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Gianfranco Donelli *Editor*

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Selection of Lactic Acid Bacteria with Probiotic Potential Isolated from the Fermentation Process of "Cupuaçu" (*Theobroma grandiflorum*)

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Abstract

In the present study, nine lactic acid bacteria isolated from the fermentation process of "cupuaçu" (*Theobroma grandiflorum*) were selected for probiotic use. *In vitro* (resistance to gastrointestinal environment, *in vitro* antagonism and co-aggregation with pathogens) and *in vivo* (intestinal colonization and *ex vivo* antagonism in germ-free mice, cumulative mortality, translocation to liver and spleen, histopathological examination of liver and ileum and mRNA cytokine gene expression during an experimental infection with *S*. Typhimurium) assays were used. Among the nine *Lactobacillus* strains isolated from the "cupuaçu" fermentation, *L. plantarum* 81 and *L. plantarum* 90 were selected as potential probiotics based on better results obtained in *in vitro* evaluations (production of diffusible inhibitory compounds and co-aggregation) as well as *in vivo* experiments (resistance to gastrointestinal environment, *ex vivo* antagonism, higher survival after enteropathogen challenge, lower hepatic translocation of enteropathogen, lower histopathological lesions in ileum

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and liver and anti-inflammatory pattern of immunological response). Concluding, *L. plantarum* 81 and *L. plantarum* 90 showed *in vitro* and *in vivo* capacities for probiotic use through different mechanisms of protection and its origin would allow an easier adaptation in an alimentary matrix for its administration.

Keywords
Probiotic selection • *Lactobacillus plantarum* • *Salmonella* Typhimurium • "Cupuaçu" fermentation • Infectious challenge

1 Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer health benefits to the host (WHO/FAO 2002). These microorganisms are generally isolated from the same host localization (gastrointestinal, vaginal or cutaneous) where they will be re-administered as pharmaceutical or food supplemented preparation to obtain some benefits. However, some probiotics do not belong to the host indigenous microbiota and have been selected from another source such as fruit (*Saccharomyces*) (Martins et al. 2005) or fermented foods (*Lactobacillus, Lactococcus, Weissella, Pediococcus*) (Bambirra et al. 2007; Saito et al. 2014; Zanirati et al. 2015; Alvim et al. 2016; Teles et al. 2016). The advantage of such probiotics lies in the way of administration as fermented or supplemented food which is already available. Additionally, they generally can confer a double protective action through antagonism against deteriorative or pathogenic agents in the aliment (cheese, sausage) as well as against enteropathogens in the host digestive tract after its ingestion (Bambirra et al. 2007).

Theobroma (Sterculiaceae) is a tropical American genus of 22 species of trees that grow in the Amazonian rainforest. The genus is noteworthy because it includes the economically important "cacao" or chocolate tree (*Theobroma cacao* L.). *Theobroma grandiflorum*, known commonly as "cupuaçu", is second to cacao in terms of economic importance (Venturieri et al. 1985). "Cupuaçu" is a medium-sized tree, usually 6–10 m and up to 18 m tall. The "cupuaçu" fruit is appreciated for its acidic and strongly aromatic pulp that surrounds the seed. The pulp of this fruit is used to prepare drinks, ice cream, liquors, jellies, and candy. Moreover, the seeds of *T. grandiflorum* have received attention because of their potential for being used as a chocolate substitute after a fermentation process (Venturieri and Aguiar 1988).

In humans, *Salmonella* is believed to cause over one billion infections annually, with consequences ranging from self-limiting gastroenteritis to typhoid fever. In contrast to the severe outcome of disease in humans, *Salmonella enterica* serovar Typhi is not virulent in most animals, including mice. However, the disease associated with *Salmonella enterica* serovar Typhimurium infection of mice closely resembles that of *S.* Typhi in humans. *S.* Typhimurium infection in mice is therefore widely accepted as an experimental model for typhoid fever in humans (Santos et al. 2001). Infection of mice with *Salmonella* induced significant clinical manifestations, tissue damage, and lethality. These manifestations are well known and described during experimental infection of murine models with *S.* Typhimurium and enteroinvasive *Escherichia coli*, and are the results of inflammation in the gut and liver of animals induced by the pathogenic bacteria through inflammation-associated signalling pathways (Eckmann et al. 2000; Guiney 2005; Huang 2009). A wide range of antibiotics are

used to treat human salmonellosis. However, genetic mutations and selective pressure have pushed *Salmonella* spp., as well as other bacteria, to become resistant or multi-resistant to antibiotics (Whichard et al. 2007). Development of alternative processes for the treatment and prevention of gastrointestinal disorders, such as probiotics, has become an attractive option.

In the present study, nine lactic acid bacteria isolated from the fermentation process of "cupuaçu" (*T. grandiflorum*) were selected for probiotic application using *in vitro* (resistance to gastrointestinal environment, *in vitro* antagonism and co-aggregation with pathogens), *ex vivo* antagonism and *in vivo* (intestinal colonization, mortality, translocation, histopathology and mRNA cytokine gene expression during an experimental infection with *S.* Typhimurium) assays.

2 Material and Methods

2.1 Microorganisms

Lactobacillus casei (one), *Lactobacillus fermentum* (three) and *Lactobacillus plantarum* (five) strains previously isolated from "cupuaçu" (*Theobroma grandiflorum*) fermentation, and pertaining to the Mars Cocoa Center Company (Mars Cocoa, Ilheus, Brazil), were used. The pathogenic indicator bacteria used for the antagonistic assay were *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 6538), *Escherichia coli* (ATCC 11229) and *Listeria monocytogenes* (ATCC 15313). All the strains were kept at −80 °C in brain heart infusion broth (BHI, Difco, Sparks, USA) supplemented with 20% glycerol (v/v).

