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Virulence gene expression of three hypervirulent *S. pyogenes* M1T1 and membrane vesicles isolation

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1. Introduction

The genus *Steptococcus* [Rosenbach, 1844] consists of catalase-negative, Gram-positive cocci which are arranged in pairs and chains and are usually facultatively anaerobic. *Streptococci* are a diverse collection of species inhabiting many body sites and they are both commensals and pathogens. In particular, nonpathogenic streptococci, are the most abundant bacterial species at the oropharyngeal level, and they have been found to exert an important role in the protection against pathogenic agents causing inflammation and infections [Tagg JR *et al.*, 2003]. Much attention has recently been devoted to the analysis of the oral microbiota to develop bacteriotherapy focused on prevention and/or treatment of upper respiratory tract infections. In this regard, a key species is *Streptococcus salivarius*, a lactic acid bacterium that is mainly encountered in the mouths of human beings. It is the first commensal bacterium that appears in the oral cavity of newborns where it colonizes the upper respiratory tract [Aas JA *et al.*, 2005] and persists there as a predominant member of the native microbiota throughout the life of its human and sole natural host [Favier CF *et al.*, 2002]. Many LAB (Lactic Acid Bacteria) strains, including *S.salivarius,* are prolific producers of bacteriocins, which are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria that kill or inhibit species closely related to the producer bacterium. Furthermore, according to several studies, large populations of *S.salivarius* efficiently adhere to oral epithelial cells, especially the papillary surface of the tongue that is a strategic location to carry out a population surveillance within the oral microbiota [Tagg JR *et al.*, 1983; Wescombe PA *et al.,* 2010]. The presence of an adhesion system such as pili, fibrils, saliva-binding proteins and host-cell-binding

proteins, together with its high competition rate, helps this species to stay in the human mouth [Nobbs AH *et al.*, 2009].

In our laboratory, during my PhD studies, we characterized one strain, *S.salivarius* 24SMBc, isolated from one healthy child that showed excellent inhibitory activity against *S.pneumoniae* and *S.pyogenes* and a potent capacity of adhesion to HEp-2 cells. The main aim of this study was to evaluate the clinical evidence of a probiotic application of *S.salivarius* 24SMBc for the prevention or reduction of recurrent medium otite (OM) children [Santagati M *et al.*, 2014]. Therefore, this strain was included in a randomized, placebo-controlled, double-blind paediatric trial that involved 100 otitis prone children. This preliminary study showed a reduction of OM episodes in children who received the intranasal administration of this probiotic with respect to children treated with placebo [Santagati M *et al.*, 2014].

The study of *S.salivarius* 24SMBc ended with the production and marketing of a new medical device, the Rinogermina nasal spray, in collaboration with D.M.G s.r.l Italy.

Streptococci, as mentioned above, include both nonpathogenic and pathogenic bacteria. In particular, *Streptococcus pyogenes* (group A streptococci, GAS) is an exclusive human bacterial pathogen. The virulence potential of this species is tremendous. Interactions with humans range from asymptomatic carriage over mild and superficial infections of skin and mucosal membranes up to systemic purulent toxic-invasive disease manifestations [Fiedler T *et al.*, 2015]. Simultaneously with the study of *S.salivarius* 24SMBc, my PhD project focalized on global regulation of virulence expression genes of three hypervirulent strains of *Streptococcus pyogenes* [Santagati M *et al.*, 2014] and their eventual production of membrane vesicles like new delivery system of virulence-associated components.

1.1 Streptococcus pyogenes

Group A streptococcus (GAS), or *Streptococcus pyogenes*, is a Gram-positive coccus and is an important human pathogen. GAS is usually present in the respiratory tract in 5 to 15% of individuals without signs of disease. As part of our flora, if defenses are compromised, *S.pyogenes* can generate a variety of suppurative infections. It is the most common cause of bacterial pharyngitis and is the cause of scarlet fever and impetigo. In the past, GAS strains were a common cause of puerperal sepsis or childbed fever. Today, group A streptococcus is responsible for streptococcal toxic shock syndrome and, recently it has gained notoriety as the "flesh-eating" bacterium which invades skin and soft tissues [Cunningham *et al*., 2000]. It is also responsible for a diverse range of clinical manifestation, such as bacteremia, cellulitis, meningitis, pneumonia and necrotizing fasciitis [Lamagni *et al.,* 2008]. GAS causes an estimated 700 million cases of mild, non invasive infections each year, of which 650,000 progress to severe invasive diseases with 25% mortality [Carapetis J.R *et al.*, 2005]. Epidemiologic studies, in 2008, showed that the resurgence of severe invasive GAS infections represented a global spread, ushering in a new pandemic, similar to that reported in the earlier part of the 20th century [Aziz *et al.,* 2008].

1.1.1 Classification of GAS

Streptococci are classified on the basis of hemolysis, colony morphology and serologic specificity. They are divided into three groups by the type of hemolysis on blood agar:

- β-hemolytic (clear, complete lysis of red cells)
- α-hemolytic (incomplete, green hemolysis)
- γ hemolytic (no hemolysis).

GAS belongs to the beta-hemolytic streptococci family and it was defined in 1933 by Rebecca Lancefield as Group A Streptococcus on the basis of its specific cell wall polysaccharides (group A, B, C, F and G) or lipoteichoic acids (group D) [Lancefield R.C, 1933]. The Lancefield classification scheme of serologic typing distinguished the beta-hemolytic streptococci based on their group A carbohydrate, composed of N-acetylglucosamine linked to a rhamnose polymer backbone. A serological differentiation based on the most abundant exposed surface antigen, the M protein, is also extensively used in infection diagnosis and, over the years, the number of serotypes has progressively increased up to 150. Streptococci were also serologically separated into M protein serotypes based on a surface protein. Serotype M1 is among the most frequent serotypes from streptococcal pharyngitis and invasive diseases and the resurgence of severe invasive GAS infections is now correlated with single clone M1T1 GAS serotype [Musser JM *et al.*, 2007]. The M1T1 strains contain the bacteriophage-encoded virulence factors extracellular streptodornase D (Sda1) and exotoxin type A (SpeA). The acquisition of prophages encoding Sda1, SpeA and the recombination of a 36kb chromosomal region encoding an extracellular toxin NAD-glycohydrolase (NADase) and streptolysin O (SLO), were a crucial factor in dissemination, potential virulence and emergence of a very abundant clone serotype M1T1 GAS that are different from M1 GAS [Musser JM *et al.*, 2005] (**Fig.1**.)

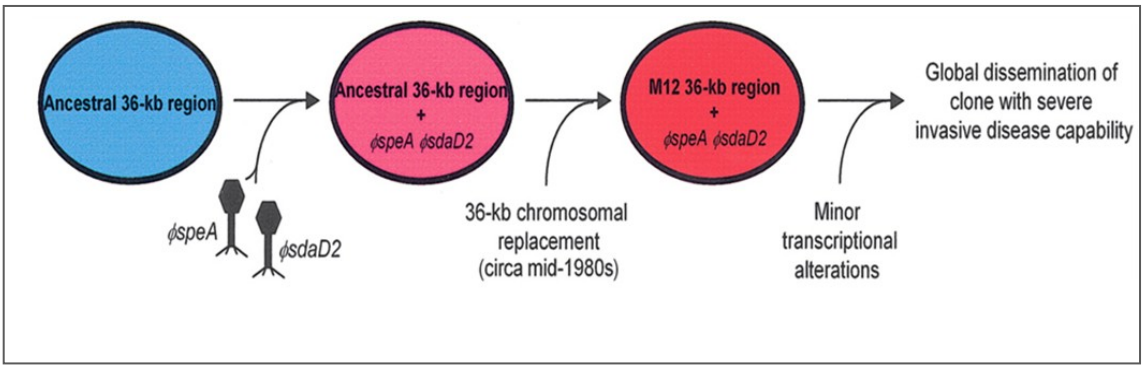
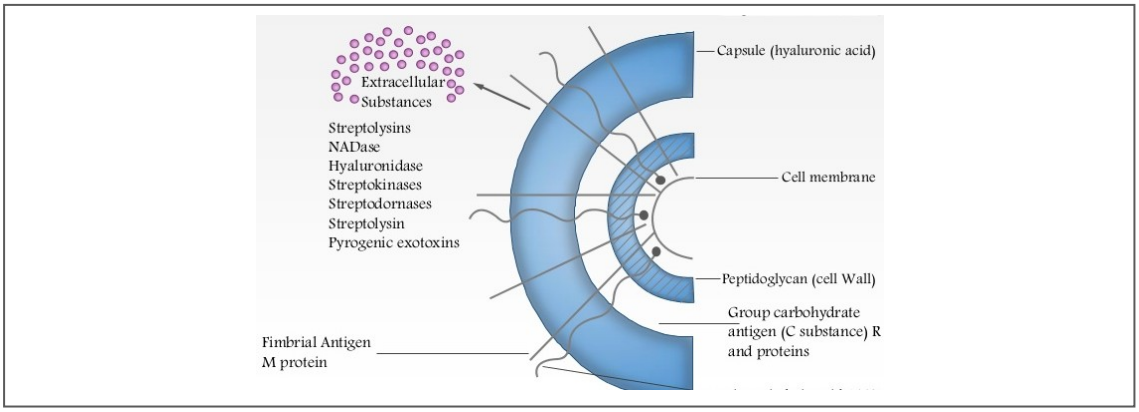


Fig.1.Paul Sumby *et al*. J Infect Dis. 2005

1.1.2 GAS Surface

Many GAS virulence factors are present on the bacterial cell surface, and of particular interest are those involved in colonization and evasion of the host immune responses. Invasive GAS disease requires successful colonization of skin or oropharynx. Initial weak interaction with mucosa, mediated by pili or lipoteichoic acid, is followed by a stronger binding lectine-carbohydrate and protein-protein interactions that confer tissue specificity. Group A Streptococcus has a highly complex and diverse cell surface, rich in antigenic components such as the polysaccharide capsule (C-substance), the cell wall peptidoglycan and many surface proteins including the M protein, fimbrial and fibronectin-binding proteins [Walker MJ *et al.*, 2011].



M Protein

The streptococcal M protein is now probably one of the best-defined molecules of the known bacterial virulence determinants. The M protein is a major surface protein and virulence factor of group A streptococci, with more than 150 distinct serotypes identified. The amino-terminal region extends from the surface of the streptococcal wall, while the carboxy-terminal region is within the membrane. The serotype specificity of GAS is determinated by N-terminal region, called the A repeat region, that is highly variable among M serotypes. The B repeat region, the mid-region, varies from serotype to serotype and the carboxy-terminal or C repeat region contains a conserved sequence shared among all of the serotypes [Fischetti *et al.*, 1989]. The M proteins are able to bind serum fibrinogen allowing the bacteria to resist phagocytosis and contain those particular epitopes that mimic human tissues, some of these are the cause of rheumatic fever, leading to an autoimmune carditis. Furthermore, M protein increases bacterial survival in neutrophils, so this protein is essential for full virulence during GAS infection. Epidemiological studies have shown a clear correlation between M-type and type of infection caused, partitioning the group *of S.pyogenes* into two classes:

- Class I (M-types: 1, 3, 5, 6, 14, 18, 19, 24) are found associated with throat infection and rheumatic fever.
- Class II (M-types: 49, 57, 59, 60, 61) produce serum opacity factor (SOF)and are associated with pyoderma and acute glomerulonephritis. [Cunningham *et al.,* 2000].

The Streptococcal Capsule

The group A streptococcal capsule is composed of a linear polymer of hyaluronic acid containing repeating units of glucuronic acid and *N*-acetylglucosamine that is produced by enzymes encoded in the highly conserved *has ABC* hyaluronan synthase operon [PH Weigel. J *et al*., 1995]. The *hasA* gene encodes hyaluronate synthase; *hasB* encodes UDP-glucose dehydrogenase [Crater DL *et al.*, 1993]; and *hasC* encodes UDP-glucose pyrophosphorylase. The hyaluronic acid capsule is required for resistance to phagocytosis [Wessels MR *et al.*, 1994] and may be an important adherence factor during host colonization because it is able to bind CD44 on epithelial cells [Wessels MR *et al.*, 1998]. Glucuronic acid and *N*-acetylglucosamine, nearly identical to the polysaccharides that are in the human host, encourage GAS evasion of the host immune response. Streptococcal isolates have different amounts of hyaluronic acid capsule that could be related to the *has* operon promotor. In the well-encapsulated strain, the promoter is more active than in a poorly encapsulated strain [Wessels MR *et al.*, 1998]. Furthermore, Levin and Wessels demonstrated that capsule production and virulence is highly influenced by a negative regulator (CsrR) that is part of a two-component regulatory system [Levin J *et al.*, 1998]. More recent work provides definitive evidence that the capsule is a major virulence determinant involved in resistance to phagocytosis in conjunction with the streptococcal M protein.

1.1.3 Streptococcal Superantigens

Streptococcus pyogenes has acquired many virulence determinants that allow it to survive within the host and they are involved in the pathogenesis of toxic shock, necrotizing fasciitis, and invasion of soft tissue and skin. The superantigens (Sags)

are the extracellular pyrogenic exotoxins A, B, C (SpeA, SpeB, SpeC) and newly discovered exotoxins such as exotoxin F (SpeF) and streptococcal superantigen (SSA) [Holm SE *et al.*, 1994]. All of these toxins act as superantigens that interact with the major histocompatibility complex (MHC) class II molecule via high zinc binding and the activation liberates large amounts of interleukins and other inflammatory cytokines such as gamma interferon and tumor necrosis factor. Furthermore, the superantigens are able to bind the beta chain (Vβ) of the T-cell subset without any processing by antigen-presenting cells. The pyrogenic exotoxin *speA* and *spec* are encoded by bacteriophages [Cunningham *et al.*, 2000], while *speB* is chromosomal and it is a cysteine proteinase that degrades vitronectin, fibronectin, and IL-1 precursor.

Streptolysin O

SLO is part of a family of cholesterol-binding cytotoxins produced by many pathogenic Gram-positive bacteria including *Streptococcus pneumoniae* (pneumolysin), *Listeria monocytogenes* (listeriolysin O) and *Bacillus* anthracis (anthrolysin) [Rest RF *et al.*, 2003]. SLO is a 69 kDa protein that oligomerizes to form large pores in the host cell membrane [Tweten RK *et al.*, 2000], and has several functions in GAS pathogenesis, including the induction of apoptosis in epithelial cells, neutrophils and macrophages [Timmer AM *et al.*, 2009]. SLO, also, prevent direct uptake of GAS into lysosomes and enhances GAS survival in the human host by interfering with both bacterial uptake and intracellular killing of GAS by pharyngeal epithelial cells.

Streptococcal inhibitor of complement, SIC

The *sic* gene, which encodes the Streptococcal inhibitor of complement-mediated lysis (SIC), is a highly polymorphic extracellular protein and putative virulence factor secreted only by M1 and M57 GAS strains. SIC is a secreted 31 kDa protein that is located in the Mga virulence regulon [Akesson P *et al.*, 1996]. Sic was shown to bind C5b67 of the membrane attack complex (MAC), thus impairing terminal complement function. In particular, SIC promotes GAS M1T1 survival and enhance the progression of invasive infection.

C5a peptidase

The endopeptidase C5a is a 130 kDa proteolytic enzyme localized on the surface of GAS strains. The C5a peptidase is encoded by a gene that is regulated by *mga* in concert with M protein [LaPenta, D *et al.*, 1994]. Peptidase cleaves the complement-derived chemotaxin C5a at its PMN-binding site [Cleary P *et al.*, 1992] and this event inhibits the recruitment of phagocytic cells to the site of infection [Cleary PP *et al.*, 1996]. The identity of the amino acid sequence of the enzyme is greater than 95% among class I and class II M protein serotypes of group A and B streptococci. Consequently, the surface of GAS presents a double barrier to the complement defenses of the host: first, the M protein, discussed above, and second, the streptococcal C5a peptidase inactivates C5a and chemotaxis.

Streptodornase D

One important distinguishing feature of global dissemination of the M1T1 GAS clone, compared to less pathogenic GAS strains, is the acquisition of a prophage (ΦM1T1Z) encoding a potent secreted DNase, Sda1[Aziz RK *et al.*, 2004]. This gene has been shown to promote MIT1 GAS virulence via

degradation of NETs. NETs are composed of DNA, granule protease, antimicrobial peptides and histones that are secreted by host neutrophils to capture and eliminate bacteria at the site of infection [Cole JN *et al.*, 2011; Buchanan JT *et al.*, 2006; Walker MJ *et al.*, 2007]. The presence of ΦM1T1Z and a second prophage that encodes the superantigens SpeA, distinguishes the GAS serotype M1T1 from its closely related M1 type.

