaccination schedule, 4) campaigns of vaccination in South Africa, Tanzania, China, Brazil and India.

To cure established HPV infections and precursor lesions of cancer, therapeutic vaccine have been developed. They elicited a cell mediated immune response targeting the tumor specific antigens (TSA) HPV E6 and E7. These proteins are oncogenic *in vivo* and *in vitro.* During the HPV infections, E6 and E7 are responsible for the cell cycle control breakdown, thought the alteration of the p53 and pRb tumour suppressors activities (6). Various forms of therapeutic vaccines have been developed and tested in animal models. Most of the vaccines induced CTLs and an effective HPV16-dependent tumor regression in animal model, nevertheless, only a few have reached the clinical trial phase. As the HPV16 mouse tumour model, kindly provided from the authors to the research community, is easy to set up, E7 was also used as a model antigen to demonstrate the efficacy of adjuvants, molecular carriers and genetic vectors as inductors or enhancers of T cell response (2). E7 was fused to a notable number of peptides and proteins, even those of HPV16 such as L1, L2 and E6 with the aim to combine HPV prophylactic and therapeutic vaccines (7).

During the last years our group has been involved in developing therapeutic vaccines for the treatment of HPV 16 dependent lesions (8-11). Recently our attention focused on the use of HPV16 E7 tumour antigen produced in *Escherichia coli* (*E. coli*). Bacteria represent a well known and cost-effective source for the production of antigens. The protein productions in *E. coli* have time, costs and labour reduced, and can be easily scaled up in industrial-scale purification. We established a purification protocol for *E. coli* expressed proteins based on the use of organic solvents, which remove most of the endotoxins. These proteins can be used in mice without any adverse effect. An anti-tumoral vaccine based on *E. coli*-derived E7 was developed in mice. The HPV16 E7 protein was purified by a one-step denaturing protocol and folded *in vitro* into a soluble form by dialysis in buffer containing DTT. In this condition E7 assembles in micro- and nanoparticles as observed by negative staining electron microscopy (EM) analysis.

Figure 4 shows representative EM micrographs of the E7 preparation samples. The protein appears dispersed on the grid as aggregates of different shape and size (Figue 4A), and clustered in compact-looking spheroidal microaggregates, the majority ranging between 100 and 200 nm in size (Figure 4B). In the same samples, E7 also appears assembled in structured particles that seem to derive from the aggregation of smaller particles (Figure 4C). These particles resemble the previously described E7 oligomers obtained by a multistep purification procedure but never used as antigen in vaccine development (12).

To investigate if this *E. coli*-derived HPV16-E7 preparation, administrated without adjuvant, was able to induce a tumour-protective immunity, groups of mice were inoculated with 10 μg of protein per mouse, 1, 2 or 3 times, at one week intervals. As a control group, mice were inoculated with a saline solution (naïve group). Two weeks after the last immunization, some of the animals were bled and killed to analyse the immune response, *in vitro*. The remaining animals were challenged with the TC-1 tumour cells and the inhibition of tumour growth in these mice was monitored for 2 months.

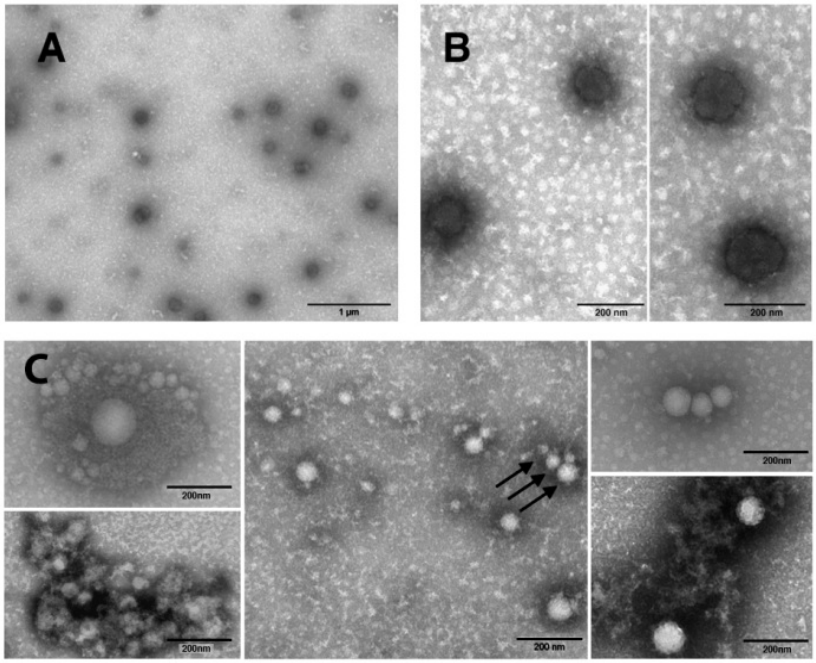

Figure 4. Electron micrograph of E7 assembled in particles: electron micrographs of the negatively stained E7 preparation samples (A); E7 particles of different shape and size (B); spheroidal microaggregates of compact aspect (C)
(highly structured E7-particles of different size are indicated by arrows; the magnitude* scale bars *are indicated)
(from Petrone et al., Journal of Translational Medicine *2011) (13)*

To analyse the humoral immune response and the animal sera of each group were pooled, then sequentially diluted to determine the antibody titres by end-point dilution in an E7-based ELISA. The anti-E7 IgG titre was 1:200 after a single immunization and progressively increased in mice immunized 2 and 3 times, reaching 1:8000 and 1:16000 respectively. The presence of IgM and IgA was analysed in comparison with the IgG. The sera show an increase of anti-E7 specific IgG already after the second protein dose; IgMs were detected only after the third E7-dose, while IgAs were never detectable (Figure 5). The animals inoculated with 3 doses of saline solution did not show any E7 specific antibody response (Figure 5A).

The therapeutic effector functions of antibodies depend on their class and subclasses therefore in order to better evaluate the E7-specific humoral immune response, the anti-E7 specific IgG1, IgG2b, IgG2c (IgG2a) and IgG3 antibody subclasses were also determined. The results show that the IgG2b level was significant after the second immunization while the level of IgG2c was significant only after the third immunization. The level of IgG1 was not significant and IgG3s were undetectable (Figure 5B).

This anti-E7 IgG isotype profile indicates that the immune response induced in vaccinated mice is a mixed Th1/Th2 type. To analyse the induction of the cell-mediated immune response in mice after 1, 2 or 3 doses of the E7 preparation, T-enriched splenocytes from mice of the same immunization group, were stimulated, *in vitro*, with the E7-specific CTL peptides and processed for T cell proliferation and γ-IFN ELISPOT assays. Splenocytes from naïve mice were pulsed with an unrelated mixture of peptides used as control.