Streptococcal exotoxin B , SpeB

SpeB, a cysteine proteinase, is the most secreted protein from *S.pyogenes*. The streptococcal cysteine proteinase is one of the best studied and the earliest identified secreted proteins from *S. pyogenes*. Much is known about its structure, function, processing, substrate specificity and regulation [Ashbaugh *et al*., 1998; Ashbaugh and Wessels, 2001]. Despite the large amount of literature on SpeB functions, the role of SpeB in GAS infections is still unknown, but recent evidence has conclusively demonstrated that SpeB is critical for the pathogenesis of severe invasive disease caused by GAS. *speB* is a chromosomal gene and is highly conserved and found in > 99% of GAS isolates, although there is a significative variation in expression levels among strains [Bohach *et al*. 1988; Ferretti J *et al.*, 1991]. The gene encodes the 398 amino acid SpeBz (40 kDa) and undergoes conversion to mature 28-kDa SpeBm form by autocatalytic truncation [Liu and Elliott 1965b; Doran *et al*., 1999]. This enzyme is able to degrade the extracellular matrix, cytokines, chemokines, complement components, immunoglobulins, serum protease inhibitors, and also cleaves and inactivates many surface-associated and extracellular GAS virulence determinants, including M1 protein, [Ringdahl U *et al.,* 2000], different superantigens, [Aziz RK *et al*., 2004; Kansal RG *et al.*, 2003],

streptokinase [Sun H *et al.*, 2005], Sda1 [Aziz RK *et al*., 2004], and SIC [Pence M A *et al*., 2010]. Furthermore, SpeBm can cleave the cell wall-anchored C5a peptidase from the bacterial surface and this causes the inhibition of recruitment of neutrophils to the site of infection [Wexler and Cleary, 1985; Berge and Björck, 1995]. As a consequence, the bacterial properties are altered, which is important for the transition from localized to systemic *S.pyogenes* infection [Cole *et al*., 2006]. Regulation of *speB* expression in GAS is extremely complicated. Maximal expression occurs from late logarithmic to stationary phase in response to environmental factors, pH and NaCl concentration. Although there are many papers on *speB* expression, the precise regulatory mechanisms that occur *in vivo* remain unclear. [Musser *et al.,* 2011]. During the different stages of an infection or response to external stresses from the environment, GAS strains are able to control transcriptional regulation of genes and protein secretion through signal peptidases. The cumulative contribution of the secreted cysteine protease SpeB to the pathogenesis of invasive GAS infection is at present unclear, and studies in various GAS serotypes and animal models have produced varying results. The Streptococcal exotoxin B is involved in bacterial phenotype shift in different ways. For example, in some *S.pyogenes* strains, deletion of the *speB* gene leads to a down regulation of gene encoding for the hyaluronic acid capsule, but the underlying molecular mechanism is not well understood [Woischnik *et al.,* 2000]. Some studies have shown that a high production of SpeB is associated with STSS, whereas others show that low SpeB production is associated with severe infection [Talkington *et al.,* 1993; Kansal *et al.,* 2000]. Musser's group demonstrated, in different papers that, using *speB* mutants in animal models, they all showed that SpeB contributes to tissue damage and bacterial dissemination

[Lukomski *et al.,* 1997, 1998, 1999]. Later, Kotb's group showed that SpeB is downregulated in invasive M1T1 isolates during infection in mice [Kazmi *et al.,* 2001]. Recently, Aziz's group, in 2004, demonstrated that GAS MIT1 WT, SpeB-positive bacteria undergo a phase–shift to the speB-negative phenotype after infection in mice [Aziz RK *et al*., 2004]. This phase switch was observed to be the result of mutations in the two-component regulatory system covR/S [Walker MJ *et al.*, 2007]. CovR/S is a global regulator, also called CsrR/S, a two component signal (TCS) transduction system that has been studied extensively because of its critical role in virulence. Normally, covR/S represses expression of about 15% of GAS genes including many involved in virulence [Federle *et al*., 1999]. Some studies have revealed a correlation between an invasive phenotype and the up-regulation of some virulent genes, coding proteins such as streptodornase, streptokinase, streptolysin O, streptococcal inhibitor of the complement and the hyaluronic acid capsule synthesis operon [Sumby P *et al.*, 2006]. At the same time, a mutation in the covR/S operon, decreases the expression of speB and consequently prevents the degradation of streptokinase and the M1 protein. The presence of these proteins promotes invasive infection and bacterial dissemination. [Cole *et al*., 2006]. Furthermore, studies of M1T1 clinical isolates from invasive disease cases, found an inverse relationship between SpeB expression and clinical severity. In particular, SpeB levels were higher in GAS M1T1 serotype isolates from non-severe invasive infections than in isolates from severe cases, such as STSS and necrotizing fasciitis. [Kansal *et al.,* 2000]. In conclusion, the precise function of streptococcal exotoxin B is extremely complex and for this reason is still unknown, because different studies have produced contradictory results [Svensson MD *et al.*, 2000; Ashbaugh and Wessels, 2001;

Lukomski *et al*., 1997, 1998, 1999]. Together the data suggest that the effects of SpeB during the interaction between host-bacteria *in vivo* will depend on the balance between the actions of several regulatory systems that control SpeB synthesis, post-translational modification, and enzymatic activity [Kansal et al., 2000] (**Fig.2.).**

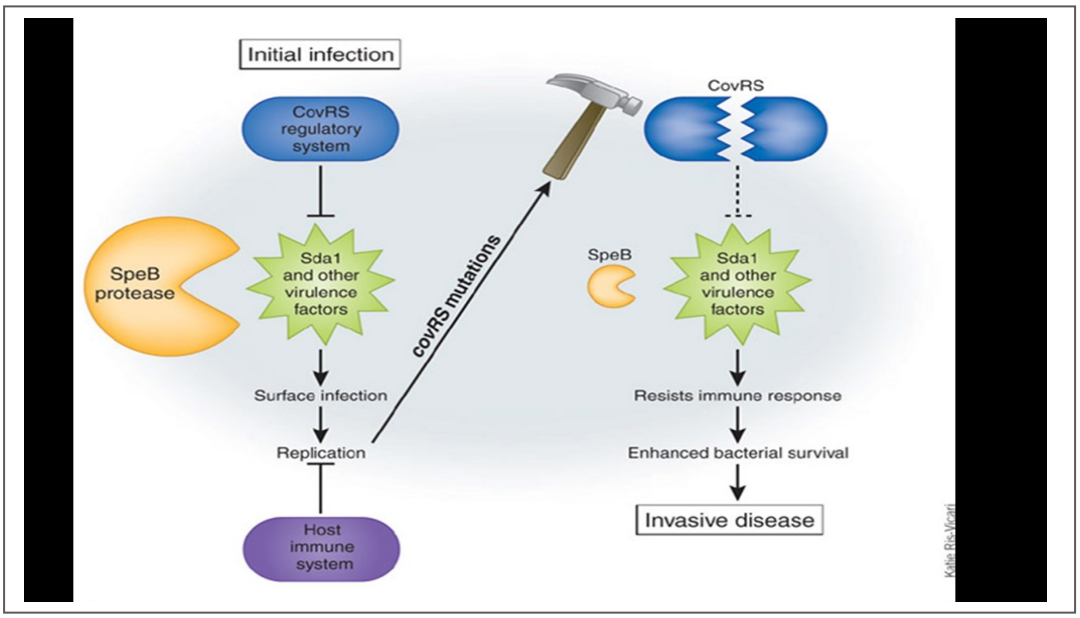


Fig.2. Model explaining mutation of *Streptococcus pyogenes* regulatory locus during invasive infection. Claire Turner & Shiranee Sriskandan .*Nature Medicine* (2007)

1.1.4 Evolutionary Origin of Clone M1T1 GAS serotype from its parental M1 Serotype

Genome sequencing, DNA-DNA microarray, PCR profiling, single-nucleotide polymorphism analysis, and several other genetic analysis techniques have highlighted the origin of a new clone, M1T1, genetically distinct from the serotype M1 strains, responsible for most recent human infections. Usually, bacteria can evolve slowly through the accumulation of point mutations or more quickly by horizontal gene transfer events, such as conjugation, transformation and prophage transduction [Ochman H *et al.*, 2000]. Nevertheless, the exact molecular mechanisms that lead to the onset of unusually virulent pathogens are still unclear. Recently,

Musser's group have demonstrated the evolutionary origin of a new clone serotype derived from its parental M1 GAS serotype. In the past, serotype M1 GAS was the pathogen responsible for most invasive diseases, such as STSS, NF, and septicemia, but during the mid-1980s, the frequency of invasive infection caused by serotype M1 GAS suddenly increased [Musser JM *et al.*, 1998]. Musser's studies have shown that there are two main events that contribute to the evolutionary origin of the hypervirulent clone M1 GAS:

- acquisition of two prophage genes: streptococcal pyrogenic exotoxin A *(SpeA)* and streptodornase D *(Sda1)*
- the recombination of a 36-kb chromosomal region from M12 serotype encoding NADase (extracellular toxin NAD^+ - glycohydrolase) and SLO (streptolysin O)

MGAS5005 is genetically representative of new serotype M1T1 strains causing contemporary infections. This clone evolved from a less virulent ancestor, SF370, through a series of recombination events. MGAS5005 was isolated for the first time in 1996 from the cerebrospinal fluid of an infected patient in Ontario, Canada, and has been used in many studies of GAS pathogenesis [Virtaneva K *et al.*, 2003; Voyich JM *et al.*, 2001; Sumby P *et al.*, 2005], while SF370 was isolated in 1985 from the infected wound of a patient [Ferretti JJ *et al.*, 2001; Smoot JC *et al.*, 2002; Beres SB *et al.*, 2002; Stevens DL *et al.*, 2000]. Consistent with the data from the DNA-DNA microarray analysis, the genomes of strains MGAS5005 and SF370 showed a strong similarity.(**Fig.3)**

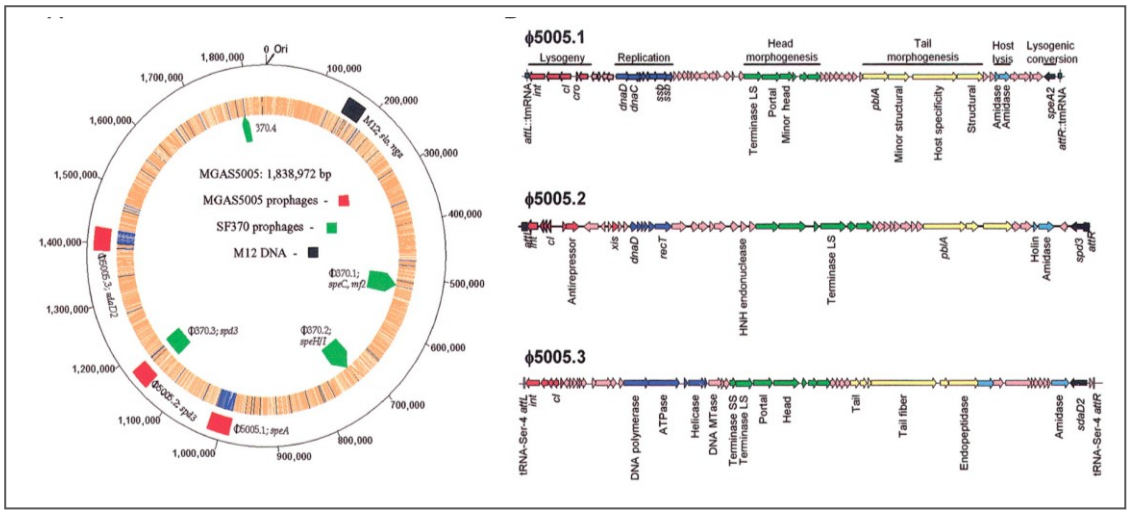


Fig.3. Atlas comparing the chromosomes of strains SF370 and MGAS5005. Paul Sumby *et al*. J Infect Dis. 2005.

Even if MGAS5005 has three genes that encode putative secreted DNases. Two of these genes are associated with prophages (*spd3* carried by prophage Φ5005.2 and *sdaD2* by prophage Φ5005.3), and one is chromosomally encoded (*spd*). DNA-DNA microarray analysis among 30 serotype M1 isolates from 6 countries [Musser *et al.*, 2005] **(Fig.4)** found a match of core genomes to 93% of the ORFs present in the genome of the reference serotype SF370 strain. [Ferretti JJ *et al.*, 2001]. The prophage regions Φ5005.1 and Φ5005.3 of MGAS5005 strain were organized in ORFs similar to the sequenced genomes of M3 and M18 serotypes [Smoot JC *et al.*, 2002; Beres SB *et al.*, 2002]. Furthermore, the genomes of MGAS5005 and SF370 differ for small insertions and deletions, and many SNPs. Musser *et al.* showed that the majority of SNPs between these two strains were localized in a 36-kb region that extends from *purA* to *nadC*, which is virtual identical to the chromosomal region present in the M12 genome serotype. Although it seems clear that lateral gene transfer was involved in the 36-kb region from M12 serotype, there are no experiments which prove the molecular mechanism [Musser *et al.*, 2005] **(Fig.5).**

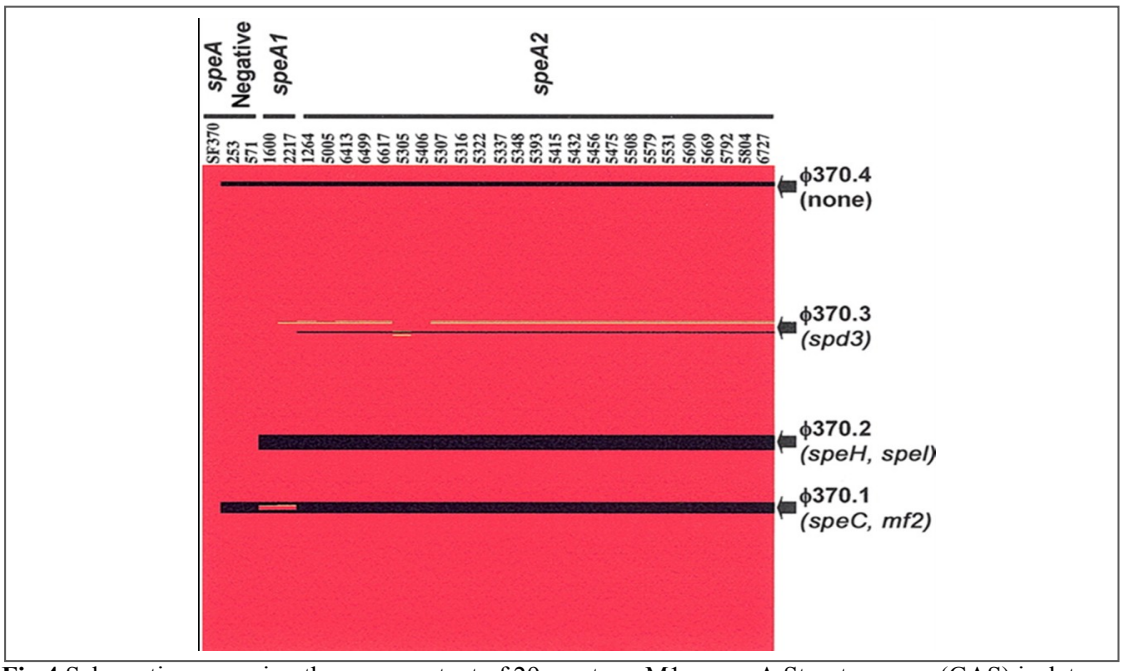


Fig.4.Schematic comparing the gene content of 29 serotype M1 group A Streptococcus (GAS) isolates with that of reference strain SF370. Paul Sumby et al., J Infect Dis. 2005

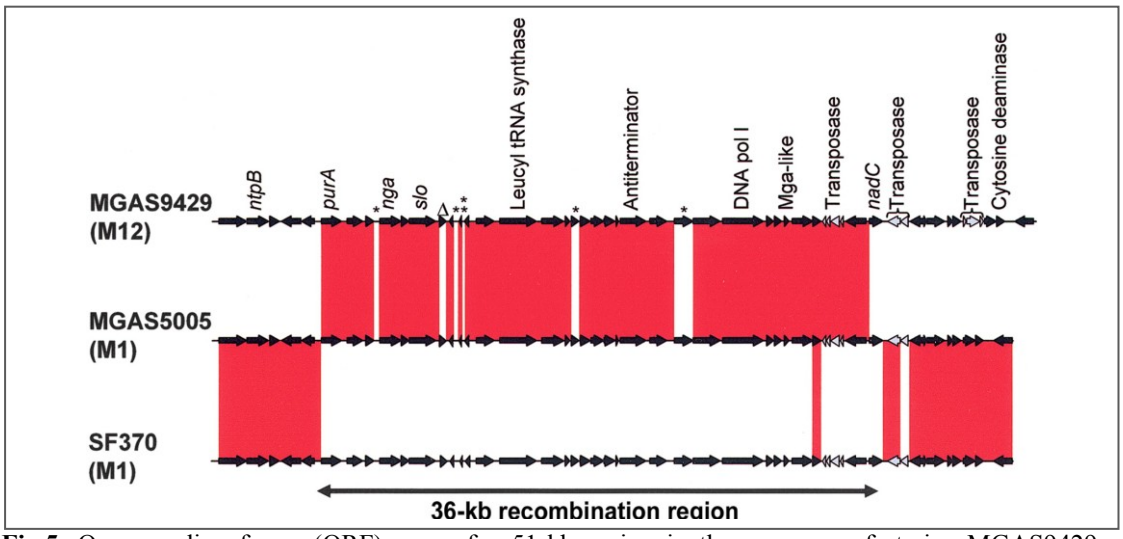


Fig.5. Open reading frame (ORF) map of a 51-kb region in the genomes of strains MGAS9429 (serotype M12), MGAS5005 (serotype M1), and SF370 (serotype M1). Paul Sumby et al., J Infect Dis. 2005

1.1.5 Disease model for invasive GAS serotype M1T1

In Group A Streptococci thirteen TCSs have been identified, but the best characterized is the CovRS system (also known as CsrRS). This Two Component regulatory System is required for survival of bacteria under

environmental stress conditions, such as low pH, iron starvation [Dalton TL *et al.*, 2004; Froehlich B *et al.*, 2009] high concentrations of NaCl, elevated temperature, and response to antibiotic stress [Sawai J *et al.*, 2007]. CovS is a sensor kinase localized on the membrane surface that is able to change the state of phosphorylation of the regulatory response, CovR [Dalton TL *et al.*, 2004], which is essential for growth under stress conditions [Froehlich B *et al.*, 2009; Sway J *et al*, 2007]. CovRS is a negative regulatory TCS that directly or indirectly influences expression of 10% to 15% of GAS genes, including several virulence factors [Levin J *et al.*, 1998; Federle *et al*., 1999; Graham MR *et al.*, 2002 and 2005; Gryllos I *et al.*, 2003; Engleberg NC, *et al.* 2001]. Mutations in the genes encoding the CovRS system in GAS serotype M1T1 affect the expression of virulence factors that are important determinants of the pathogenesis of these invasive bacterial strains. Phosphorylated CovR seems to negatively regulate many genes, including *speB, HasA, ska, Sda1* and *sagA* [Heath A *et al.*, 1999; Engleberg NC *et al.*, 2001-2004], but the mechanism by which CovR function is not yet fully understood. Sumby *et al.*, using an Affymetrix expression microarray, analyzed nine strains of serotype M1, including six from patients with pharyngitis and three from invasive disease episodes. This study showed a completely different transcriptome cluster based on the analysis of the microarray data **(Fig. 6A**), which were designated pharyngeal transcriptome profile (PTP) for the six pharyngitis isolated, and invasive transcriptome profile (ITP) for the three invasive isolated [Sumby *et al,* 2006]. All PTP isolates had wild-type covR/S genes, while all ITP isolates had either a mutated covR or covS gene. The two transcriptomes differ by

approximately 10%, and the genes with differences include multiple known and putative virulence-associated genes. Furthermore, GAS isolates with ITP profile were recovered from mice that were subcutaneously with PTP GAS, proving a bacteria shift phenotype in vivo-selected [Kazmi *et al.,* 2001]. The increased virulence of ITP GAS in the bacteremia model was linked to up-regulation of many factors that inhibit PMN function, including capsule, Sic, C5a peptidase, and other important virulence factors, whereas the level of *speB* mRNA was downregulated. SpeB, as we have already described above, degrades fibronectin, vitronectin, and other host molecules [Aziz RK *et al.*, 2004; Rasmussen M *et al.*, 2002; Kapur V *et al.*, 1993], and also cleaves some bacterial proteins. Thus, decreased SpeB production may play a key role in the increased virulence of ITP GAS by preserving GAS virulence factors [Aziz RK *et al.*, 2004; Raeder R *et al.*, 1998; Wei L *et al.*, 2005]. However, the *in vivo* consequences of this *in vitro* observation are unclear. Taken together, all these data hypothesize that CovS regulates CovR to enhance the repression of one subset of genes *speA*, *hasA* and *ska,* while at the same time reducing the repression of a second subset of genes *speB*, *grab* and *spd3* (encoding a streptodornase) **(Fig. 6B**), [Trevino J *et al*, 2009]. For this reason, it has been proposed that phosphorylated CovR represses promoters of the first gene subset, whereas non-phosphorylated CovR represses the second gene subset [Trevino J *et al* , 2009; Churchward G *et al.*, 2007]. Kensal *et al.* reported a similar phenomenon that M1T1 AP (hypervirulent animal-passeged,with$SpeB^{-}$/ $SpeA^{+}$/ Sda^{high}phenotype), which are hardly detected in a WT population ($SpeB^{+}$/ $SpeA^{-}$/ Sda^{low}phenotype), become the bacterial majority community *in*

vivo, maybe because they have a survival advantage due to over expression of certain virulence genes. In conclusion, the identification of covRS mutations in highly virulent GAS serotypes isolated from human patients with severe disease suggests a key role for survival and persistence in distinct environmental niches [Aziz RK *et al*., 2010], in contrast to the wild-type phenotype, which is better adapted for the initial stages of the infection. Although it is still unclear how this highly complex system works, it is clear that there is a link between ΔCovRS and the hypervirulent strain. Probably, in the future, we will need detailed structure-function studies and more analyses of the ways that different stimuli can affect CovS and consequently CovR, to better understand the exact molecular mechanism.

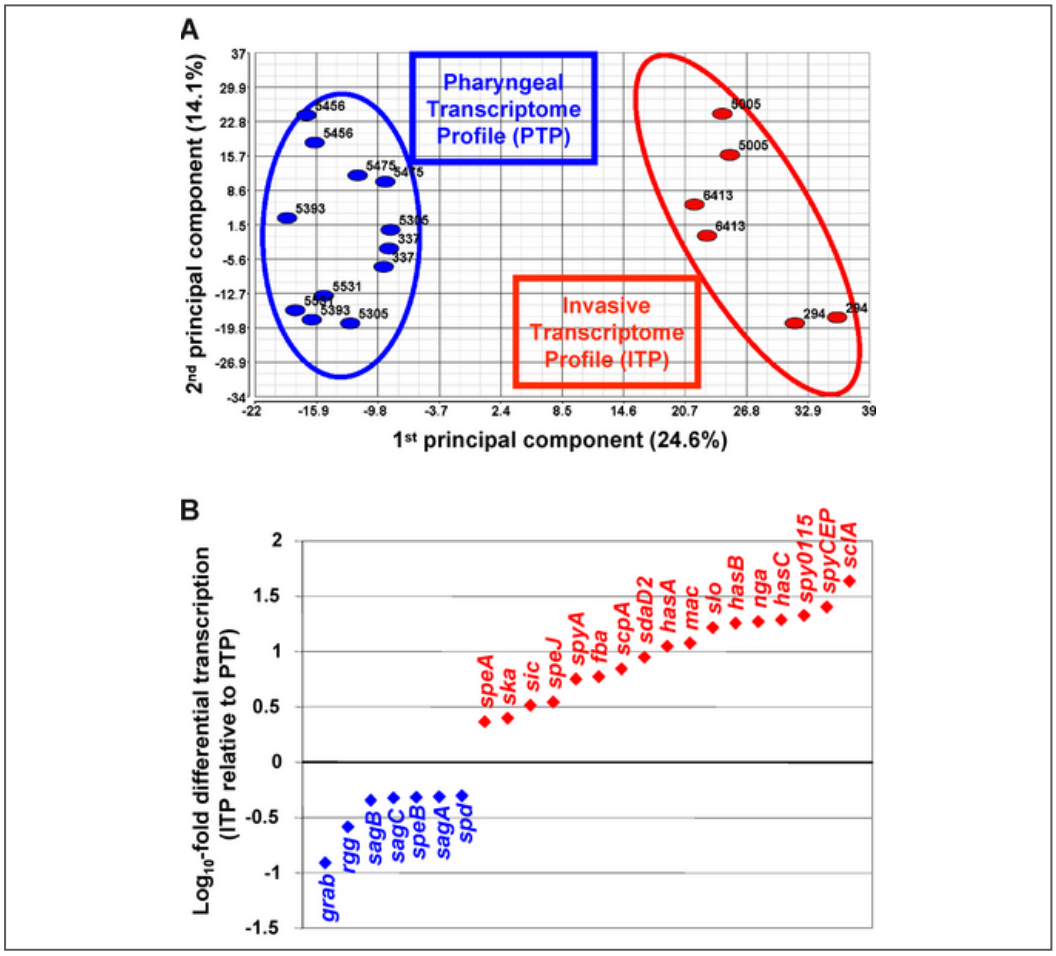


Fig.6A-B. Differential Gene Expression of ITP and PTP GAS. Sumby P. Plos, 2005.

1.2 Bacterial Membrane Vesicles

1.2.1 Background and history

Bacteria have evolved over the millennia to survive in a wide variety of environments. Regardless of living location, the microbes have had to evolve different tools to grow in environments subject to change. One such tool that facilitates microbe-microbe, microbe-host and microbe-environment interactions is the production of membrane vesicles (MVs). The first discovery of MVs was made over five decades ago, and it is now clear that the production of OM vesicles has been observed for a wide variety of Gram negative bacteria in all stages of growth as well as in a variety of growth conditions, such as infected tissues, with *Pseudomonas aeruginosa* and *Escherichia coli* because they are the most studied bacteria model. Vesicles were discovered first by T.J. Beveridge and colleagues, who is still considered the undisputed pioneer of the biological roles of MVs and mechanism of MV formation. The content, composition and purpose of these structures were unknown. For most of the time, the study of MV formation by prokaryotes has focused only on Gram negative bacteria. Recently, attention has turned to determining if MV production is also possible in Gram positive bacteria. Now we know that the production of spherical membranous vesicles is shared among all three branches of the tree of life: eukaryotes, Gram negative and Gram positive bacteria [Lee EY *et al.,* 2009; McBroom AJ *et al.*, 2005; Rivera J *et al*., 2010], archaea [Ellen AF *et al.*, 2010], fungi [Oliveira DL *et al.***,** 2010], and parasites [Silverman JM *et al.,* 2008-2010]. The release of MVs plays an integral role in cell physiology and the pathogenesis of infection. As the OMVs are known to

induce the immune system, they have been recognized as promising agents to be used as vaccines. Recently, one successful example is a vaccine for meningitidis caused by *Neisseria meningitidis* [Findlow *et al*., 2006; Boutriau *et al*., 2007; Williams *et al.,* 2007]. Besides direct medical applications, the study of OMVs is also an opportunity to better understand the physiology of bacteria.

1.2.2 What are MV Vesicles?

Bacterial membrane vesicles are closed spheroid particles produced by both Gram positive and Gram negative bacteria. The outer membrane (OM) vesicles of Gram-negative bacteria have a heterogeneous size from 10 to 300 nm in diameter, while the MVs secreted by Gram-positive bacteria have a smaller diameter between 20 to 150 nm [Dorward and Garon, 1990; Lee *et al*., 2009; Rivera *et al*., 2010]. In general, MV vesicles reflect the composition of the outermost portion of the membrane from which they derive, thus the OM vesicles derived from Gram-negative bacteria containing LPS, glycerophospholipids, and OM proteins as well as enclosed periplasmic components [Gankema H *et al*., 1980; Hoekstra D *et al*., 1976; Horstman AL *et al*., 2000; Kadurugamuwa JL *et al*., 1995; McBroom AJ *et al*., 2005] **(Fig.7)**. Just as there are differences in the envelope structure among Gram positive and Gram negative bacteria, MVs are completely different in composition in these two types of microorganism. Gram positive MVs originate from the cytoplasmic membrane and proteomic studies show they are composed mostly of cytosolic protein, cytoplasmic membrane-associated protein, as well as some secreted protein [Gurung *et al*., 2011].

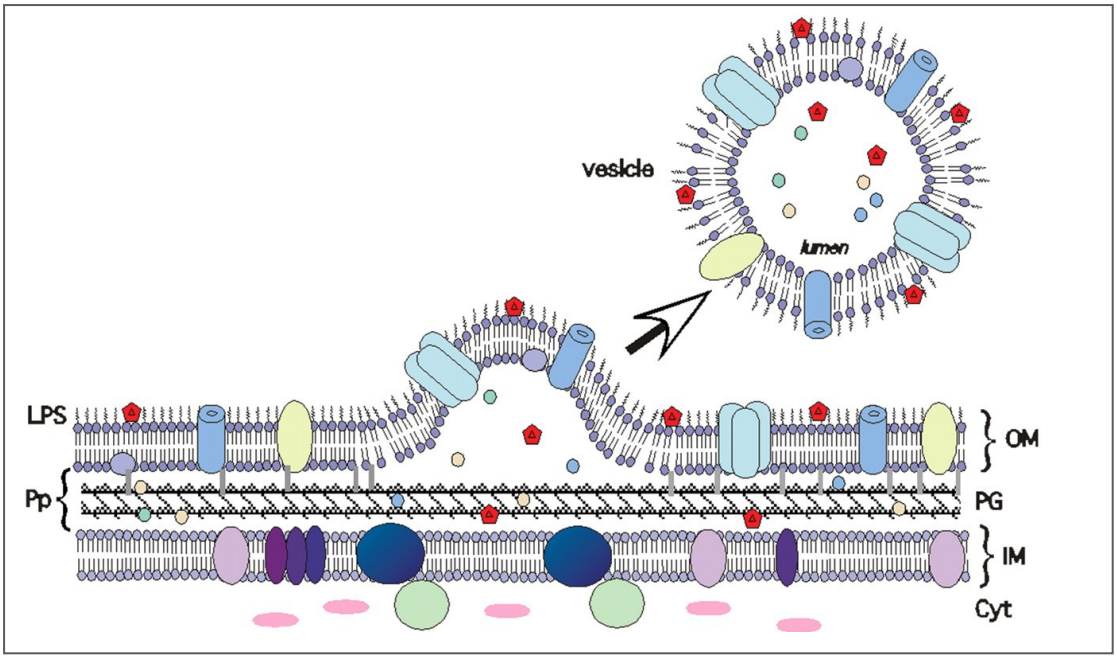
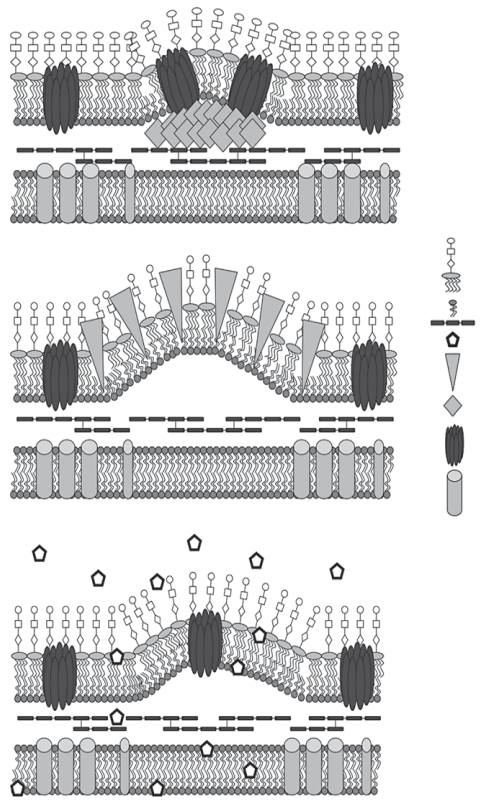


Fig.7. Model of vesicle biogenesis. (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol. Meta J. Kuehn, 2005. Genes & Development.

1.2.3 Regulation of vesicle Biogenesis

OMVs are secreted by bacteria both in liquid and on solid media, as well as *in vivo.* The goal of many OMV studies has been to understand how OMVs are formed and how this process is regulated, but is still poorly understood. Initially it was thought that the formation of OMVs was a physical process associated with the turnover of cell OM [Haurat *et al.,* 2011]. Although this idea is still held by some, recent discoveries show that there is likely to be an elaborate mechanism behind the biogenesis of OMVs. There are several reports indicating that vesicles form as a response to membrane stress [McBroom AJ *et al*., 2007], however, the fact that membrane vesicles are released even when the cells grow without external stress makes it difficult to think that MV biogenesis is exclusively a stress response. For Gram-negative OMVs, several models of biogenesis have been hypothesized based on architectural features of the envelope and vesiculation mutants [Beveridge *et al*., 1999; Deatherage *et al.,* 2009; Kulp and Kuehn, 2010]. Three mechanisms have been

proposed for vesicles biogenesis: (i) the accumulation of misfolded protein in the periplasm, may result in a mechanic force that literally pushes out the outer membrane and eventually leads to the release of OMVs; (ii) some external agents that could intercalate into the outer membrane and as a consequence membrane packaging is disturbed; (iii) some small molecules that normally are secreted through the cell envelope could change the regulation of genes, causing an increment or decrement of levels of OMVs (**Fig.8**).