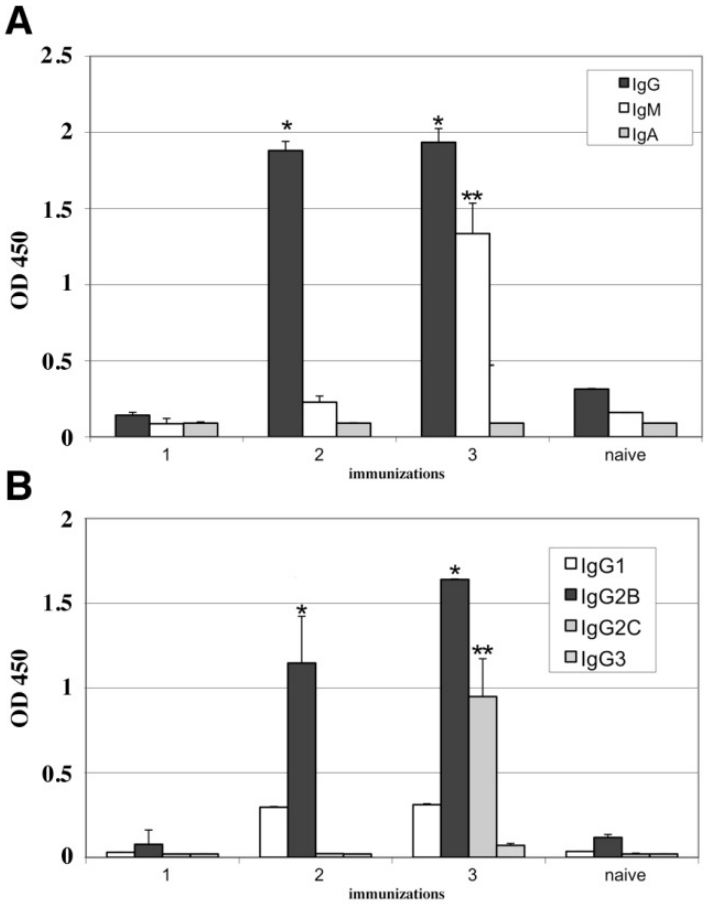

Figure 5. Analysis of the E7-specific antibody response:
ELISA results showing the anti-E7 IgG (black bars), IgM (white bars) and IgA (grey bars) reactivity of the pooled mice sera samples either naïve or immunised with 1, 2 or 3 E7 doses (A);
ELISA results showing the anti-E7 IgG1, 2b, 2c and 3 isotype reactivity of the pooled mice sera samples (B)
(from Petrone et al., Journal of Translational Medicine *2011) (13)*

The results are shown in Figure 6. Splenocytes from mice immunised with 2 and 3 doses of E7 showed a high Stimulation Index (SI) suggesting that specific T clone selection occurred after E7 peptide stimulation (Figure 6A). Splenocytes from naïve mice and from mice immunised once showed non-significant SI. Conversely, in the γ-IFN ELISPOT assay (Figure 6B) only the splenocytes of mice that received 3 E7 doses, stimulated with the E7-specific CTL peptides, showed a significant level of E7-specific γ-IFN producing cells (Figure 6B).

To evaluate the efficacy of the E7 preparation as inductor of anti-tumour immunity, the mice immunised with 1, 2 or 3 doses of E7 were challenged with the TC-1 tumour cells, and the tumour growth was monitored for two months after the challenge. Mice vaccinated with three doses of E7 particles were fully protected from tumour growth. Only 40% of the mice immunised with 2 doses of E7 were tumour free, whereas the mice immunised with 1 dose and the naïve mice developed a palpable tumour within 4 weeks of tumour-monitoring, after the challenge with TC-1 cells (Figure 7).

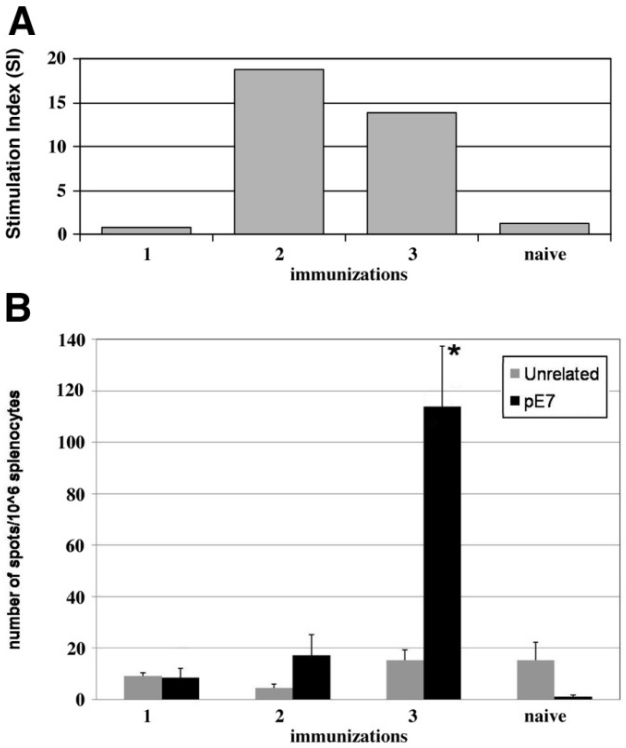

Figure 6. E7-specific cell mediated immune response. T cell proliferation assay (panel A); T cell proliferative response of C57/BL6 naïve and mice immunised with 1, 2 o 3 E7 doses. Panel B. IFN γ-secreting cells from naïve mice and mice immunised with 1, 2 o 3 E7 doses. Cells were stimulated with two CTL E7 peptides (black bars) or with an unrelated peptide (grey bars)
(from Petrone et al., Journal of Translational Medicine *2011) (13)*

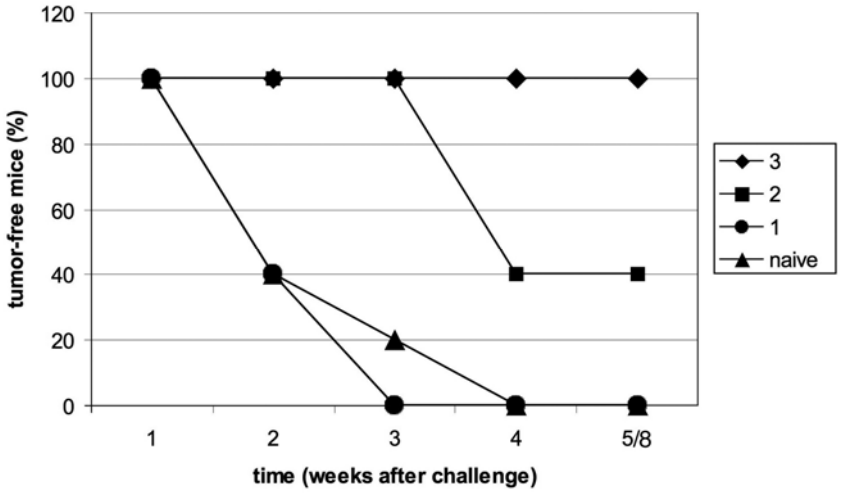

Figure 7. Mice naïve or vaccinated with 1, 2 and 3 doses of E7 were challenged with 1x10^5 TC-1 tumour cells and the tumour growth was monitored weekly
(the percentages of mice without tumour are indicated)
(from Petrone et al., Journal of Translational Medicine *2011) (13)*

In conclusion we show, for the first time, the use of recombinant HPV16 E7, assembled *in vitro* into particulate form, to induce protective immunity against a HPV16-related tumour in an HPV16 mouse model. E7 particles, used without adjuvant, are excellent stimulators of the immune system. In C57BL6 mice, the E7 preparation induces anti-tumour immunity sustained by both humoral and cell-mediated immune responses. This E7 protein (derived from *Escherichia coli*) without adjuvant could represent, along with the recently proposed *E. coli*-derived HPV antigens a low cost constituent for the development of a new generation of HPV16 vaccines, which combine prophylactic and therapeutic activities.

In the attempt to develop an antitumoral HPV16 monodose vaccine this E7 preparation, in particles form, was adsorbed on lamellar crystals of Poly-L-lactide (PLLA). PLLA is a biodegradable polymer used in the production of nano- and microparticles for the controlled release of drugs. Poly-L-lactide is produced from polymerization of lactic acid obtained from microbial fermentation of corn starch or sugarcane feedstocks. It is a type of bio-plastic that can be processed into fiber and film like most thermoplastics. Recently, PLLA particles have been used in delivery of antigens for a rational manipulation of the immune system for vaccine development. Controlled release of antigens from biodegradable polymers is a promising technology for reducing the number of immunizations in vaccine development.

PLLA single crystals were crystallized from 0.25% (w/v) p-xylene solution (mw ≈ 152 x 103 g·mole-1) at 90°C. To generate functionalized particles (APLLA) free amino groups were introduced onto the PLLA lamellar single crystals. Morphological features of both native PLLA and modified APLLA were analysed by Scanning Electron Microscopy (SEM) (Figure 8) (14).

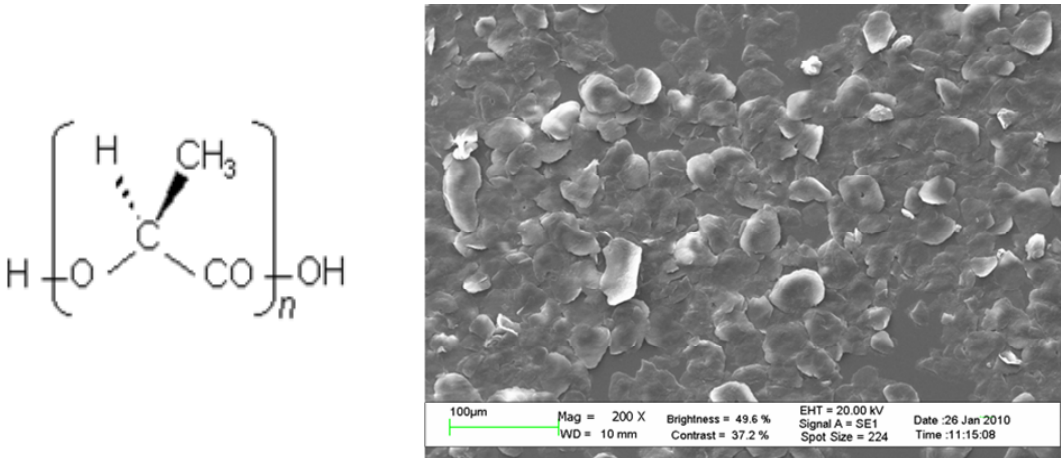

Figure 8. Skeletal formula of the Polylattide (left side) and SEM of the PLLA single crystals synthesised for the study (right side)

Both crystals showed hexagonal shape and a 2d-micro-1d-nano-sized lamellar structure with a mean lateral dimension of about 30 µm and a thickness of about 11-12 nm.

The *E. coli*-derived HPV16-E7 in particles form was adsorbed onto either PLLA or APLLA at 4°C in Tris-NaCl buffer by 48 h incubation in rotator. The two particles adsorbed similar quantities of E7, as assessed by a Coomassie stained SDS-PAGE quantization (data not shown). In order to study the immune response induction, E7-PLLA and E7-APLLA samples were administered to C57BL76 mice using a prime-boost immunization schedule. The results showed that 100 mg/per mouse of both the particles administered by intraperitoneal or subcute injection

were not toxic for the animals. The analysis of the antibody response showed that the E7-APLLA crystals induces and higher titre of anti-E7 IgG antibodies (1:16000), compared the E7-PLLA crystals (1:2000), which showed a lower antibody titre even compared the E7 without crystals (1:4000). Unfortunately, the analysis of the cell-mediated immune response, by proliferation and γ-IFN ELISPOT assays, did not show any detectable E7-specific immune response in splenocytes of mice receiving either E7-APLLA or E7-PLLA crystals. We concluded that a prime-boost regimen of E7 immobilised on lamellar crystals induce only a specific antibody response.

In order to evaluate any possible T cell induction by the E7 immobilised on poly-L-lactide crystals, a tumour protection experiment was performed using the TC1/HPV16 mouse tumour model. The immunization schedule was as that known to be effective with the E7 in particles without crystals. It consists of 3 immunizations of the antigen at 1 week interval. After one week last immunization, mice were challenged with the TC-1 tumour cells and the tumour growth was monitored for 2 months. Three groups of mice were immunised respectively with: 1) 10 μg/mouse of E7-PLLA; 2) 10 μg /mouse of E7-APLLA; 3) 10 μg/mouse E7 in particles form. Mice received in each immunization about 30 mg polylattide/mouse. A fourth group of animals was immunised with a saline solution and used as a control group. The analysis in mice sera of the E7-specific antibody response by end point dilution assay showed that the higher titre was found in mice immunised 3 times with the APLLA (1:32000). Interestingly mice received the E7-PLLA showed a lower antibody titre (1:4000) than the mice immunised with the E7 in particles (1:8000).

The analysis of the cell mediated immune response by γ-IFN ELISPOT assay showed that the higher number of γ-IFN producing cells were obtained with splenocytes of mice immunised with 3 doses of APLLA absorbed with E7. The lower immune stimulation of the T cell response was obtained with splenocytes from mice immunised with E7-PLLA, analogous to that observed for the stimulation of the humoral immune response. In mice immunised with the E7 without PLLA the quantity of γ-IFN producing cells was in the average expected (Figure 9).

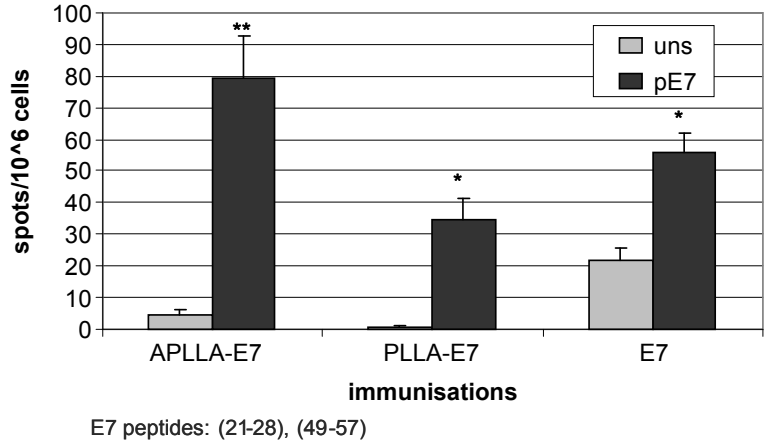

Figure 9. ELISPOT. IFN γ- ecreting cells from splenocytes of mice immunised with APLLA-E7, PLLA-E7 o E7. Cells were stimulated with two CTL E7 peptides (black bars) or with an unrelated peptide (grey bars)

In tumour protection experiment, the mice immunised with either aminolysed-PLLA-E7 or E7 were fully protected from the tumour challenge. Naïve mice and mice immunised with either APLLA or PLLA developed a palpable tumour within four weeks from the tumour cells challenge (Figure 10).