Andrew J.Manning Meta J.Kuehn,2013

Fig.8. Possible Mechanisms of OMV Induction. Three possible models to explain how OMVs form in response to different stimuli. **A**) The accumulation of misfolded or overexpressed protein in the periplasm may result in a physical force on the outer membrane, causing the envelope to separate, the outer membrane to bulge, and eventually lead to the release of the OMV. **B**) External agents that act at the outer membrane could intercalate into the outer membrane, and disturb the packing of the outermost membrane. This disruption may lead to a change in the curvature of the membrane generating a bulge that eventually could bud off completely. **C**) A naturally secreted small molecule which can pass through the cell envelope could result in a change in the regulation of genes, causing an increase or decrease in levels of OMVs.

In contrast to OMVs, the mechanisms and regulation of MVs of Gram positive bacteria have not yet been completely understood. OMV biogenesis is often considered to be stress regulated, in fact, OMV levels are modulated by altering levels of envelope proteins, temperature, and quorum sensing signals, and antibiotic treatment has been demonstrated to influence several aspects of vesiculation. For

example, gentamicin and mitomycin affect the secretion and composition of OMVs in Gram-negative bacteria. The level of Shiga toxin-associated OM vesicle production by *Shigella dysenteriae* increased with mitomycin [Dutta S *et al*., 2004]. Release of MVs by *Pseudomonas aeruginosa* increased approximately threefold after exposure of the organism to four times the MIC of gentamicin [Kadurugamuwa JL *et al*., 1995]. The antibiotic ciprofloxacin interacts with DNA synthesis and leads to an SOS response, which was found to be involved in increasing OMV levels. Vesiculation was found in both nonpathogenic and pathogenic species, under a range of growth conditions, including in liquid broth and on agar plates in the laboratory [Deatherage BL *et al.,* 2009; Silverman JM *et al.,* 2008], in biofilms [Schooling SR *et al*.,2006], upon infection with bacteriophages [Loeb MR *et al*., 1974], and in pathogenic organisms growing within an animal host [Feldmesser M *et al*., 2001; Fiocca R *et al.***,** 1999, Marsollier L *et al.,* 2007, Necchi V *et al.,* 2007]. In general, pathogenic microbes produce more OMVs than nonpathogenic ones, such as enterotoxigenic *E.coli* (ETEC) that produces about 10-fold more vesicles than nonpathogenic *E.coli* [Horstaman and Kuehn, 2002].

1.2.4 Function of MVs

Several studies have shown many different functions for bacterial membrane vesicles. The main idea is that vesicles are necessary for the survival of the bacteria that produce them. MV release is essential for promoting interactions between microbial cells and between eukaryotic host cells and microbes, including communication, release of antigens, and secretion of virulence factors **(Fig. 9).**

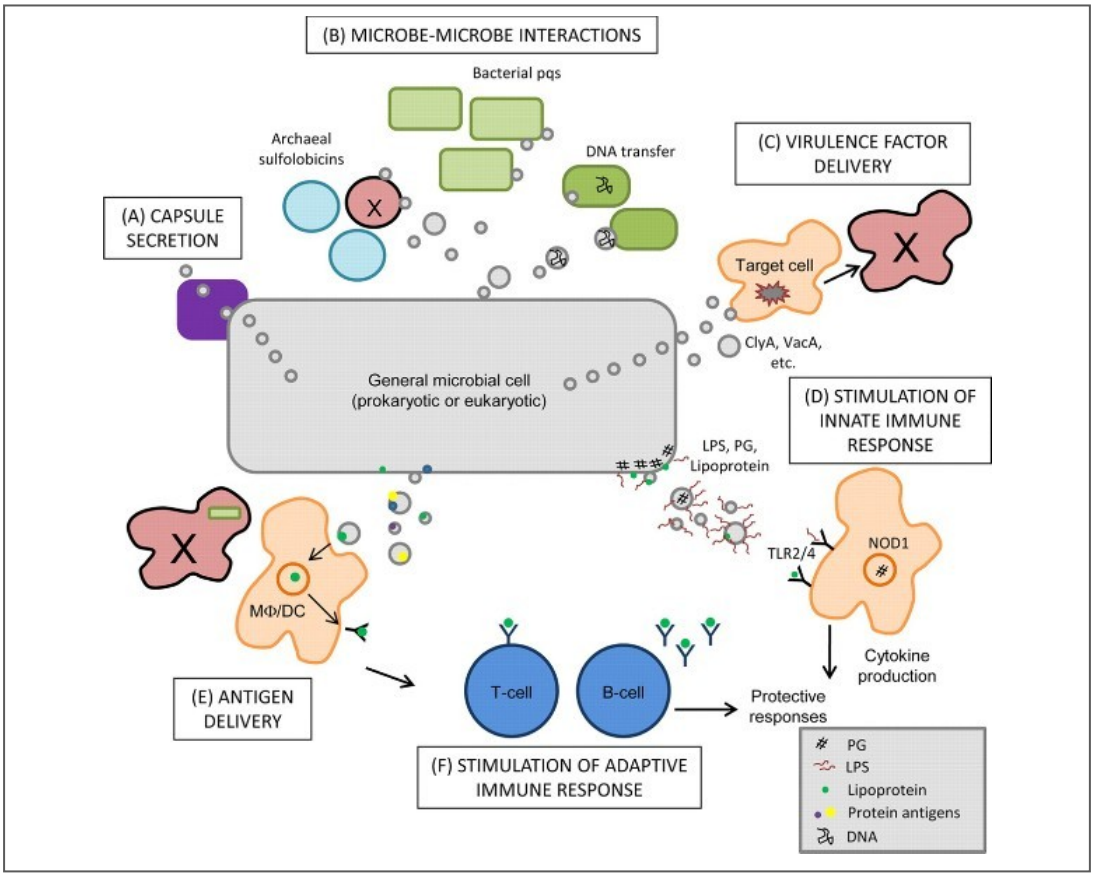


Fig. 9. Biological impact of MV release. Brooke L. Deatherage, and Brad T. Cookson *Infect. Immun.* 2012.

OMVs produced from one bacterium can kill other competing microbes in the same niche, and in this way bacteria destroy coinfectors to prevent competition for limitated nutrients. The predatory nature of MVs was described in *P.aeruginosa* that has the ability to kill other Gram negative as well as Gram positive bacteria [Li et al., 2008]. In a recent study, Vasilyeva *et al* found that Lysobacter spp. XL1 secreted bacteriolytic enzymes in OMVs [Vasilyeva NV *et al*., 2008]. This activity of OMVs shows that they might be capable of distinguishing between self and non-self cells in a mixed community, thus vesiculating bacteria may have a survival advantage in mixed-population infections thanks to their capacity to eliminate competing bacterial strains. Furthermore, OMVs play a role in biofilm formation and maintenance. Biofilm formation is characterized by the expression of genes responsible for

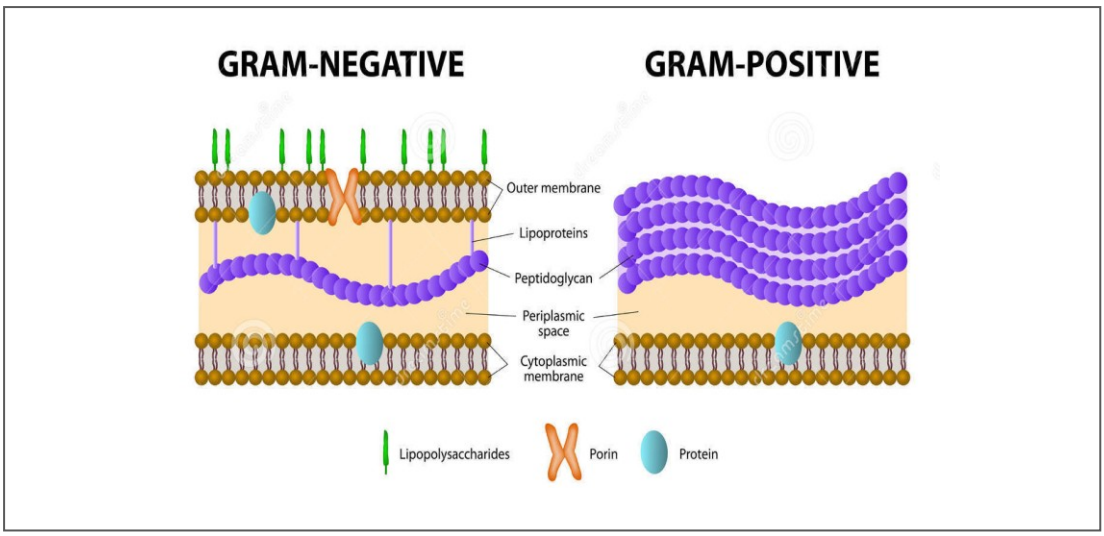
exopolysaccharide production and co-aggregation of cells. Analyses of OM vesicle components have demonstrated that vesicles contain a wide variety of virulence factors. These virulence factors include protein adhesins, toxins, enzymes and antigens such as lipopolysaccharide (LPS). Kadurugamuwa and Beveridge showed that MVs liberated from *Pseudomonas aeruginosa* contain many virulence factors and are important in disease pathogenesis. Factors packaged into *P.aeruginosa* MVs include phospholipase C, proteases, alkaline phosphatases, and hemolysins. Although to date the real benefit to transport toxin and virulence factors by MVs is still unclear, Beveridge proposed two advantages: (i) the virulence toxin inside the MVs could be more concentrated and more focused to target cells; (ii) virulence factors transported by MVs may protect from degradation and recognition by host factors or microorganism. In addition, vesicles can facilitate the horizontal transfer between bacteria and this contributes to genetic diversity and bacterial survival. For example, *M.catarrhalis* which carry β-lactamase within MVs, is able to transfer resistance-antibiotic genes to *S.pneumoniae* and *H.influenzae* and to promote survival of these bacteria in the presence of antibiotic amoxicillin [Shaar *et al.,* 2001b]. Recently, Fulsundar *et al*., have shown that OMVs from *Acinetobacter baylyi* were found to transfer small DNA fragments to *E. coli* [Fulsundar *et al*., 2014]. Vesicle surface factors can mediate adhesion with eukaryotes and, as a consequence, vesicle materials can be internalized. The presence of bacterial membrane vesicles during infection processes has been observed in many human samples and infected tissue. Several reports of clinical isolates of *H.pylori* show OMVs in contact with epithelial cells. [Fiocca *et al*., 1999; Keenan *et al.,* 2000]. In

studies of *S.aureus* and *B.anthracis*, MVs were found to be lethal to host cells [Gurung *et al.,*2011; Rivera *et al*.; 2010].

The mechanism of adherence and internalization is still the subject of many studies, however, OMV adherence has been studied in molecular detail for heat-labile enterotoxin (LT) of enterotoxigen *E.coli* [Horstman and Kuehn, 2002]. It is clear that vesicles contain compounds that are recognized by eukaryotic cells in the innate and acquired immune response pathways. Alaniz *et al.*, demonstrated that OMVs from *Salmonella enteric* serovar *Typhimurium* are potent stimulators of proinflammatory cytokine secretion. *Salmonella* OM vesicles determine the increased expression of tumor necrosis factor alpha and interleukin-12. The ability of bacterial membrane vesicles to trigger inflammatory response to pathogens has led to the development of immunogenic vaccines. Many studies have focused on investigating the potential of OMVs as vaccines for pathogens including *Neisseria meningitides*. A study carried out in Cuba showed that the OMV vaccine had a promising efficacy of 83-94% [Sierra GV *et al.,* 1991]. More recently, a vaccine containing three *N.meningitides* surface antigens was developed in order to provide broad protection and minimize the risk of escape through mutations. New bacterial and viral disease are emerging, and at the same time there is a decline in the efficacy of antibiotics. For this reason, the application of OMVs holds some promise in this context.

1.2.5 MVs in Gram positive bacteria

Despite the fact that the discovery of membrane vesicles in Gram-negative bacteria goes back at least 40 years, MVs have been overlooked in Gram-positive bacteria. This lapse might be attributed to the very different composition of the cell wall between these bacteria.



Recently, it has been demonstrated that MVs can be produced by Gram-positive bacteria, such as *S.aureus* and *B.anthracis* [Lee *et al*., 2009; Rivera J *et al.,* 2010]. Similarly, MVs shed from both strains are bilayer spherical vesicles, but the size is smaller than OMVs from Gram-negative bacteria. For the first time, Lee and colleagues showed that MVs from *S.aureus* contain many virulence factors and toxins, such as adhesins, proteolysin, coagulase and many other related enzymes that are implicated in infections of humans [Lee *et al*., 2009]. A few years later, Gurung *et al.* demonstrated that *S.aureus* secretes membrane vesicles into the extracellular milieu during *in vivo* infection and the protein A, one of the toxins from *S.aureus*, can be efficiently delivered by intact MVs, whereas the protein A from lysed MVs is unable to entry the cytosol of the host cell [Gurung M *et al.,* 2011]. *Streptococcus pneumoniae* also produces membrane vesicles, although these vesicle are biologically and biochemically different from the plasma membrane from which they derive [Olaya-Abril A *et al*., 2014]. In particular, MVs are more enriched in lipoproteins and transmembrane proteins than in the plasma membrane. Furthermore, MVs do not have lipoteichoic acid, a typical molecule on the surface of Gram-positive bacteria. Proteomic analysis showed that MVs have a different fatty acid

composition: short-chain saturated fatty acids, conferring much more fluidity than the plasma membrane, consist of large amount of long-chain fatty acids. This might be, at least in part, an explanation for the biogenesis of membrane vesicles from the very hard cell wall of Gram-positive bacteria [Mercier R *et al*., 2012]. More recently, a large amount of lipoproteins organized in vesicles was isolated from *Streptococcus pyogenes* [Biagini *et al.,* 2015]. Proteomic analysis of the vesicles revealed that they were composed of phospatidylglycerol and lipoproteins. Bacterial lipoproteins are possible vaccine candidates because they are a major class of cell-surface proteins that are sensed by Toll-like receptors. Although, MVs shed by this bacterium are composed of lipoproteins, those structures do no act in a pathogen-associated molecular pattern [Biagini *et al.,* 2015]. Taken together, these resultst seem to indicate that MVs serve as a transport system for virulence-associated components. During my PhD project, I tried to check, for the first time, if *Streptococcus pyogenes* can also be a producer of membrane vesicles and whether this could explain the hyper virulence of some *Streptococcu*s strains due to the delivery of some toxins within MVs.

2. *Study Aim*

During my PhD at the Microbial Molecular Antibiotic Resistance (MMAR) laboratory my research line focused on molecular characterization and evaluation of genetic expression in different growth phases of three hyper virulent strains of *S.pyogenes*, isolated in different Italian hospitals, that were responsible for serious cases of acute necrotizing pneumonia that led to the rapid death of patients due to respiratory failure, sepsis and necrotizing hemorrhagic pneumonia [Santagati M, 2014]. Simultaneously to the molecular characterization of clinical isolates, we assessed a production of membrane vesicles (MVs) by *Streptococcus pyogenes* during growth, hypothesizing that these structures could vehicle some toxins to explain the hyper virulence of our sample used in this study.

In this study, three strains of *S.pyogenes* were included:

1. RM1, isolated in Umberto I Hospital, Rome
2. RMG1, isolated in Agostino Gemelli Hospital, Rome
3. CT1, isolated in Vittorio Emanuele Hospital, Catania

The phases of the trial plan were:

I. Molecular identification of *emm*-typing and MLST determination

II. Determination of the antibiotic resistance profile and virulence gene content by PCR and sequencing

III. Gene expression study of some virulence toxins in different growth phases by Real-Time PCR

IV. Isolation and analysis of membrane vesicles by TEM

3. Materials and Methods

3.1 Bacterial strain and growth condition. All GAS serotype M1T1 strains were previously isolated from different patients with an invasive GAS infection [Santagati M, 2014]. One clinical strain (CT1) was isolated from the emergency department (ED) of Vittorio Emanuele Hospital in Catania, Italy, in July 2012. RM1 was isolated from the ED of the University of Rome Medical Center. RMG1 came to the ED of the Catholic University Medical Center in Rome in February 2012. All of these strains were tested for susceptibility to antibiotics and for the presence of virulence genes by the PCR method [Santagati M, 2014].

We also used two representative GAS M1 serotypes, SF370 [Ferretti, J. 1991] and serotype M1 strain MGAS5005 were obtained from the American Type Culture Collection (ATTC No.BAA947) [Graham MR, 2002]. All of these strains were grown *in vitro* in Brain Heart Infusion broth with 0.2% yeast extract at 37°C with 5% CO_2.

3.2 DNA extraction. For each sample, all colonies grown on Columbia Agar Base, plus 5% horse blood were collected and washed with 1 ml of a solution of 0.9 % w/v NaCl. Then, microbial DNA was extracted and purified through a QIAcube Extractor using the QIAamp DNA Mini kit (Qiagen, Limburgo, NL).The DNA concentration (absorbance at 260 nm; A_{260}) and the purity (A_{260}/ A_{280}) were calculated using a BioPhotometer D30 (Eppendorf, Hamburg, Germany).

3.3 Molecular gene identification. The molecular identification of strains, *emm*-type and virulence genes was performed by PCR assays. *emm* typing was performed by PCR using protocols and the database of the Centers for Disease Control and Prevention (Atlanta, GA, USA; www.cdc.gov/ncidod/biotech/strep/M-

ProteinGene_typing.htm). Strains were tested for multiple virulence genes (**Table 1**) by using the PCR assay [Santagati M, 2014].

Gene*	Primer name†	Primer sequence, 5′→3′	Amplicon size, bp
slo	**MS442**	GGTAACCTTGTTACTGCTAATGCTGA	400
	MS443	TAATGGAAATATCGACTGGTGTAGTG‡	400
speA	**speA-fw**	CTCAACAAGACCCCGATCCAAG	500
	speA-rew	ATTTAGAAGGTCCATTAGTATATAGTTGC‡	500
speB	MS384	GGCATGTCCGCCTACTTTACCGA	800
	MS385	CAGGTGCACGAAGCGCAGAAG‡	800
speC	MS410	TACTGATTTCTACTATTTCACCTATCATC	447
	MS411	TCTGATTTTAAAGTCAATTTCCTGG‡	447
speG	MS412	GCTATGGAAGTCAATTAGCTTATGCAG	448
	MS413	CCGATGTATAACGCGATTCCGA‡	448
speI	speI-up	GGTCCGCCATTTTCAGGTAGTTT	516
	speI-rew	ACGCATACGAAATCATACCAGTAG‡	516
speJ	MS414	CACTCCTTGTACTAGATGAGGTTGC	508
	MS415	ACGCATACGAAATCATACCAGTAG‡	508
sagBC	**sagBC-fw**	GCAGCTAGTTGCTCAACATTTAATG	600
	sagBC-rew	CATAGGCAGTCGCCTGATTCC‡	600
prtF	MS400	CGGAGTATCAGTAGGACATGCGGA	882
	MS401	CTCCCACCAACATTGCTTAATCCA‡	882
PAM	**PAM-fw**	GCAGACGACGCTAGAAATGAAGTA	900
	PAM-rew	CCTGCTTGTGGTGCTTGACCTTTAC‡	900
sof	MS402	ATGCCTGGTTGGGTATCTTCGGT	406
	MS403	AGAGAACAAAACGTTCTGCGCCTA‡	406
ssa	**ssa-fw**	GTAGTCAGCCTGACCCTACTCCAGAAC	621
	ssa-rew	ACTGATCAAATATTGCTGCAGGTGC‡	621
sda1	**MS431**	GGGTCTATAAGAAAAGTGGGCAAAG	439
	MS432	TGATCGTAAAGGTGGGATGCAGTA‡	439

**slo,* streptolysin O; *speA*, streptococcal pyrogenic toxin A; *speB*, streptococcal cysteine protease; *speC*, streptococcal pyrogenic toxin C; *speG,* streptococcal pyrogenic toxin G; *speI*, streptococcal pyrogenic toxin I; *speJ;* streptococcal pyrogenic toxin J; *sagBC*, streptolysin S-associated gene B/C protein; *prtF,* fibronectin-binding protein; *PAM*, plasminogen-binding protein; *sof,* serum opacity factor; *ssa,* streptococcal superantigen; *sda*1, streptodornase D.
†Primers indicated in **boldface** were created with the VectorNT program (Invitrogen, Carlsbad, CA, USA). Other primers were described by Santagati et al. (*11*).
‡Reverse primers.

Tab.1: Primers used for identification of virulence genes by PCR for *S.pyogenes* isolates [Santagati M, 2014].

3.4 RNA extraction. Total RNA was isolated from GAS grown to the mid-exponential phase (optical density at 600nm [OD_{600}] of 0.4) and to stationary phase (optical density at 600nm [OD_{600}] of 1.0). Cells were harvested by centrifugation and the bacterial pellet was re-suspended in 100 µl of diethylpyrocarbonate (DEPC) treated water. 100 µl of lysozyme 20mg/ml (Sigma) was added and incubation continued for 30 min at 37°C. After incubation we added 1ml of TRIZOL Reagent (Life Technologies) and incubation continued for 30 min at RT. After incubation 0.2 ml of chloroform was added per 1 ml of TRIZOL Reagent. The samples were mixed vigorously and then centrifuged at 12,000 × g for 15 min at 4°C. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 ×

g for 10 min at 4°C. The pellet was air dried and dissolved in DEPC treated water. Contaminating genomic DNA was removed from each RNA sample using Turbo DNase (Ambion) and verified by PCR. The RNA was quantitated by A_{260} measurement.

3.5 RT-PCR and qRT-PCR. 10 µg of total RNA was converted into complementary DNA using hex nucleotide primers ImPRO-II Reverse Transcriptase Kit (Promega) according to the manufacturer's instructions. Quantitative real-time PCR assays were performed using QuantiFast Probe Master mix in a Rotor-Gene instrument (QUIAGEN). Transcription of the gyrase subunit A *(gyrA)* was not affected under a variety of *in vitro* experimental conditions hence, *gyrA* expression was used to normalize *in vitro* TaqMan data. TaqMan primers and probes for genes of interest are shown in **Table 2**. Primers and probe (0.25 µM forward and reverse primers and 0.1 µM probe) was prepared, then 0.5 µL of each primer and 0.2 µL of probe were used in a final PCR reaction volume of 20 µL, containing 10 µL of 2X QuantiFast Probe PCR Master Mix (Qiagen, Germany), and 7.8 µL water and 2 µL of the genomic cDNA was added. The thermal profiling of the reaction included an initial denaturation step at 95ºC for 4 min followed by 40 cycles of annealing-extension step at 60ºC for 30 s. The performance of primer and probe sets was tested using theRotor-Gene Q system (Qiagen, Germany), and raw data were analyzed by the Rotor-Gene Q Software 2.1.0.9. All experiments were performed in triplicate on two separate days. Expression analyses were performed using the relative expression software tool REST2009 (Relative Expression Software Tool).

GENE	PROBE/PRIMERS	SEQUENCE	FLUOROFOR
gyrA	gyrA-Tq gyrA-up Tq gyrA-rew Tq	ACCACTGAGCCATACGAACCAT TCGTCGTATTCTCTATGG CAAGCATATGGCGATAAC	FAM
covS	covS-Tq covS-up Tq covS-rew Tq	TGCCATACGGTCAGCCTCAT GGCATATTGGTCTCTTACA GTACGCGAATCATGTCTA	FAM
covR	covR-Tq covR-up Tq covR-rew Tq	CAACATTAGTCTCAACGGCTTCATCAT CGTGAATATGATTTGCTTAA CGGAGATAACGAATATAGAC	FAM
speB	speB- Tq speB-up Tq speB-rew Tq	ATTCTAGGATACTCTACCAGCGGAT GAAGCAATGTTTTCTTTACC GAGGATTTGTTATCGTTTCA	HEX
sagA	sagA-Tq sagA-up Tq sagA-rew Tq	TACAGCAGCAACAGCAGCCT GTAGCTGAAACAACTCAA CCAGTAGCAATTGAGAAG	FAM
slo	slo-Tq slo-up Tq slo-rew Tq	CCTTGTTACTGCTAATGCTGAATCG GCAGCTCTTATCATTGGTA GTCGTTGTGGTTTCTGTA	FAM
sic	sic-Tq sic-up Tq sic-rew Tq	TAGACCAGCCATATTGAGACCAGA CTGGAGATGGTTTGTCTA GGCCATTCTTCTTTATCG	FAM
hasA	has-Tq has-up Tq has-rew Tq	AGCACAGACCTATCCGTTATCAGAA GATGCCGAGTCATTATTAG CGACAAATATCCACTTCTC	FAM
ska	ska- Tq ska-up Tq ska-rew Tq	AACAGTCAAGTCGGTCCAGC ACTGTGTTTGCATTAAC CAGGTTGTGATGTTAGATC	FAM
sda1	Sda1- Tq Sda1-up Tq Sda1-rew Tq	AGAGCCACTGAATCCGACTACAAG GTGGGTGGTATTCTTATTTC NCTTCTTCTTAAGCTATCG	FAM
speA	speA- Tq speA-up Tq speA-rew Tq	CCTCCGTAGATACATGGACTCCTT TCACGATTTTAATGTTTCA TCCCTTCATGATTTGTTAC	FAM

Tab.2: Primers/Probes used for TaqMan assay

3.6 MV Isolation protocol. MVs were isolated from supernatant culture following the method of Way et al. [Wai S.N, 2003]. Briefly, *S.pyogenes* RM1 strain was

grown in 1 L BHI-medium and cultivated overnight (Optical Density, OD_{600} ~ 0.9/1.0). After removal of the bacterial cells by centrifugation at 3500 rpm for 10 min at 4°, the supernatant fraction was sequentially filtered through 0.45 µm and 0.22 µm vacuum filters (PVDF Millipore) to remove residual bacteria and cellular debris. Preparations of membrane-derived vesicles were checked for absence of bacterial contamination by cultivating small aliquots on blood agar.

The filtered supernatant was then concentrated by ultrafiltration with Stirred Cell Model 8200 (Amicon) using a 100 kDa Ultrafiltration Disc PLHK (Millipore), and was centrifuged at 150,000 x g for 2h at 4° (Beckman L8-70M Ultracentrifuge, SW28 rotor). The pellet was washed with phosphate-buffered saline (PBS), centrifuged again (150,000 x g for 2h at 4°), and resuspended in 200µl of PBS.

3.7 Purification of MVs. Fractionation of MV preparations was performed by density gradient centrifugation essentially as described by H.Chutkan [H.Chutkan, 2013]. OptiPrep (60% stock, Sigma-Aldrich) solution was added to the pellet containing MVs at a ratio of 1:3 (by volume) to adjust the vesicle preparation to 45% OptiPrep. 2 mL of vesicles were pipetted into 45% OptiPrep on the bottom of a 12.5-mL Ultraclear centrifuge tube, and then different Optiprep/HEPES layers were sequentially added as follows: 2 ml 45%, 2 ml 40%, 2 ml 30%, 2 ml 20%, 1ml 15%, and 2 ml 10%. Gradients were centrifuged at 120,000 x g for 4h at 4°C in an SW 41 Ti rotor (Beckman Instruments Inc.), and fractions of equal volumes (2/1 ml) were removed sequentially from the top. The MV fraction of 10%, 15% and 20% and those of 30%, 40% and 45% were polled together and was added tenfold the sample volume of DPBSS. MV fractions were centrifuged at 38,400 x g for 4h at 4°C to

remove the OptiPrep Solution. Vesicle pellets were resuspended in 200µl of DPBSS (**Fig.10**).

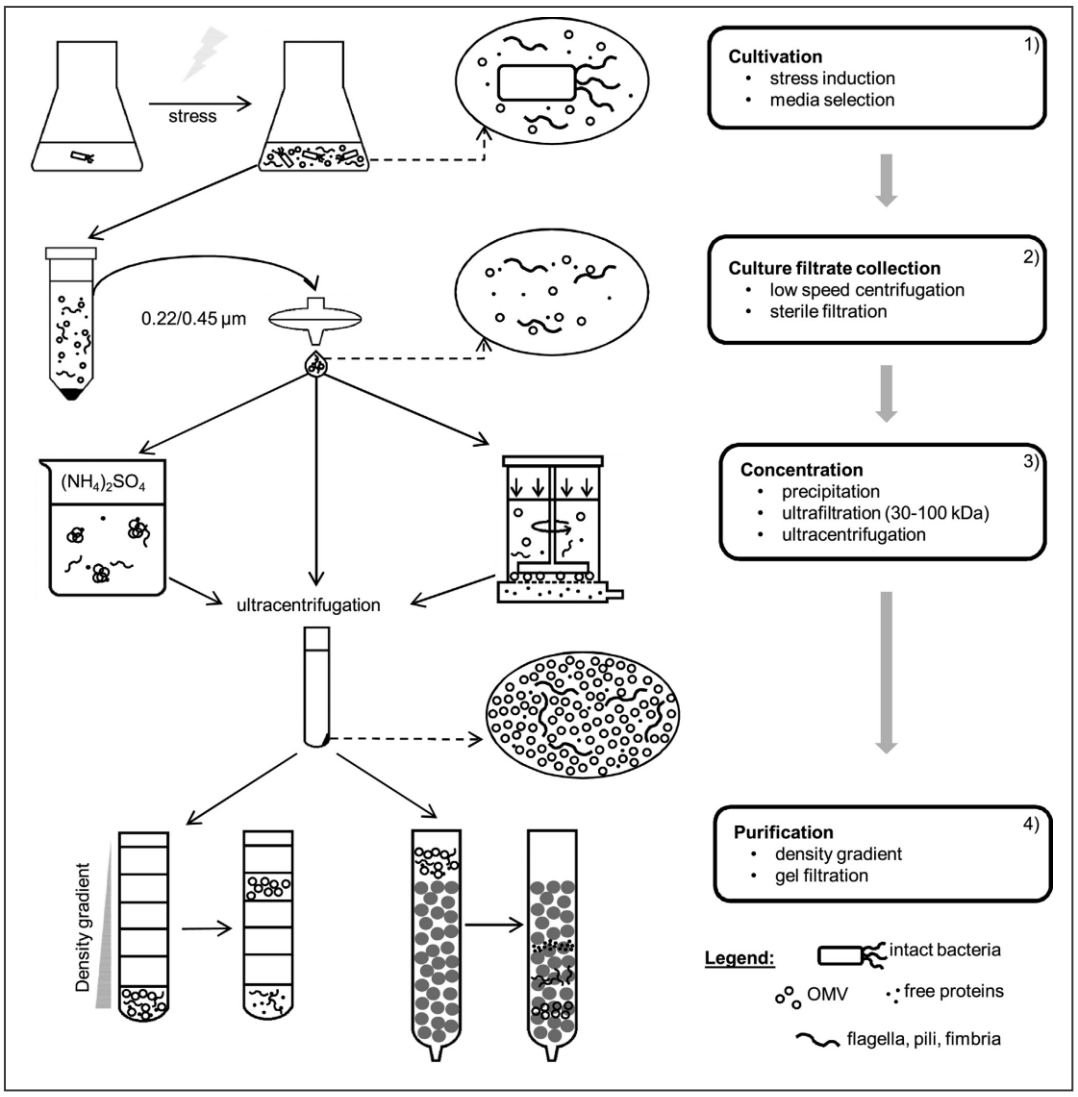


Fig.10. OMV preparation workflow. (1) Cultivation in liquid media (2) Removal of intact bacteria (3) Concentration of the culture filtrate (4) Purification. Klimentová J, 2014. *Microbiological Research.*

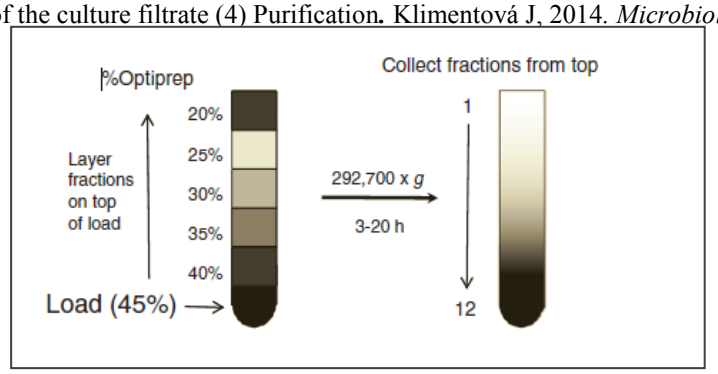


Fig.11. Schematic of ultracentrifuge tubes loaded with the vesicle preparation in the 45% OptiPrep at the bottom of the tube. Halima Chutkan, 2013. *Molecular Biology*, vol. 966.

3.8 Scanning Electron Microscopy (SEM) analysis.

Bacteria for scanning electron microscopy were washed in 0.1 M sodium cacodylate buffer, applied to a poly L-lysine-coated coverslip, fixed with 2.5% glutaraldehyde, dehydrated, sputter coated and viewed on a Philips XL 30 ESEM at 30 kV.

3.9 Transmission Electron Microscopy (TEM) analysis.

The membrane vesicle pellets (20 µl) were fixed in 60 µl of 3% formaldehyde at 4°C. A drop (5 µl) of the above suspension was layered on a formvar-coated copper grid (Electron Microscopy Sciences, Fort Washington, PA) and allowed to dry for 20 min to absorb exosomes. The grids were rinsed side down 2x2 min in PBS, fixed with 2% glutaraldehyde in PBS for 5 min at room temperature, rinsed 2x2 min with water and therefore were negatively stained with 4% uranyl acetate for 5 min and allowed to air dry. The observations were carried out using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

3.10 Dynamic Light Scattering (DLS). DLS measurements were performed with Zetasizer Nano ZS (Malvern, Herrenberg, Germany). Solvent-resistant micro cuvettes (ZEN0040, Malvern, Herrenberg, Germany) were used for experiments with a sample volume of 150µL. The count rates obtained were then corrected for the attenuator used. For each sample, two measurements were averaged **[**Palmieri, 2014]

4. Results

4.1 Molecular identification of emm-typing and MLST determination

The *emm*-typing determination of all isolates included in our study was conducted by PCR using protocols and the database of the Centers for Disease Control and Prevention (CDC). All three GAS strains were *emm*-type 1 and the analysis of allelic profiles obtained showed that strains RMG1 and CT1 belong to ST28 sequence typing, while strain RM1 is a new single locus variant (slv) of ST28, which was deposited by us on the MLST database and called "ST648" [Santagati M, 2014]. Furthermore, determination of the clonal complex, which was performed using the algorithm eBURST, showed that all strains with ST28/ST648 belong to the Clonal Complex 28 (CC28) [Santagati M, 2014].