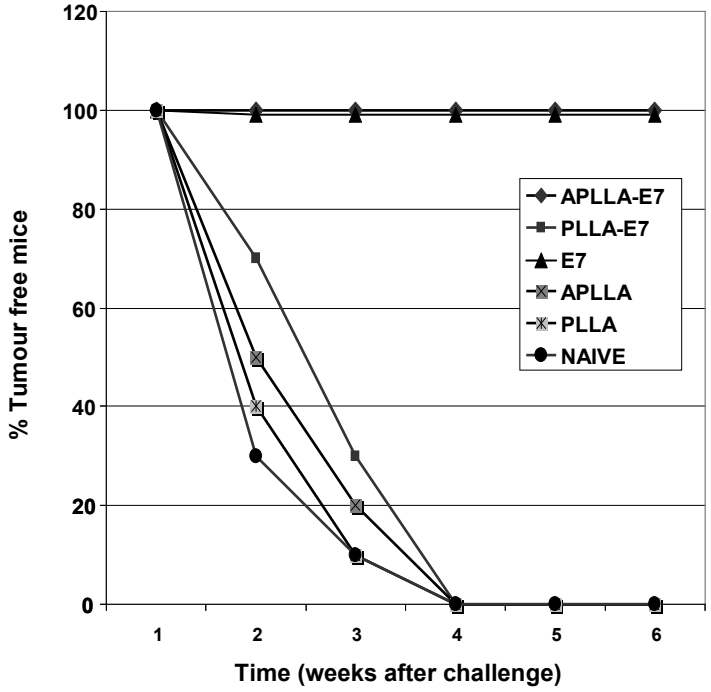

Figure 10. Mice vaccinated with 3 doses of E7-APLLA, E7-PLLA, E7, APLLA, PLLA and naïve were challenged with 1x10^5 TC-1 tumour cells and the tumour growth was monitored weekly

The experiments performed so far using as antigen the E7 adsorbed on polylactide crystals show that aminolysed-PLLA increases the E7 immunogenicity in term of humoral and cell mediated immune response. Conversely the unmodified PLLA does not show similar activities.

The adjuvant effect on the T cell response of the polylactide crystals depends from the immunization schedule, in fact only three doses of antigen adsorbed on the particles, are efficacious in the induction of a tumour protective immunity.

New experiments using APLLA-E7 are currently ongoing, increasing the dose of protein and particles injected per mouse in the attempt to develop a monodose vaccine inducing a T cell mediated immune response.

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MEASLES ERADICATION: THE ROLE AND ACTIVITIES OF THE NATIONAL REFERENCE LABORATORY FOR ELIMINATION OF MEASLES AND CONGENITAL RUBELLA

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Introduction

According to the goals of World Health Organization (WHO) in the European Region (1), Italy implemented a National Elimination Plan (NEP) for measles and congenital rubella in 2003, in order to reach the objective by 2010. The aim of the NEP is to reach at least 95% vaccination coverage for one dose of MMR (Measles, Mumps and Rubella) vaccine in children in their second year of life, to perform catch up vaccinations of older children and adolescents, and to administer a second dose of MMR vaccine to 5-6-year-old children. According to the NEP, physicians have to report suspected cases of measles within 12 hours and laboratory diagnostic testing of blood, saliva and urine specimens is recommended for all sporadic cases. Case report forms are collected centrally at the Ministry of Health and the Istituto Superiore di Sanità (ISS, the National Health Institute in Italy).

Measles is a contagious human disease caused by Measles Virus (MV). Symptoms include high fever, conjunctivitis, coryza, cough, the appearance of Koplik spots on the buccal mucosa and a maculopapular rash. Vaccination programs have dramatically reduced the incidence of measles on a global scale (2, 3). Measles transmission has been interrupted in the region of the Americas, and the Eastern Mediterranean, European and Western Pacific Regions have established elimination targets for the near future. Despite the success of global measles vaccination programs, measles is still responsible for an estimated 245,000 deaths each year (4) with most of these deaths occurring in developing countries. Sustained measles outbreaks continue to occur in developed countries that have failed to maintain a high level of population immunity (5). In 2002, the WHO Regional Committee for Europe adopted a resolution to eliminate indigenous measles and rubella in the 53 Member States in the Region by 2010. Elimination is defined as a situation in which sustained virus transmission cannot occur and secondary spread from importation of disease will end naturally without intervention. Key strategies to achieve this goal are: achieving and sustaining high coverage (≥ 95%) with two doses of measles and at least one dose of rubella vaccine through high-quality routine immunisation services; providing a second opportunity for measles immunisation through Supplemental Immunisation Activities (SIA) in susceptible populations; using the opportunity provided by measles SIA to target populations susceptible to rubella with combined measles and rubella-containing vaccine; and strengthening measles, rubella, and Congenital Rubella Syndrome (CRS) surveillance through rigorous case investigation and laboratory confirmation of all suspected cases (6).

The regional strategy encourages rubella vaccination opportunities, including supplementary immunisation activities, for all rubella-susceptible children, adolescents and women of child-bearing age. All national SIA conducted in the eastern part of the WHO European Region have

included rubella vaccine. In addition, rubella vaccination is part of the routine immunisation schedule all member states. Since 1998, measles incidence in the WHO European Region has declined from 110 cases per 1,000,000 population to historically low levels of ≤ 10 cases per 1,000,000 in 2007 and 2008. In 2008, 29 member states reported a measles incidence of less than one per 1,000,000 population, selected as one of the indicators for monitoring progress towards elimination. This progress is based on high immunisation coverage achieved through a routine two-dose schedule for measles-containing vaccine and SIA to reach susceptible populations. The estimated regional coverage for the first dose of measles vaccine increased from 88% in 1998 to 94% in 2008. Moreover, reported coverage for the second dose ranged from 62% to 99% in 2008. From 2000 to 2008, at least 17 countries conducted nationwide SIA, reaching approximately 54 million people. Surveillance has been strengthened by improving case investigation procedures, expanding case-based reporting and increasing laboratory testing.

As part of laboratory-based surveillance for measles, genetic characterization of circulating wild-type viruses provides an important tool for mapping transmission routes, documenting the elimination of endemic strains, and distinguishing vaccine reactions from wild-type infections (2, 6). In a given country or region, a genotype is considered endemic if it is consistently associated with outbreaks over an extended time period. On the contrary, the identification of multiple genotypes associated with a limited number of outbreaks and/or sporadic cases is more consistent with multiple importations of virus than with endemic virus circulation (7).