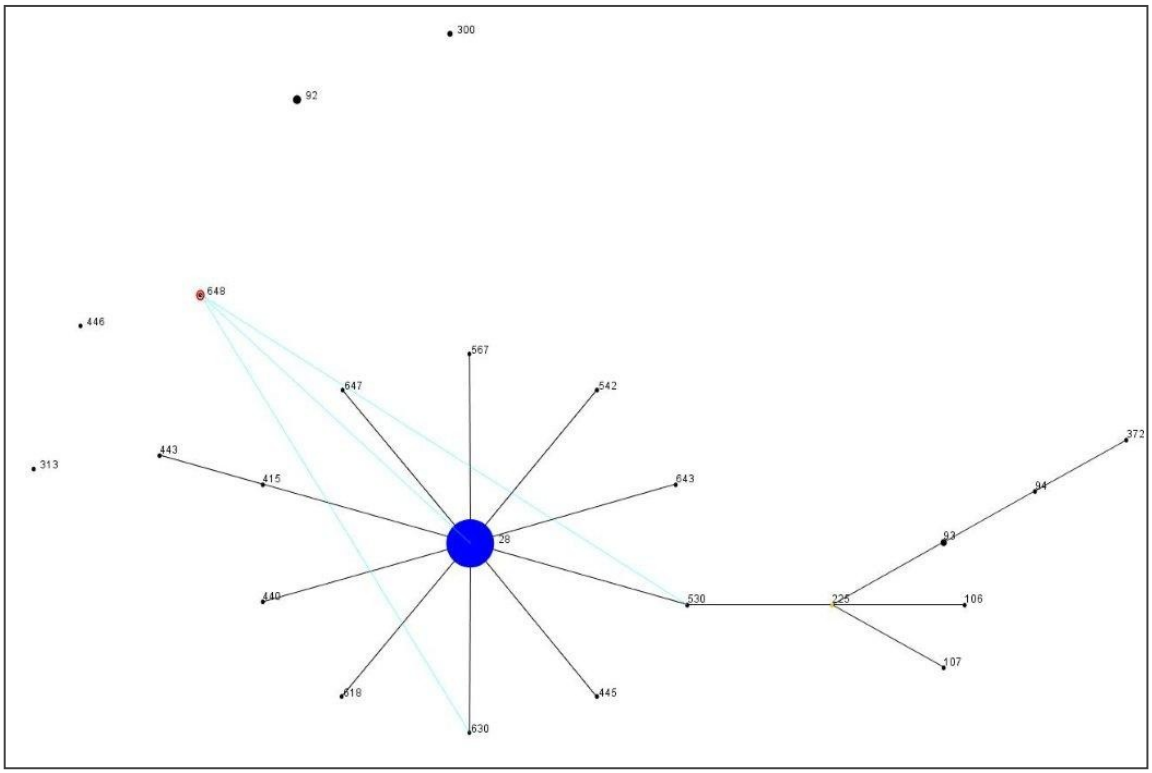


Fig.13: Eburst of CC28; *S.pyogenes* RM1

4.2 Determination of antibiotic resistance profiles and virulence gene content

All isolates were susceptible to erythromycin, tetracycline, amoxicillin, penicillin, and clindamycin by Etest (bioMérieux, Marcy l'Etoile, France). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints (www.eucast.org/clinical_ breakpoints) [Santagati M, 2014].

The virulence profile of our isolates was determined by PCR assay and all primers used were designed with the VectorNTI program (Invitrogen, Carlsbad, CA, USA) and are described in **Tab.1.**

To realize our aim, we introduced two different control strains into the study: *S.pyogenes* SF370, belonging to the M1-Type, (GenBank No.AE004092) and the hypervirulent MGAS5005 strain (GenBank No. CP000017.2) with serotype M1T1 responsible for serious invasive infections.

The results obtained showed that the three clinical isolates, RM1, RMG1 and CT1 were identical, in terms of the *spe* genotype (*speA*+, *speB*+, *speC*–, *speG*+, *speI*–, *speJ*+, *smeZ+),* to the reference strain MGAS5005 M1T1 and [Santagati, 2014] they were also positive for *sagA* and *sda1*. All the three GAS strains tested demonstrated the absence of *ssa*, *prf* and *sof;* while the SF370 strain was negative for *speA* and *sda1* and positive for *speC* (**Tab.3**).

Strains	emm-type	CC	ST	*speA*	*speB*	*speC*	*speG*	*speJ*	*speI*	*sagA*	*sagBC*	*smeZ*	*slo*	*ssa*	*sda1*	*pam*	*sof*	*prtF*
SF370	M1	28	28															
5005	M1																	
RM1	M1	28	648															
RMG1	M1	28	28															
CT1	M1	28	28															

Tab.3: Virulence gene content of three clinical isolates (black) and two reference control strains (red).

4.3 Gene expression study of some virulence toxins in different growth phases by Real-Time PCR

The expression of virulence genes in GAS is controlled by *covR*S and changes during growth-phase. To investigate gene expression levels of most virulence toxins implicated in GAS infections, we chose three growth phases: OD 0.4 and 1.0 at $A_{600\ nm}$ and O/N time points, which coincide with the exponential phase ($A_{600\ nm}$ = 0.4) and the stationary phase ($A_{600\ nm}$ = 1.0 & over night,O/N). To check for variation in individual RNA isolations, total RNA was isolated from triplicate cultures at each time point.

To determine the virulence expression profile of our three clinical isolates, we used to different reference strains: SF370 which represents the model of invasive but not aggressive GAS infection; and MGAS5005 strain, a serotype M1T1 organism that is genetically representative of contemporary isolates and that has been used extensively in pathogenesis research.

Our results showed that *covS* and *covR*, a sensor kinase and intracellular regulator of the CovRS system, were downregulated in exponential phase in CT1, RMG1 and SF370 *S.pyogenes* and upregulated in OD:1 phase with exception of SF370 in which expression levels of *covS* remain low during the stationary growth phase, while those of *covR* increase. Instead, levels of *covS/R* transcripts were upregulated both in the model of GAS invasive infections, MGAS5005, which in one of the strains, RM1, which was the clinical isolate that caused a more aggressive infection. RM1 and MGAS5005 strains showed the same behavior with an upregulation of this locus during all growth phases.

The transcription of *speB* and *sagA*, according to other papers [Chuan, Chiang-Ni *et al.,* 2009], was restricted to the stationary phase for all isolates, except for CT1 which always presents low levels of *speB* gene.

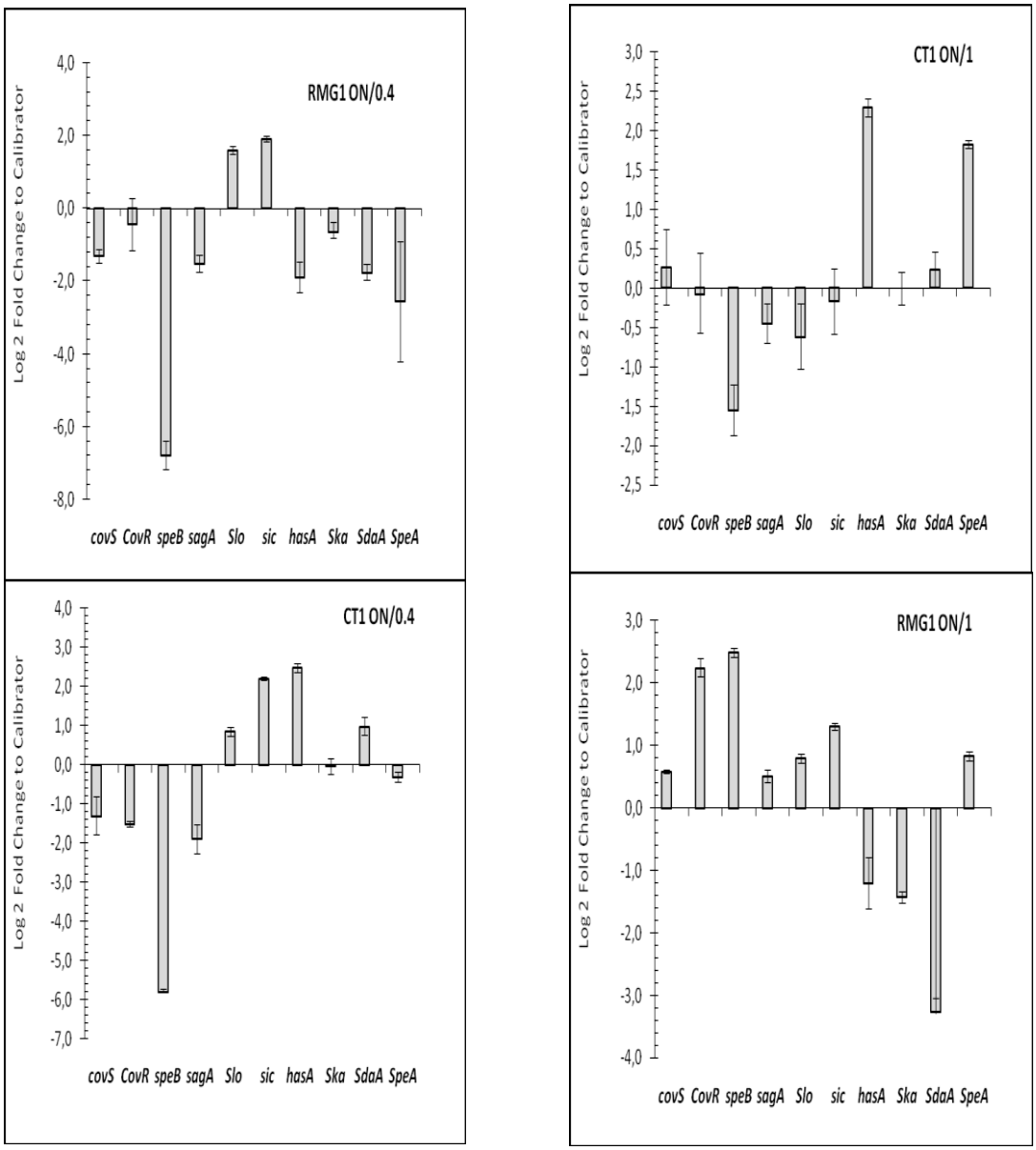
In contrast, expression of *slo* was restricted to the exponential phase for all clinical isolates, but not for MGAS5005 and SF370, which downregulated both *sl*o and *sic* genes. High levels of *sic* gene were observed in CT1 and RMG1 strains during their exponential phase; during the stationary phase just in CT1 GAS strain a lower expression of *sic* was observed, which remained constant for RMG1 in both phases.

RM1 increased the expression of *sic* gene in the stationary phase with an opposite behavior to the *slo* gene.

The *hasA* gene encodes hyaluronate synthase, which is required for resistance to phagocytosis, and was upregulated during all growth phases just for CT1 and RM1 strains, for the other strains this gene was downregulated. Regarding *ska* gene, we cannot describe significant changes in expression levels of our clinical isolates. An important characteristic of the hypervirulent globally disseminated MIT1 clone of GAS is the presence of two prophage-encoded genes, *sda* and *speA*. We observed high levels of *sda* transcripts in all growth phases and in all our samples of M1T1 GAS, except for RMG1; while the transcription of *speA* was restricted for most of the stationary phase, but not for MGAS5005, which unexpectedly expressed a low level of *speA* gene.

According to our analyses, none of the clinical isolates has an expression profile of virulence determinants comparable to the two different models of GAS strains, SF370 and MGAS5005. In fact, levels of two regulated genes (*covS/covR*) along

with genes encoding the toxins *speB* and *sagA*, greatly increase during their stationary phases for all of our clinical isolates. (fig. 14; Tab. 4)



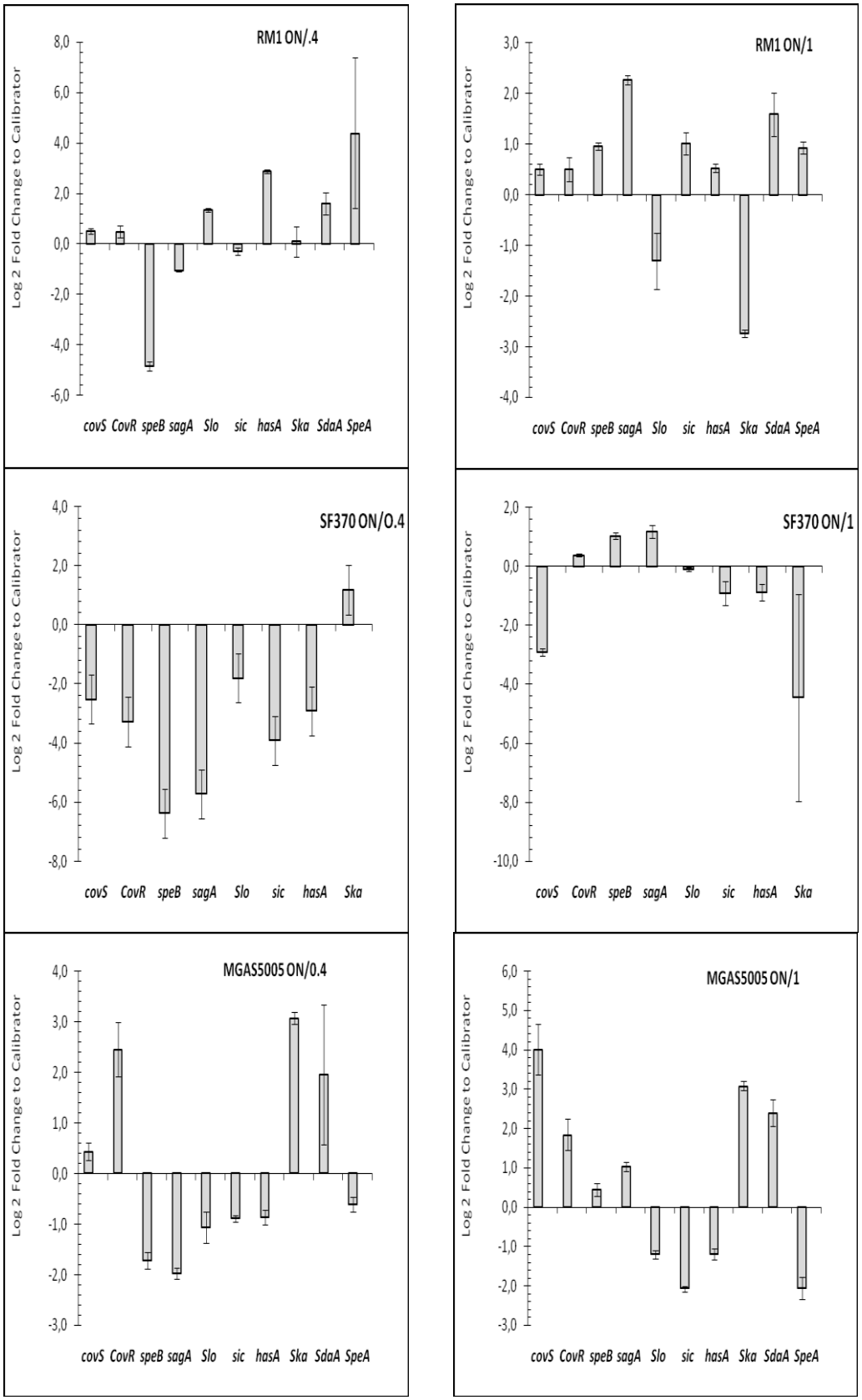


Fig.14: Study of gene expression of *S.pyogenes* by TaqMan Real-Time PCR;

Strains	*covS*		*covR*		*speB*		*sagA*		*slo*		*sic*		*hasA*		*ska*		*sda*		*speA*	
	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1	ON/O.4	ON/1
CT1																				
RMG1																				
RM1																				
5005																				
SF370																				

Legend : downregulated

upregulated

no difference

Tab. 4: Analysis of GAS in vitro transcript levels. Aliquots were removed for RNA extraction at equivalent OD600 values: 0.4; 1.0 and O/N.
Relative abundance of CovR/S regulated transcripts determined by TaqMan assays. Data shown are fold changes in transcript (O/N culture strain relative to 0.4 culture and O/N relative to 1.0 culture) normalized to *gyrA* transcript amounts for selected genes.

4.4 Secretion of MVs from Gram positive bacteria, S.pyogenes

We investigated the production and release of MVs by *S.pyogenes* RM1, which was the S.pyogenes to have a new single locus variant (slv) of ST28. First, we tested whether the bacteria released membranous material into the culture medium by filtering and ultracentrifuging the supernatant when the bacterial culture reached an OD_{600} = 09/1.0, which corresponded to the late-stationary phase (fig. 15). In the first step, MVs were isolated using a combination of centrifugation and ultrafiltration to eliminate residual cells and cell debris. In the second step, MVs were purified using OptiPrep gradient density centrifugation to remove protein aggregation. After OptiPrep gradient density, we combined two OptiPrep layer fractions from the bottom to the top of the gradient: 45%, 40% and 30%; 20%, 15% and 10%. We checked the presence of spherical vesicle-like structures in the putative MV fractions by TEM after preparing the samples. In both fractions membrane vesicles were observed with heterogeneous size from 165nm to 800nm. Dynamic Light Scattering analysis of two fractions confirmed heterogeneous size, with a peak of 623 nm for the fractions of 20%, 15% and 10%; (fig. 16 e) while a peak was observed of 800nm for the fractions of 45%, 40% and 30% (fig. 16 e).

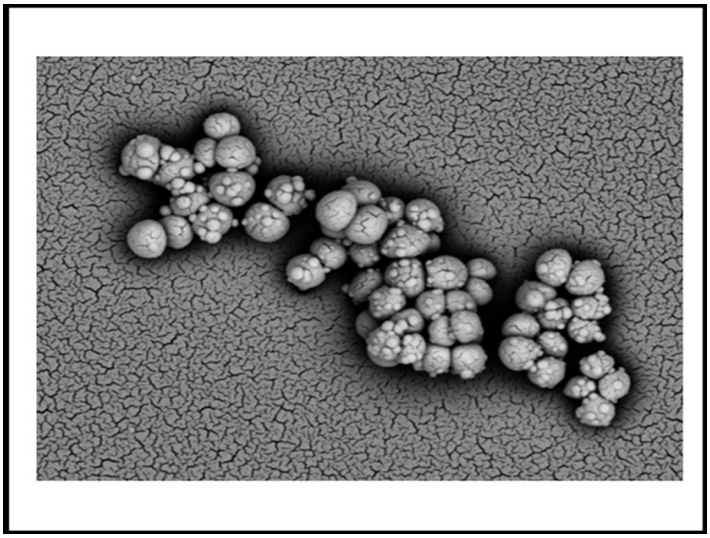
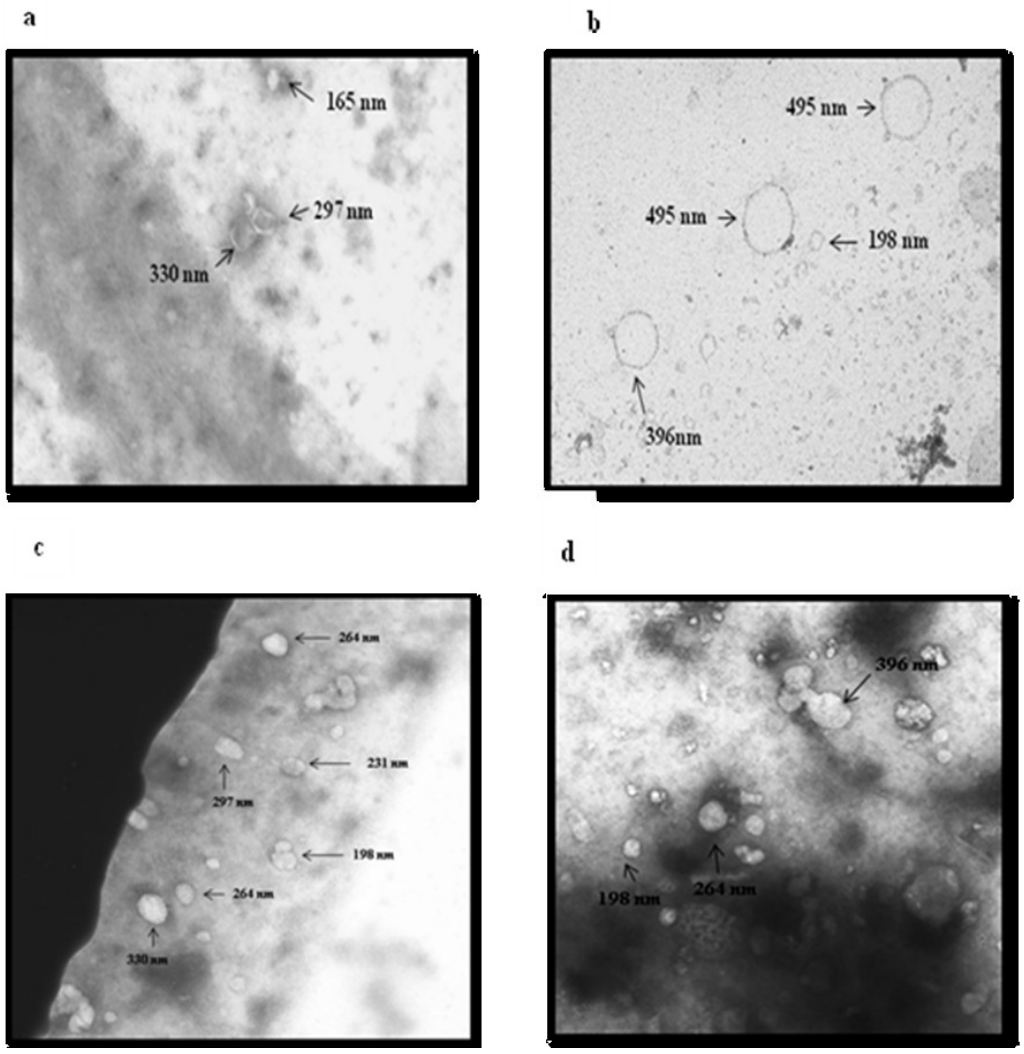


Fig. 15: MVs shed from *S.pyogens;* This scanning electron image by Sem shows the formation of MVs on the cell surface.



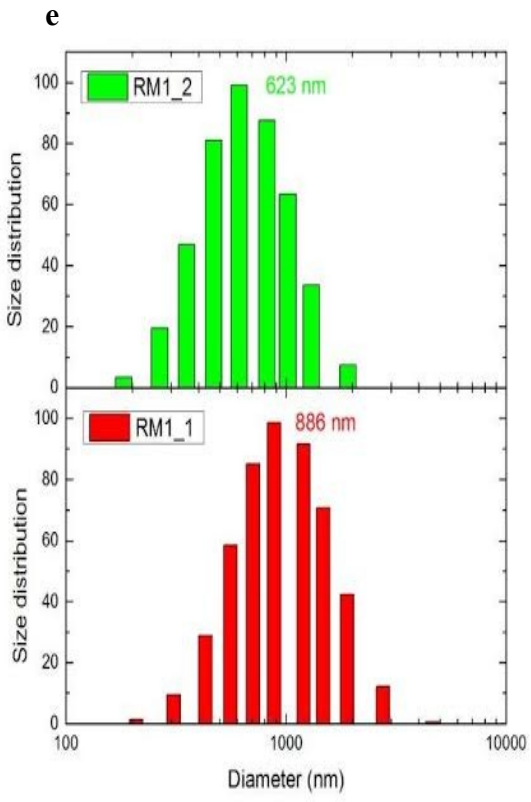


Fig. 16: *S.pyogens* MVs after Optiprep gradient density; Fractions 20%, 15% and 10% (**a** and **b**); Fractions 45%, 40% and 30% (**c** and **d**); Size distribution of MVs measured with dynamic light-scattering shows the diameter range of 165–800 nm. RM1_2 (green): Fractions 20%, 15% and 10% with a peak of 623nm; RM1_1(red): Fractions 45%, 40% and 30% with a peak of 886nm (**e**).

5. Discussion

Streptococcus pyogenes M1T1 cause a wide variety of human pathological infections ranging from uncomplicated pharingytis to necrotizing fascitiitis and streptococcal toxic shock syndrome. Worldwide, GAS causes an estimated 650,000 invasive infections and 150,000 deaths annually [Carapetis J.R *et al.*, 2005]. Increased reports of severe GAS disease in recent decades have been attributable to the emergence of a new globally-disseminated clone of the M1T1 serotype [Cole JN *et al.*, 2011]. Whereas antibiotic therapy is normally efficient against non-invasive GAS disease, severe *S.pyogenes* group A infections are more complicated to treat because the pathogenesis of *S.pyogenes* group A infections reflects the complex interplay between host and bacteria, depending on production and secretion of several molecules that block phagocytosis, degrade immunoglobulins, inhibit complement activation, and facilitate invasion of human tissue [Cunningham *et al.*, 2000]. Genomic analysis of more than 3600 *S.pyogenes* strains belonging to serotype M1 distinguished two main groups: SF370-like and MGAS5005-like, the latter characterized by a hypervirulent profile related to the acquisition of phage-associated virulence factors by horizontal gene transfer mechanisms [Fiebig A, 2015]. Invasive bacterial disease requires the presence of virulence factors that facilitate the microbe's interactions with host tissues and subvert the defenses of the innate immune system. In the *emm1* GAS clone, progression to systemic infection is also favored by mutations in the two-component covRS regulator, which enhance resistance to subepithelial innate immune defenses and facilitate deep-tissue penetration. These mutations markedly alter the transcriptional profiles of invasive GAS isolates compared with those of pharyngeal mucosal isolates [Churchward G *et*

al., 2007] characterized by strongly upregulating *sda1* transcription and markedly downregulating the expression of the gene encoding the cysteine protease *SpeB*.

In the present study, we characterized three clinical isolates of M1T1 GAS clone isolated from different Italian Hospitals evaluating their virulence content, their expression profile in different growth phases and investigating the ability to produce membrane vesicle structures as a new strategy to delivery virulence factors. Analysis of *emm*-typing showed that all GAS isolates (CTI, RMG1 and RM1) belonged to ST28 sequence typing, while strain RM1 showed a new single locus variant (slv) of ST28, which was deposited in the MLST database and called "ST648" [Santagati M, 2014]. First results were consistent with the severity of infections reported by patients, which is typical of GAS MIT1 strains. One important feature of MIT1 clone compared with less phatogenic GAS strains is the acquisition of two prophage encoding *Sda* and *SpeA* toxins. The former has been shown to promote MIT1 GAS virulence via degradation of DNA-based neutrophil extracellular traps (NETs), allowing the bacteria to escape from neutrophils and thus facilitating the spread of bacteria [Buchanan JT *et al.*, 2006]. Moreover, *Sda1* is an essential driving force for *Cov*R/S mutation and SpeB-switching in some strains. SpeB allows accumulation and activation of the broad-spectrum host protease plasmin on the microbial cell surface, thereby promoting infection spread to normally sterile sites. Superantigen SpeA also contributes to augmenting dissemination and virulence potential. Analysis of genetic virulence content of the three clinical isolates showed a comparable profile between themselves and to the reference strain MGAS5005, confirming the presence of *sdA, speA* and other genes implicated in virulence (**Tab.3**).

The expression profile of the most virulence toxins implicated in GAS infection diseases was obtained by TaqMan Real-Time assay. Our analysis demonstrated that not all of our clinical isolates have the same virulence expression profile despite all belonging to the M1T1 serotype. For CT1 and RMG1 a maximum level of *CovS* transcripts in stationary phase was observed; while RM1 showed similar results to MGAS5005, where the levels of both *CovS* and *CovR* transcripts increased simultaneously to their growth. Indeed, Chuan, Chiang-Ni *et al.* demonstrated that CovR expression at the stationary phase was associated to *emm*1/ST28strains [Chuan, Chiang-Ni *et al.,* 2009]. However, one of the most notable changes in gene expression during bacteria growth is the remarkable downregulation of the major streptococcal protease, SpeB, and the consequent increase of most extracellular factors, including *slo, sic, hasA* and *sdA.* In fact, when SpeB is normally expressed, it is able to degrade fibronectin, vitronectin to facilitate tissue invasion and spreading within the host [Kapur *et al*., 1993] and at the same time some virulence toxins are degraded (Sic, M protein, Ska, and Sda) maybe to evade initial innate host defenses [Aziz *et al.,* 2008]. Thus, our results, showing an inverse correlation between low transcript levels of SpeB and high levels of *hasA, sic, sda* and *slo* genes, are in agreement with current literature [Nelson C et al., 2011]. In particular, the production of cytotoxin streptolysin O (SLO) prevented internalization of GAS into lysosomes of pharyngeal epithelial cells during the first step of GAS invasion [Sumby P *et al.*, 2005], this may be a reason why, in our clinical isolates, the *slo* gene is highly upregulated during the exponential phase while s*peB* is downregulated. Furthermore, we observed that *speB* expression is identical to *sagA* for all of our strains. SagA toxins are known to cause epithelial cell cytotoxins, encouraging tissue necrosis

[Engleberg NC *et al.*, 2004], but we do not have an explanation about the correlation between *speB* and *sagA* genes. Besides, all of our isolates showed an upregulation during the stationary phase *of speA* (**Tab.4**).

Another aim of our study was to evaluate the ability of *S.pyogenes* membrane vesicle production. For many decades, it was assumed that production of membrane vesicles was a capacity just of Gram-negatives, which produced and release vesicles derived from outer membrane [McBroom AJ *et al.*, 2005] and only more recently was discovered in Gram-positive bacteria [Lee EY *et al.,* 2009; Rivera J *et al*., 2010]. For these reasons, we first investigated whether hypervirulent *S.pyogenes* RM1 was able to produce MVs. When thin sections of GAS RM1 were analyzed with SEM, MVs were clearly visible on the surface of *S. pyogenes*. We also demonstrated, for the first time in this strain, that production and release of MVs were active processes and increased from the late exponential phase to the stationary phase (data not shown). Similarly, MVs shed from *S.pyogens* were bilayered spherical vesicles. The size range of streptococcal MVs, observed by TEM, were with a heterogeneous size from 165nm to 800nm while Gram-negative OMVs were spherical and bilayered structures with an average diameter of 20–300 nm as described by Beveridge et al. [Beveridge et al., 1999]. The size range of MVs of RM1 strain were largerer than we expected, thus, those results were then confirmed by Dynamic Light Scattering analysis and the two fractions of MVs derived from OptiPrep purification, presented two different peaks: 623 nm for the fractions of 20%, 15% and 10%; 800nm for the fractions of 45%, 40% and 30%, in agreement with TEM observations. It has been reported that MVs act as transporters of toxin cargos that are delivered to host cells

[Thay B, *et al.*, 2013], so we can assume that *S.pyogenes* MVs play a role in the delivery of virulence factors to host cells.

In conclusion, studies on possible mutations that occur in CovR/S system are ongoing in our research group because this could increase virulence and pathogenesis of our clinical M1T1 isolates. Furthermore, we have to consider that different severities of disease progression caused by clinical M1T1 isolates depend not only on regulatory gene mutation, but also on host conditions, explaining the remarkably different virulence expression profile of our clinical isolates. Moreover, studies on membrane vesicles of *S.pyogenes* strains will take us one step closer to an integrated view of their functions and compositions as well as applications of these vesicles such as new delivery systems.

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ORIGINAL ARTICLE

Colonization, safety, and tolerability study of the *Streptococcus salivarius* 24SMBc nasal spray for its application in upper respiratory tract infections

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Abstract *Streptococcus salivarius*, a non-pathogenic species and the predominant colonizer of the oral microbiota, finds a wide application in the prevention of upper respiratory tract infections, also reducing the frequency of their main pathogens. In this pilot study, the primary objective was to evaluate the safety and tolerability of a nasal spray, *S. salivarius* 24SMBc, as a medical device in a clinical study involving 20 healthy adult subjects. The secondary aim was to determine the ability of colonization assessed by molecular fingerprinting. Twenty healthy adult subjects, aged between 30 and 54 years, without a medical history of recurrent otitis media, were enrolled. All patient characteristics fulfilled the inclusion criteria. All subjects were treated daily for 3 days with the nasal spray containing *S. salivarius* 24SMBc at a concentration of 5×10^9 colony-forming units (CFU)/ml. The persistence of *S. salivarius* in the nasopharynx was investigated by the antagonism test and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). The tolerability and safety were clinically assessed by clinical examinations during treatment. Our results demonstrate the capability of *S. salivarius* 24SMBc to colonize the rhinopharynx tissues in 95 % of subjects and persist in 55 % of them after 6 days from the last dose of the formulation, maintaining a concentration of 10^5 CFU/ml. The treatment was well tolerated by all healthy patients and no adverse effects were found. The topical application of streptococcal probiotics is a relatively undeveloped field but is becoming an attractive approach for both prevention and therapy, especially for pediatric age patients. *S. salivarius* 24SMBc possess characteristics making this strain suitable for use in bacteriotherapy.