In order to support case ascertainment, a National Reference Laboratory was also established at the ISS. The role of our laboratory is to confirm outbreaks/cases by IgM serology and to determine the measles virus genotype for molecular epidemiological purposes (8, 9).

Method

RT-PCR (nested) genotyping of MV

In ISS National Reference Laboratory for Measles, Mumps, and Rubella, a total of 43 samples were examined for the presence of viral nucleic acid (RNA) by Reverse Transcription (RT) and nested Polymerase Chain Reaction (PCR). Thirty-four of samples obtained from 16 patients were analysed for MV. Viral RNA was extracted from the supernatant. For the detection of MV, a highly conserved part of the MV nucleoprotein gene was reverse transcribed and amplified by nested PCR (10). The 450 nucleotide PCR product corresponding to the C-terminal part of the nucleoprotein ORF was used for MV genotyping. The PCR products were sequenced and compared with the WHO viruses reference strains (11).

Results and discussion

A total of 172 samples were collected from different regions of the Italy between May 2002 to December 2007. This representative set of sequence was genotyped by comparing the sequences coding for the carboxyl terminus of the nucleoprotein (456 nucleotides) with the sequences of the WHO reference strains. The results indicated that for 39 out of 41 specimens collected in 2002, either just before or during the peak of the outbreak, belonged to genotype D7 and 2 to genotype A.

During 2003, seven cases were caused by genotype D7 and one by the genotype A. All three 2004 positive samples belonged to the genotype C2. Then twenty-eight (22%) of the measles

sequences detected from chains of transmission in Italy during 2002-2004 belonged to genotype D7. Three (3%) of the 33 sequences obtained from clinical specimens were placed in genotype A and three were placed in genotype C2. All 21 sequences of year 2002 classified as genotype D7 were closely related to each other showing no more than 0,3% nucleotide heterogeneity overall, suggesting a common origin of the epidemic.

Three specimens, two by 2002 and one by 2004 respectively, had nucleotide sequences in genotype A, which contains all vaccine viruses. A comparison of the sequences with the vaccine strains and with the wild-type genotype A strains has revealed closed relation (genotypes distribution is showed in Figure 1).

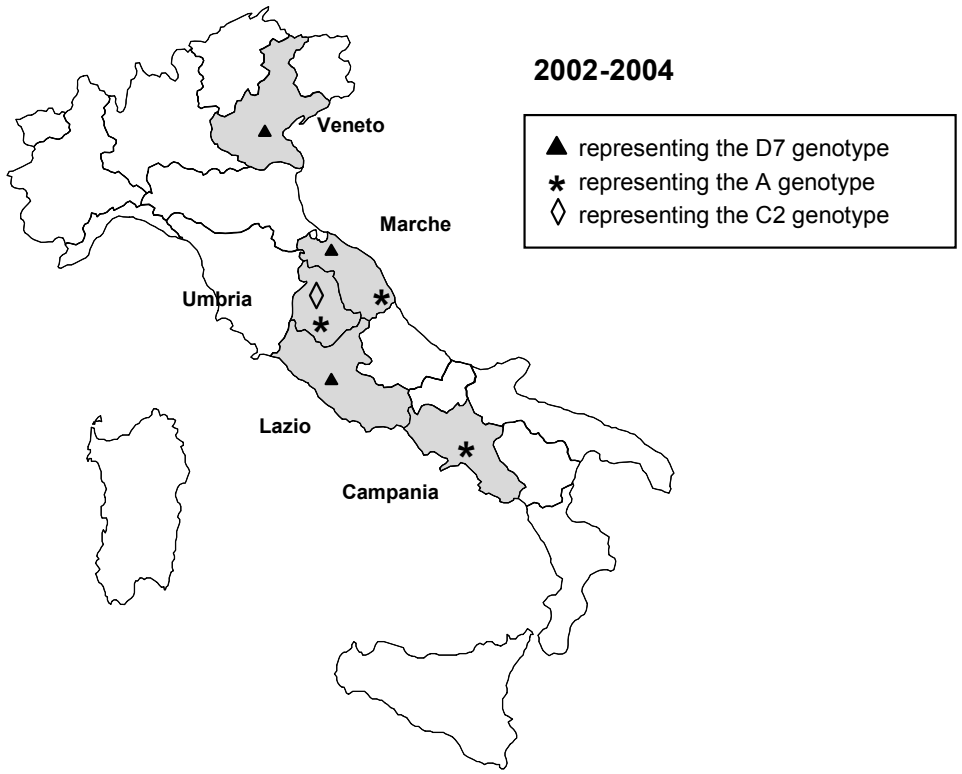

Figure 1. Measles distribution in Italy during the years 2002-2004

In the year 2005 the laboratory surveillance of cases was interrupted and restarted in the year 2006.

During 2006 the sequences analysis showed 59 out 75 specimens belonged to genotype D4 and 16 to the genotype B3. In the year 2007 the sequences of cases showed 29 specimens belonged to genotype D4, 35 belonged to genotype B3, 6 belonged to genotype D5 and one belonged to genotype D8. The majority of MVs collected between 2006 and 2007 belonged to genotype D4 (Figure 2).

In view of the goal of measles elimination, it is of great importance to assess the circulation of wild-type measles virus (MV). Genetic analysis is indispensable in understanding the epidemiology of measles, it showed that the strains circulating from year 2002 to 2008 were genotype A, D7, D4, B3, D5. All Italian measles strains were closely related at the same strains circulating in European countries.

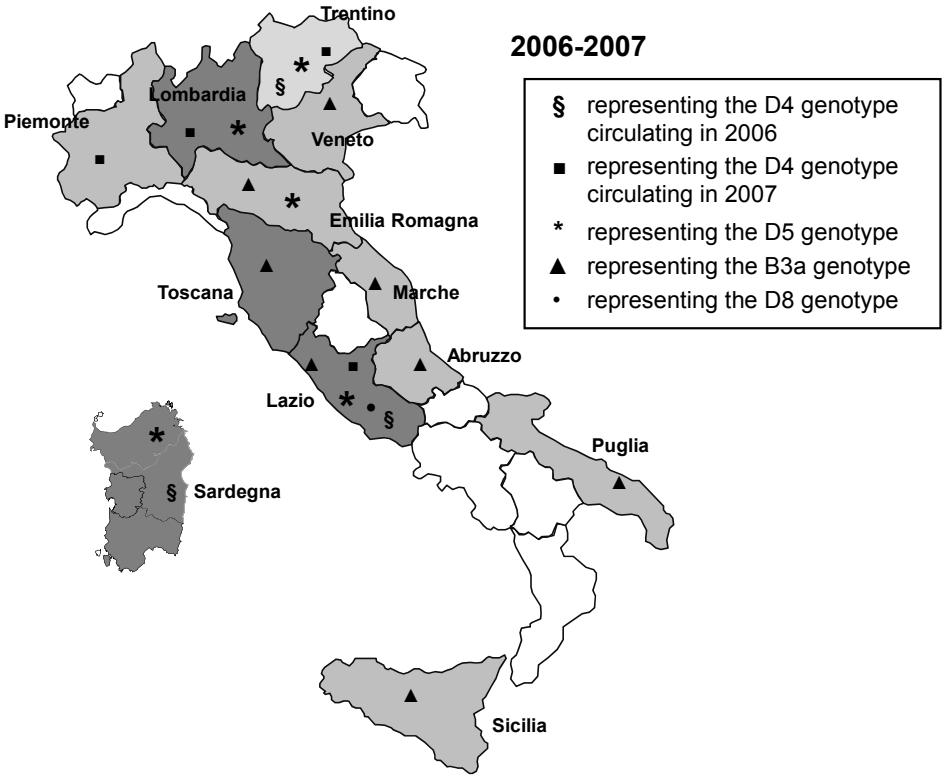

Figure 2. Measles distribution in Italy during the years 2006-2007

Countries in the measles elimination phase, such as Italy, need to make increased efforts to obtained appropriate specimen from each chain of transmission.

Genetic characterization of wild-type measles viruses provides a means to study the transmission pathways of the virus and is an essential component of laboratory-based surveillance. Laboratory-based surveillance for measles and rubella, including genetic characterization of wild-type viruses, is performed throughout the world by the WHO Measles and Rubella Laboratory Network, which serves 166 countries in all WHO regions. In particular, the genetic data can help to confirm the sources of virus or suggest a source for unknown-source cases as well as to establish links, or lack thereof, between various cases and outbreaks.

If Italy wants to achieve the goal of eliminating measles by 2015 (12, 13), its utmost priority should be to convince reluctant parents and health professionals about the critical importance of universal measles vaccination. Indeed, improvement in the education of health professionals and the general public concerning the risks of non-immunity to vaccine preventable diseases will play an important role in advancing the measles elimination program.

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COMMUNICATION AND RESEARCH: A POSSIBLE INTEGRATION

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In the twentieth century it was widely believed that infectious diseases were no longer to be considered among the most important problems in public health. In countries with advanced economies, in fact, a drastic reduction of such diseases has been achieved thanks to advanced scientific research, the use of vaccines and the introduction of antibiotics. However, at the beginning of the third millennium, new infectious diseases, in particular Human Immunodeficiency Virus (HIV) and related opportunistic infections such as tuberculosis, have demonstrated the world's population susceptibility to infection. Even today, infectious diseases by multiple etiologic agents (viruses, bacteria, fungi and protozoa) are the leading cause of death and the main determinant of disability or socio-economic hardship for millions of people around the world. Infectious diseases affect 365 million people, most of whom live in developing countries. More than 14 million people die annually due to these diseases.

In such a complex scenario the RCT (psycho-socio-behavioral Research, Communication, Training) Unit of the Department of Infectious, Parasitic and Immune-mediated Diseases (MIPI) of the Istituto Superiore di Sanità (ISS, the National Institute of Health in Italy) is working to reach these goals:

- integrating psycho-social approach with biomedicine in order to meet the health needs of the population and provide appropriate responses in relation to infectious diseases (psycho-socio-behavioral research);
- delivering scientific and personalized information on HIV infection and other Sexually Transmitted Diseases (STD) considering the complexity of psychological variables, social and cultural rights of individuals (phone counselling on HIV/AIDS and STD);
- translating the results of studies and research on infectious diseases, with particular reference to STD, in effective messages helping people to both improve the quality of life and choose healthier lifestyles (communication).
- promoting and spreading knowledge and expertise on communication and relational issues through training programs addressed to National Health Service (NHS) operators, Non-Governmental Organizations (NGOs), Voluntary Associations (VA) and Community of Migrants (CM) (training).

Role of RCT Unit

The partnerships between health professionals and experts in the biomedical research, human/social sciences, the psycho-socio-behavioral research and communication/training has allowed to develop, share and implement some precious strategies to promote and protect health, preventing HIV infection, tuberculosis and other STD. The commitment of the RCT Unit team initially focused on the HIV phone counselling activity and more recently on STD. Such counselling activity provided the RCT Unit researchers elements and indicators to engage also in other areas, in response to some of the institutional functions of the MIPI Department.

The RCT Unit working group – in addition to the daily HIV/AIDS and STD phone counselling activities – is currently working on:

- scientific research in the psycho-socio-behavioral area;
- training/intra-extramural update courses for NHS staff and VA operators;
- health education aimed to the youth population and vulnerable groups (migrants, women);
- consultation and collaboration with research institutes, ministries and local governments concerning the health of migrant populations

HIV/AIDS and STD phone counselling

Within the context of HIV, AIDS and STD information intervention, counselling has proved to be a precious operational tool. This method, which involves an operator and a properly trained person/user, is characterized by the application of knowledge, personal qualities or skills such as active listening and empathy, as well as professional communication strategies and techniques aiming at both the activation and reorganization of the people's potential (empowerment). The goal is to make choices and changes in situations perceived as difficult by the individual himself or to tackle, in an active way, problems and difficulties relating to different personal situations. HIV/AIDS and STD phone counselling activities are located inside the RCT Unit of the ISS. This service of phone counselling (known as *Telefono Verde AIDS* (TVA), established in 1987 by the National Commission for the fight against AIDS and co-financed by the Ministry of Health and the ISS itself, was the first nationwide experience of public AIDS Helpline, engaged in both primary and secondary prevention of HIV infection and addressed to Italian and foreign general population. From March 2007 to December 2008 a "transcultural" phone counselling activity was started thanks to the work of highly skilled cultural mediators, able to answer in seven languages (English, French, Romanian, Spanish, Arabic, Chinese and Russian). During these years, the RCT Unit working group has been coordinated by a scientific responsible, collaborating with researchers and consultants coming from different areas, and with different expertise and overseen – periodically – by a supervisor. The positive impact of this approach resides in the opportunity to answer directly to the person/ user, through a personal interview able to give the right specific and scientific information which will be transformed into personalized messages, necessary to promote the implementation of both healthy changes in the people's behaviors and the implementation of Life-skills designed to avoid risky behaviors. Gathering such wide and different experience has allowed us to develop a standardized phone counselling method to:

- supply scientifically accurate information on HIV infection, AIDS and sexually transmitted infections through a custom interview;
- gather information on specific population groups (such as the reasons leading those people to get an HIV test, acceptability of a vaccine against HIV, etc.);
- make telephone surveys.

This expertise has been used to intervene both in emergency situations, caused by morbid events causing health alarm in the population (Necrotizing Fasciitis, Ebola Virus, Bioterrorism) and in fields concerning drug addiction, organ transplants, heat waves and rare diseases, more recently, in the recruitment procedures for the testing of anti-HIV vaccine based on TAT protein.

The HIV/AIDS and STD phone counselling (800861061 available only from Italy) is done anonymously and it is free of charge from Monday to Friday, 13.00 to 18.00. During the last 24 years this precious service has been able to get and answer about 671.823 calls for a total of over 1.762.369 questions, with an average of 120 calls a day (1-7).

Scientific research in the psycho-socio-behavioral area

Since the early 90s, extensive research have been carried out in the psycho-socio-behavioral domain funded by the European Commission and the Ministry of Health with the help of the NHS structures, universities, research institutes, NGOs and VA.