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Introduction

In recent years, there has been increasing evidence indicating beneficial effects of probiotics in the prevention and treatment of many diseases, especially in the gastrointestinal tract, preserving intestinal epithelium by maintaining its microbiota and modulating immune response [1–4]. Until now, few studies have been addressed to the use of probiotic strains in upper respiratory tract infections (URTIs) and some studies suggested clinical advantages for the host after probiotic administration [5–7]. The strategy of using a bacterial species belonging to the healthy human oral microbiota as an oral probiotic for URTIs offers great benefits for the host, contributing to the recolonization process, re-establishing microbial balance, and reducing the level of potential pathogens. As regards to potential pathogens, *Streptococcus salivarius* species is considered the predominant "safe" colonizer, capable of fostering a more balanced, health-associated oral microbiota, interfering with potential pathogens; thanks to these characteristics, it is suitable for use as an oral probiotic [8]. Nasopharyngeal colonization plays an essential role in the pathogenesis of URTIs and, in particular, in recurrent acute otitis media (rAOM), acting as a reservoir for mainly respiratory pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* [9, 10]. Rebalance of the nasopharyngeal microbiota is a new strategy for the prevention of AOM based on the interaction and competition between potentially pathogenic and commensal bacteria. The

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alteration of the nasopharyngeal microbiota and the absence or the low concentration of α-hemolytic streptococci may correlate with the recurrence of acute episodes [11, 12].

The first clinical use of oral streptococcal probiotics concerning treatment of halitosis and/or *S. pyogenes* infections was reported by Tagg and co-workers, attributing this ability to the presence of *S. salivarius* K12, belonging to the normal commensal flora of the nasopharynx and producer of the *sal*A/B bacteriocin that is responsible for the inhibition of *S. pyogenes* species [13, 14].

Our group has already studied a strain of *S. salivarius*, 24SMBc (DSM23307), selected from a healthy child [15], for its probiotic characteristics and for its remarkable ability to interfere with URTI pathogens. In particular, this strain can be used as an excellent application in the prevention of rAOM in infants and children, having a strong inhibitory capacity versus *S. pneumoniae*, one of the main pathogens responsible for rAOM [15]. It is well known that rAOM is the most common infection in children, responsible for most antibiotic prescriptions in early childhood. Over 80 % of children experience at least one episode in the first three years of life and about a third has three or more episodes [16]. The treatment of AOM has a significant impact on child health, healthcare costs, and the development of antimicrobial resistance. In only 10–20 % of children can AOM result in recurrence and/or persistence with complications, such as impaired hearing, behavior disorders, and surgical interventions [17].

The main aim of this pilot study was to evaluate the safety, the human tolerability, and persistence of *S. salivarius* 24SMBc, used as a new nasal spray formulation.

Materials and methods

Study design

The objective of this pilot study (performed from 2011 to 2012) was to evaluate the safety and tolerability of *S. salivarius* 24SMBc nasal spray used as a medical device and its ability to colonize and persist in the human rhinopharynx of healthy patients. Twenty healthy adult volunteers, aged between 30 and 54 years, including male and female subjects (12 male and 8 females), without a medical history of recurrent otitis media, were enrolled after informed signed was obtained. The nasal spray formulation contains *S. salivarius* 24 SMBc at a concentration of 1×10^9 suspended in a water solution with dimethicone, without gas. The product was tested preliminarily for its stability at 25 °C and at 4 °C for 1 month, confirming the original concentration. All patients were treated with cefpodoxima (200 mg twice daily for 6 days) before the nasal spray administration, to reduce the level of other oral streptococci and to favor 24SMBc colonization. The nasal spray was administered four times per day at intervals of about 4 h (concentration per day 8×10^9) for 3 days, excluding the night, by two puffs in each nostril. The study protocol was approved by the ethical committee of L'Unità Operativa Complessa (UOC) di Otorinolaringoiatria—ASP 3 CT, P.O. Acireale, Italy. Because of the safety assessment of the study, our ethical committee suggested only an adult patient population. This study was conducted according to the principles of the Helsinki Declaration (protocol no. MED/SEC/2011/1; rev. 01del 05/07/2011).

Patient selection

The subjects enrolled in this study met the following inclusion criteria: aged between 30 and 54 years old of both sexes and healthy.

Exclusion criteria were: morphofunctional disorders of the nasal passages with intranasal airflow predisposing conditions such as to determine the genesis of inflammatory diseases; objective endoscopic mucosal atrophy and obvious deficit of mucociliary clearance; vasomotor hypertrophic inflammatory diseases, both type-specific and non-specific; metabolic diseases (diabetes), cystic fibrosis, asthma gastroesophageal reflux; a clinical history of recurrent inflammation and/or recurrent URTIs; rhinosinus inflammatory and/or acute oropharyngeal diseases; already using inhaled treatment and/or antibiotics in the 30 days prior to enrollment; treated with immunosuppressants; suffering from chronic renal failure; hypersensitive to cephalosporins; and patients who were pregnant and/or lactating.

Microbiologic analysis of samples

The microbiologic evaluation was performed by rhinopharyngeal (inferior nasal turbinates) swabs obtained after antibiotic treatment (T_0) and at 2 h, 4 h, 24 h, and after 6 days of nasal spray treatment, labeled as T_1, T_2, T_3, and T_4, respectively. Each rhinopharyngeal swab was plated directly onto Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5 % horse blood to determine the total microflora, and onto Mitis Salivarius Agar (Difco Laboratories), a selective medium for α-hemolytic streptococci. Cultures were incubated overnight at 37 °C in 5 % CO_2 air atmosphere. In addition, all swabs were cultured to determine the presence of other pathogens according to standard laboratory procedures.

Test for antagonism activity and molecular fingerprinting

To evaluate the presence of *S. salivarius* 24SMBc, each morphologically distinct colony grown in Mitis Salivarius Agar was tested for bacteriocin-like inhibitory substance (BLIS) production using a deferred antagonism test [18]. The indicator strains were representative strains of URTIs including AOM pathogens [15]. The molecular fingerprinting of

S. salivarius 24 SMBc was performed on each BLIS-positive colony. The profile was performed by random amplified polymorphic DNA (RAPD) analysis using primers as described previously [19].

Clinical assessments

The clinical evaluation was performed by examination using a rhinolaryngoscope (Pentax FNL-10RP3) through both nasal cavities and by rhinomanometry (Rhinospir PRO, Sibel, Barcelona, Spain). The endoscopic examination (Fig. 1: patient 13, endoscopic evaluation post-treatment) by this method was evaluated as normal by the examination of trophism and the color of the mucous membranes of the whole rhinopharyngolaryngeal tract, as well as the presence and characteristics (hyper or normal morphology) of any secretions (serum or purulent) throughout the upper airway [20, 21].

Safety determination of the preparation was assessed on the basis of objective assessment and on the analysis of the following clinical parameters: body temperature (measured in the morning); blood sample for testing of inflammatory markers [white blood cell count, platelet count, VES, polymerase chain reaction (PCR)]; intensity of any symptoms (runny nose, sneezing, cough) as subjectively perceived according to the Jackson score (0–3); recording of headache, myalgia, and earache.

Results

Safety and tolerability of *S. salivarius* 24SMBc nasal spray formulation

All subjects reported no symptoms associated with the nasal spray administration (runny nose, sneezing, coughing, headache). Side effects and/or undesirable effects were not observed at all. The body temperature (measured in the morning) showed no clinically significant changes, and inflammatory markers did not change after treatment with inhalation of the product in any of the volunteers enrolled in the study. Physical examination of the upper airway showed no signs of infectious diseases throughout endoscopic surveillance.

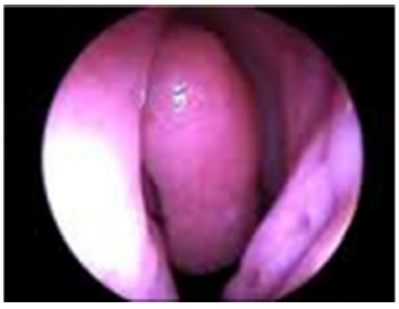

Fig. 1 Endoscopic evaluation post-treatment of patient 13 performed using a rhinolaryngoscope (Pentax FNL-10RP3) through both nasal cavities

Microbiological evaluation and colonization

A total of 100 samples (T_0, T_1, T_2, T_3, T_4) from the 20 subjects were analyzed. All samples were negative at T_0 (i.e., after antibiotic treatment), with the exception of 001 and 009, which were colonized with *S. aureus* (about 50 CFU/ml). The averages of the total microflora population determined on Columbia blood agar after 2 h, 4 h, 24 h, and 6 days from nasal spray administration were approximately from 20 to $>10^6$ CFU/ml for all samples, while on Mitis Salivarius Agar, it was approximately from 10 to 10^4 CFU/ml; only very few samples showed no growth of α-hemolytic streptococci on Mitis Salivarius Agar (Table 1). In addition, samples 001 and 009 at T_3 and T_4 were colonized with *S. aureus*, at low concentrations, remaining unchanged with respect to T_0 (i.e., 50 CFU/ml) and samples 002, 012, 013, and 015 at T_4 were colonized with coagulase-negative staphylococci (CoNS) at bacterial concentrations of approximately from 30 to 100 CFU/ml. The staphylococcal colonization could have interfered with the streptococcal adhesion processes.

The deferred antagonism test to evaluate $BLIS_S$ production and RAPD-PCR analysis for molecular genotyping were applied to each α-hemolytic streptococcal colony to determine the level of colonization of the specific *S. salivarius* 24SMB in the human upper respiratory tract of the volunteers.

The same colonies, showing typical characteristics of *S. salivarius* 24SMBc, i.e., large, soft, and pale blue, were analyzed by the antagonism test against *S. pyogenes* and *S. pneumoniae* groups [15]. All strains tested showed the same *S. salivarius* 24SMB inhibitory activity profile, i.e., strong activity against *S. pneumoniae* and *S. pyogenes*, and no inhibition of oral streptococci. All streptococcal colonies with a positive antagonism test, assayed by RAPD analysis, provided a unique fragment pattern typical of our *S. salivarius* genotype.

Figure 2 shows that the genomic profiles obtained by both OPA3 and OPA18 were identical for all colonies tested.

All these results confirmed that 19 out of 20 patients (95 %) were colonized at least in the first 4 h after nasal spray administration, and 11 out of 20 (55 %) colonizations persisted for at least 6 days from the last dose of the formulation.

Table 1 Levels of *Streptococcus salivarius* 24SMBc colonization over time (2 h to 6 days)

Patient	Age (years)	Bacteria count (CFU/ml)	T_1, 2 h	T_2, 4 h	T_3, 24 h	T_4, 6 days	Persistence of *S. salivarius* 24SMBc at various times
001	45	Total count	$>10^6$	$>10^5$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	65	10	10^4	10^4	T_1, T_2
002	20	Total count	$>10^6$	10^2	3×10^2	10^5	–
		α-hemolytic streptococci count	10^3	20	–	–	T_1, T_2
003	18	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	50	30	10^2	10^2	T_1,T_2,T_3,T_4
004	28	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	30	10	10	20	T_1,T_2,T_3,T_4
005	19	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	–
		α-hemolytic streptococci count	10^2	40	50	30	T_1,T_2,T_3,T_4
006	26	Total count	2×10^2	$>10^6$	20	50	
		α-hemolytic streptococci count	50	4×10^2	10	10	T_1, T_2
007	24	Total count	$>10^6$	$>10^6$	10^4	$>10^6$	
		α-hemolytic streptococci count	10^4	10^3	10^2	10^3	T_1,T_2,T_3,T_4
008	54	Total count	$>10^6$	10^4	$>10^6$	$>10^6$	
		α-hemolytic streptococci count	10^4	10^3	10^3	10^2	T_1,T_2,T_3,T_4
009	31	Total count	$>10^6$	10^5	10^5	10^5	
		α-hemolytic streptococci count	10	30	–	10^2	T_1,T_2
010	38	Total count	$>10^6$	10^5	10^5	$>10^6$	
		α-hemolytic streptococci count	10^3	10^2	10	10^2	T_1,T_2,T_3,T_4
011	30	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^2	50	10^2	70	T_1,T_2,T_3,T_4
012	27	Total count	10^5	10^5	10^4	10^5	
		α-hemolytic streptococci count	20	50	10	80	T_1,T_2
013	23	Total count	$>10^6$	10^5	10^5	10^4	
		α-hemolytic streptococci count	10^2	5×10	10^2	8×10	T_1,T_2
014	32	Total count	$>10^6$	10^5	10^5	10^5	
		α-hemolytic streptococci count	9×10^3	5×10^2	70	10	T_1, T_2
015	43	Total count	$>10^6$	$>10^6$	10^5	10^6	
		α-hemolytic streptococci count	–	10	80	50	–
016	42	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		α-hemolytic streptococci count	10^2	50	50	30	T_1,T_2,T_3,T_4
017	52	Total count	10^6	10^6	10^5	10^4	
		α-hemolytic streptococci count	–	20	10	20	T_2,T_3,T_4
018	36	Total count	10^6	10^6	10^5	10^4	
		α-hemolytic streptococci count	10^2	80	10	50	T_1,T_2
019	33	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^3	10^2	10	10	T_1,T_2,T_3,T_4
020	24	Total count	10^6	10^5	10^6	10^6	
		α-hemolytic streptococci count	10^2	50	10^2	70	T_1,T_2,T_3,T_4

Discussion

There is great interest in the role of the microbiome in the complex equilibrium between a healthy state and progression towards disease. Many studies have addressed this role, above all in gastrointestinal-related diseases and oral pathologies, but only a few have supported a beneficial microflora involvement in URTIs [2, 22–24].

The new approach to use "friendly bacteria", which means to use harmless bacteria to displace pathogenic organisms—by bacterial interference—thus preventing colonization of pathogenic bacteria, is gaining ever more interest, finding applications in many fields [25]. Among studies involving upper

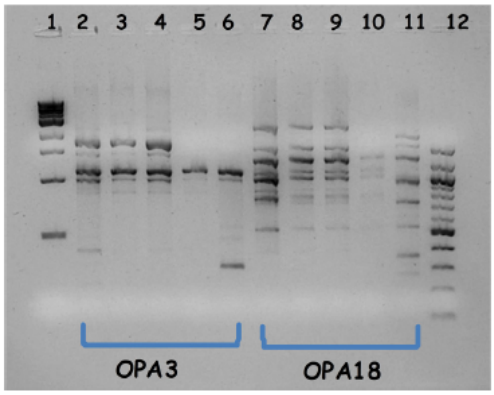

Fig. 2 Random amplified polymorphic DNA (RAPD) fingerprinting with OPA3 and OPA18 primers. Lane 1: Marker 1 kb; lanes 2 and 7: *S. salivarius* 24SMBc; lanes 3 and 8: patient 3 at T_1; lanes 4 and 9: patient 4 at T_1; lanes 5 and 10: patient 6 at T_3; lanes 6 and 11: patient 15 at T_3; lane 12: marker 100 bp

respiratory tract diseases, only a few have pointed out the possibility of using α-hemolytic streptococci as prophylaxis against rAOM and URTIs using a single strain such as *S. salivarius* K12 in oral tablets [26, 27] or α-hemolytic streptococci group (*S. mitis*, *S. oralis*, and *S. sanguis*) in a nasal spray [28, 29], and, more recently, by using *S. sanguinis* and *Lactobacillus rhamnosus* strains [30]. However, there is still only relatively limited clinical evidence on the role of oral probiotics on health improvement. An important aspect of this "bacteriotherapy" approach is the recolonization of the rhinopharynx with healthy flora, as the pharyngeal microbiome has an essential role in the airway linings to protect against many infections [31].

Many studies have highlighted the close correlation between the reduction of potential pathogens and the presence of commensal streptococci [28]. Evidence has shown that a healthy microbiota confers protection against URTIs and a lack or reduction of α-streptococci, especially those with antagonist activity against otopathogens, has been correlated with a higher incidence of re-infections in patients with streptococcal pharyngotonsillitis. Furthermore, it has been found that children who are prone to otitis media are colonized with a lower concentration of α-streptococci compared with those who are not prone [11]; in these cases, probiotics can confer natural protection against infections and, in some cases, become a new prophylaxis. In this context, *S. salivarius*, a non-pathogenic species and predominant colonizer of the oral microbiota, finds a wide application in the prevention of URTIs.

The current study, conducted on healthy volunteers, demonstrated that the administration of a dose of 8×10^9 CFU per day of *S. salivarius* 24SMBc [15] as a nasal spray was well tolerated in all volunteers, and there were no side and/or undesirable effects; in addition, 95 % of volunteers were colonized by *S. salivarius* 24SMB and 55 % remained colonized until the sixth day after the last administration, whereas low-rate staphylococcal colonization in six samples could have interfered with *S. salivarius* 24SMBc colonization.

In conclusion, the primary endpoint of our study—the nasal spray safety and human tolerability—as well as the secondary endpoint in terms of persistence of colonization, were largely achieved in all cases treated.

The application of oral probiotics is a relatively undeveloped field but is becoming an attractive approach for prevention and therapy, especially for pediatric age patients. *S. salivarius* 24SMBc possess characteristics making this strain suitable for use in bacteriotherapy.

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Conflict of interest The authors declare that there is no conflict of interest.

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