The main research areas in which the team is committed are:

- identification and testing of intervention models to improve adherence to HIV screening tests (8-10);
- promotion and protection of foreigner's health with particular reference to HIV/AIDS infection, tuberculosis, human papilloma virus and STD. In this scenario, the Italian National Focal Point (Infectious Diseases and Migrant) has been working since 1997 through the coordination of RCT Unit and the involvement of 70 experts of public network, NGOs and VA from different regions of North, Central and Southern Italy (11-14);
- evaluation of interventions to prevent infectious diseases addressed to foreign people and evaluation of HIV/AIDS counselling in cross-cultural context (15-17);
- analysis of the quality of life of people living with HIV, with particular reference to the issues related to the need for parenthood;
- study of the attitudes and socio-demographic characteristics of people with HIV risk behaviours and people affected by HIV (18-21);
- network of 23 HIV/AIDS phone counselling services over the national territory (1, 3).

Training

Since 1991, some researchers of RCT Unit are engaged in intra-and extramural training/ update activities on issues of effective *vis à vis* and telephone communication and counselling for NHS, NGO and VA psycho-social and health operators within different areas. More than 8,000 operators have been trained through the years. Learning or improving professional counselling skills may be an important educational tool for implementing effective communication of health professionals dealing directly or indirectly, with any issue related to infectious diseases. Through the application of the andragogical learning theory of the American scholar Malcolm Knowles, the training and the personal experience of individual health professionals become a central element of both the person and the professional figure. The aim is to encourage new cognitive schemata, conceptual elaborations and links to practical experience, allowing not only a quick and accurate response to individual requests but also a more specific action in relation to the complexity of the different topics.

In this specific context, the basic assumption of this kind of training is the actual availability and the inner motivation of the operator to establish a strong relationship with the other, based on trust, cooperation, unconditional acceptance, authenticity and empathy (22-24).

Health education activities

The intra and extramural health education activities aimed at both secondary/high schools and university students has been carried out in collaboration with regional services through seminars and peer education interventions in order to make a concerted prevention action within the field of STD targeting both the young Italian and foreign people (25-27).

Consultancy and collaboration activities

No less important have been those consulting activities carried out primarily with the Ministry of Health and with other Italian universities which have led to a fruitful collaboration. As for the Ministry of Health, the RCT Unit contribution was largely developed in some information and educational campaigns for the fight against AIDS as well as in the development of some phone counselling services in health emergency situations; furthermore, a Public Relations Office has been created for the Ministry itself while in the university field some intervention tools and techniques for the prevention of infectious diseases, have been identified.

Conclusions

The work done by the team of the U.O. RCF since 1987 has allowed us to increase scientific research in a field of social sciences which is unfortunately not fully developed in Italy yet; we have been also been able to transform these studies and research results into effective messages for the general population in order to guide people to better behaviors and lifestyle, promoting health and improving the quality of life. The control of infectious diseases is a challenge to the modern paradigm of biomedical sciences, requiring innovative prevention models and necessary partnerships between different disciplines or professional areas. Therefore, we believe that the presence of the RCF operational unit within the Department MIPI, may represent not only a concrete experience of an integrated approach to the study of infectious diseases, but also an operative model to tackle those health issues involving both the individual and the entire community.

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RESEARCH ACTIVITY ON TICK-BORNE DISEASES IN LATIUM REGION

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Introduction

Ticks (Ixodida: *Ixodidae*) are arthropods belonging to Chelicerata Subphylum, Aracnida Class, as spiders, scorpions and mites. About 900 species of ticks are known in the world and all of them are ectoparasites. Moreover such Arthropods are obligatory aematophagous as they stay on the host for a certain period of time for the bloodmeal essential for growth and reproduction. Italian Fauna ranges 2 tick families, *Ixodidae* or "hard ticks" (ranged in 6 genera: *Ixodes*, *Boophilus*, *Hyalomma*, *Rhipicephalus*, *Dermacentor*, *Haemaphysalis*), and Argasidae or "soft ticks" (ranged in 2 genera *Argas* and *Ornithodoros*) to which 36 species belong (Checklist of Italian Fauna). In the last decades, Tick-Borne Diseases (TBD) are dramatically spreading.

The growth of global relations and global warming are a health threat to the introduction and spread of vector-borne diseases in Europe, such as TBD. Among the arthropods, ticks are highly efficient vectors of many pathogens of viral, bacterial and protozoan nature that cause diseases now considered emerging basing on the increased reported cases in animals and humans.

Recently, also in Italy as elsewhere, the chances for humans to contract TBD increased and in particular the high number of dogs as pet taken in public parks, the rearing livestock activities, provide new opportunities for encounter between man and ticks. The low host specificity and the ability to transmit pathogens (both veterinary and human interest) since the immature stages of larva and nymph explains their high efficiency as vectors of infectious diseases for which the possibility of transmission is directly proportional to the length of stay of the tick on the host.

The main infectious bacterial diseases transmitted by ticks, with epidemiological importance in our country are the Rickettsiosis, Lyme disease and the relapsing fever. In southern Europe, including Italy the human Rickettsiosis are caused mainly by *Rickettsia conorii* and *Coxiella burnetii*. Among TBD virus Crimean-Congo Hemorrhagic Fever (CCHF) is responsible for a disease that causes high death rates among people, transmitted by many tick species mostly belonging to the genus Hyalomma. Fortunately, this disease rarely occurs in humans, although in animals may be more common. Sporadic outbreaks of CCHF have been reported in 30 countries in Africa, the Middle East, Asia and Europe.

Recently the research activity on ticks (temporary hung up in the last years) has been started again in the Department of Infectious, Parasitic and Immune-mediated Diseases of the Istituto Superiore di Sanità. Since 2011 until now, we carry out 4 topics 3 out of them completely "money free", voluntarily, and 1 only supported by a small fund by the Fondazione Cassa di Risparmio di Civitavecchia (CARICIV Foundation) useful for mission repayment. Such research lines are here reported and described in their preliminary results, some of them already published on peer reviews or presented at national and international congresses.

Occasional record of *Ornithodoros coniceps* in Latium

The first record of *Ornithodoros (Alectorobius) coniceps* (Canestrini) was reported for Italy in 1877, inside the interstices of the ancient mosaics at S. Marco Basilica in Venice. Afterwards only few discoveries of the species are reported for Italy; the last record is dated back to 1984, in L'Aquila town (Abruzzo Region). The present study shows the data of a survey carried out as a result of a massive infestation by *O. coniceps* in an ancient villa in Anzio town (Latium region) recently restored. In the past decades the villa has been fallen into disrepair, becoming an occasional shelter for wild animals mainly pigeons, that colonized the whole building for generations. This case appears worthy of note because it is the first record of this species after more than 25 years in Italy. A total of 136 specimens were collected by three methods: manual, mechanical aspirators and Wilson traps. Wilson trapping indicates positive O. coniceps tropism for CO_2. It is important to point out that ticks belonging to Ornithodoros genus could be vectors and reservoirs of different species of Borrelia (*B. hermsi, B. perkeri* e *B. turicata*).

This study was published on the peer-review *Experimental and Applied Acarology* (Khoury *et al*., 2011).

Bionomics of ticks in Insugherata natural reserve in Rome and detection of pathogens potentially transmitted

Since 1997 the Insugherata Natural Reserve has been an important protected area of Rome (about 740 ha), characterized by different biotopes and a significant biodiversity. This park is also an important area for human recreational activity, archaeological remains and pastures for sheep breeding. After a preliminary tick survey in 2010 followed by a bacteriological analysis, in order to investigate the tick composition and distribution and the possible presence of pathogens potentially transmitted, an annual acarological and bacteriological research was implemented during 2011. Three collection sites (woodlands and pasture) were selected and biweekly monitored. Free living ticks were sampled by dragging a white flag (about 1 m^2) for 15 minutes/operator, morphologically identified and stored at -80°C.

For bacteriological analysis, a first Real-time PCR was performed using gltA gene to distinguish Rickettsiae species belonging to both Spotted Fever Group (SFG) and to Typhus Group (TG). A following Real-time PCR using ompB gene allowed discriminating the species within SFG. In order to identify among 15 rickettsiae of the SFG, the ompA gene was amplified and sequenced. For Borreliae and Ehrlichiae genera were used ospA and 16S rRNA genes, respectively. A total of 189 specimens were collected: *Rhipicephalus turanicus* (77.2%), *Ixodes ricinus* (13.2%), *Dermacentor marginatus* (8.5%) and *Haemaphysalis punctata* (1%). *R. turanicus* was widespread in all sites, especially in pasture and occurred since the end of March, reaching then high densities in the following two months. *I. ricinus* and *H. punctata* were collected only in woodlands: the first species was always present from January to April with slight abundances, whereas the second one appeared only in the half of January with two specimens. During the first four mounths, *D. marginatus* was present in the ecotonal area between woodland and the adjacent grassland with decreasing densities. In samples collected in 2010, 1 out of 8 *R. turanicus* females resulted positive for *Rickettsia massiliae* (100% of identity with sequences in GenBank). Until now 40 ticks of the 2011 sample were analyzed for pathogen detection: 7 *I. ricinus* and 8 *R. turanicus* specimens were found positive for Rickettsiae

belonging to the SFG and 1 *I. ricinus* resulted positive for *Ehrlichia sp.* The pathogen identification at species level is ongoing. This research represents a significant contribution to the knowledge of the tick fauna and of the potential risk of introduction and spread of tick-borne diseases in a natural area of Rome. As *Rickettsia conorii,* the main agent of Mediterranean spotted fever, *Rickettsia massiliae* belongs to the SFG and represents an important etiological agent of tick-borne human spotted fever rickettsiosis. In Italy this pathogen was at first isolated in Sicily in 1985 and then identified only in 2005 in a blood human sample. In 2008 this rickettsia was identified in *R. turanicus* collected in Sardinia. In conclusion these preliminary findings seem very encouraging and this kind of approach could be extended to other areas.

The results about acarological aspects of this study have been presented as posters at *XXIII Congresso Nazionale di Entomologia*, held in Genoa on 13-16 June 2011 (Toma *et al*., 2011a; Khoury et al., 2011). The whole study ranging also molecular data on bacteria has been presented in the same year at the *VII Ticks and Tick-borne Pathogens Conference* in Zaragoza (Toma *et al.*, 2011b; Di Luca, 2011).

Preliminary data on tick-borne disease pathogens in migratory birds in Italy

Ticks are vectors of viral, bacteric and protozoarian pathogens responsible of emerging diseases worldwide. Apart from the economic foreign trade and transport of things and people, the passive transport by migratory birds allows the spread of various tick species worlwide. In April 2010 in Latium region (Italy) we started collecting ticks on birds caught during seasonal bird ringing activities, and our data were compared with acarological records collected on birds from Sardinia region (Capoterra, nearby Cagliari) as interesting crossroads for vectors and pathogens circulation. In Latium region such activity was carried out in Castel di Guido and Paliano, nearby Rome and in Ventotene and Ponza Islands (Central Tyrrhenian Sea).

Ringing activities were carried out on Ventotene and Ponza in April and May, when huge numbers of migrating passerines stage on the small islands during their spring migration. Several migrants land at these stopover sites directly from North African coast, after non-stop flights of up to 14-16 h across the sea. In Paliano the ringing activity was in October, while in Castel di Guido in the earlier September during the post-breeding period which coincides with beginning of autumn migration for migrants. Ticks were collected directly from the birds and they were identified as morphology, then stored at -80°C in order to search for bacteria using molecular tools. For bacteriological analysis, a first Real-time PCR was performed using *glt*A gene as molecular marker to distinguish *Rickettsia* species belonging to both Spotted Fever Group (SFG) and to Typhus Group (TG). A following Real-time PCR using *omp*B gene allowed to discriminate the species within SFG. To identify Rickettsie of the SFG, the ompA gene was amplified and sequenced. For *Borrelia* and *Ehrlichia* were used molecular markers ospA gene and 16S rRNA gene. In Latium sites 126 ticks were collected on 39 birds belonging to 14 species: 3 partial migrants, European, and 11 longdistance migrants. All collected ticks were at early stages, for the main part: 122 nynphs belonging to genus *Hyalomma*; 2 nymphs and 2 larvae belonging to *Ixodes* genus. In Sardinia 2 females of *Ixodes festai* were collected on Common Blackbird. The molecular analysis showed positivity for *Rickettsia sp*. in 4 ticks belonging to genus *Hyalomma*: one was found on a Common Blackbird and three on Nightingale, trapped in Castel di Guido. Data reported showed that the prevailing ticks on partial and long-distance migratory birds are the nymphs of *Hyalomma*, that represents the genus comprising the most competent species for Crimean-Congo Haemorragic Fever virus

(CCHF). The occurrence of 4 ticks positive for *Rickettsia sp*. confirms that bird migration could be a way of spread for tick-borne diseases. Therefore is noteworthy that the occurrence of *Ixodes festai*, unofficially reported for Sardinia has been here confirmed and observed on Common Blackbird as a new host species.

Field inquire on ticks in Tarquinia outskirts

Within the series of studies aimed to deepen the ixodidic Fauna knowledge in Latium Region, in 2011 we started a research in the surrounding of Tarquinia (Viterbo Province), in collaboration with the University of Tuscia in Viterbo, the Università Agraria of Tarquinia, the Istituto Zooprofilattico di Lazio e Toscana, supported by CARICIV Foundation. We selected 4 sites carachterized by woodlands, pastures and ecotonal situations that were monitored every 15 days for ticks collections by dragging.

Such just started study pointed out the occurrence of a various ixodidic Fauna, as we found 4 specie out of 18 specimens: *Dermacentor marginatus* 1 male, *Hyalomma marginatum marginatum* 2 females, *Ixodes ricinus* 7 nymphs, *Rhipicephalus sp*. 4 males and 4 females. The still ongoing research will be carried out in order also to complete it with molecular data. Such studies demonstrate how collaboration among ISS Departments, ISS and other Research Institutes and associations can be useful and effective; in spite of this positive aspect is impossibile to ignore that voluntary studies will be very limited and so the participation to international projects is desirable in order to achieve higher targets.

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