2.2 Animals

For experiments involving survival, translocation and histopathological and immunological determinations, conventional (CV) 6 weeks-old Swiss mice of both sexes were obtained from the Centre for Animal Care of the Federal University of Minas Gerais, Brazil. Germ-free (GF) 6–8 weeks-old Swiss mice (Taconic, Germantown, USA) were used for intestinal colonization and *ex vivo* antagonism evaluations. GF mice were housed in flexible plastic isolators (Standard Safety Equipment Company, McHenry, USA) and handled according to established procedures. Experiments were carried out in micro-isolators (Uno Roestvaststaal, BV, Zevenaar, The Netherlands). For all the animals, water and commercial autoclavable diet (Nuvital, Curitiba, Brazil) were sterilized by steam and administered ad libitum. Mice were maintained in a ventilated animal caging system (Alesco Ltda., Campinas, Brazil) with controlled lighting (12 h light, 12 h dark), humidity (60–80%) and temperature (22 ± 1 °C). All experimental procedures were carried out according to the standards set forth by the Brazilian College for Animal Experimentation (COBEA 2006). The study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CEUA/UFMG, protocol No 24/2015).

2.3 *In Vitro* Evaluation of Resistance to the Gastrointestinal Environment

A *Lactobacillus* culture in de Mann, Rogosa and Sharpe broth (MRS broth, 48 h, 37 °C) was centrifuged (4000× g, 5 min, 5 °C), the supernatant removed and the pellet washed twice with PBS buffer (pH 7.4). A volume (5 ml) of this suspension was mixed with the same volume of a solution simulating the gastric environment and containing CaCl2 (0.22 g/l), NaCl (16.2 g/l), KCl (2.2 g/l), NaHCO3 (1.2 g/l) and 0.3% (w/v) porcine pepsin (Merck, Darmstadt, Germany), and then adjusted to pH 3.0 with 1 N HCl (Vinderola et al. 2011). Aliquots of 0.1 ml were taken immediately after mixture (before pH adjustment) and after 90 min of incubation at 37 °C, and plated onto MRS agar for viable cell counts after

incubation at 37 °C during 48 h in an anaerobic chamber (Forma Scientific Company, Marietta, USA), containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. To simulate the intestinal environment, *Lactobacillus* pellet, obtained as described above, was resuspended in the original volume in 0.3% (w/v) bovine bile solution (Ox-Gall, Oxoid, UK) supplemented with 0.1% (w/v) pancreatin at pH 8.0. Aliquots of 0.1 ml were taken before and after an incubation period of 180 min at 37 °C, and plated onto MRS agar for cell counts after anaerobic incubation at 37 °C during 48 h. All the assays were performed in triplicate, and the results were expressed as $\log_{10}$ of colony forming units (CFU)/ml.

2.4 Determination of Antagonistic Activity

2.4.1 *In Vitro* Antagonistic Assay

The antagonistic activity of the *Lactobacillus* isolates was evaluated by the double layer agar diffusion assay as described by Teixeira et al. (2012). Aliquots of 5 µl from *Lactobacillus* cultures containing 10^9 CFU/ml were spotted onto plates containing MRS agar (Difco). After incubation for 24 h in the anaerobic chamber, lactobacilli were killed by exposure to chloroform for 30 min and the residual chloroform was allowed to evaporate for an equal period of time. The presence of antagonistic substances was revealed by overlaying the plate with 3.5 ml of BHI soft agar medium (0.75%) supplemented with 10 µl of cultures of indicator bacteria (10^9 CFU/ml). After incubation for 24 h at 37 °C, under aerobic conditions, the presence of inhibition halo around the *Lactobacillus* spot was observed and the diameter of the inhibitory zone determined with a digital calliper (Mitutoyo Digimatic Caliper, São Paulo, Brazil).

2.4.2 Supernatant Culture Assay

The antagonism test using a culture supernatant, as proposed by Hütt et al. (2006) with modification, aimed to evaluate the presence of substances produced by lactobacilli cultures responsible for antagonism against pathogens. After centrifugation (5000× *g* for 5 min) of *Lactobacillus* growth culture in BHI broth, the supernatant was collected and sterilized by filtration on a 0.22 µm Millex filter (Millipore Merck). Then, an 1% inoculum of *S*. Typhimurium ATCC 6538 was added to this supernatant and incubated for 18 h at 37 °C. After incubation, decimal serial dilutions were plated onto MacConkey agar (Difco) and incubated for 24 h at 37 °C before colony counting. The use of BHI broth instead of MRS broth for the growth of lactobacilli, as described in the original method, aimed to reduce the influence of acid production on a possible antagonistic effect by the supernatant.

2.4.3 *Ex Vivo* Antagonistic Assay

The assay to detect *ex vivo* antagonist effect of *Lactobacillus* isolates was conducted by the double layer agar diffusion assay (Vasconcelos et al. 2003) 6 days after a mono-association of the selected *Lactobacillus* with GF mice, and *S*. Typhimurium ATCC 6538 was used as the indicator bacterium. Faeces from three mono-associated mice were collected by anal stimulation and placed in the centre of a Petri dish containing MRS agar (Difco) and then incubated at 4 °C for 24 h. After this period, the dish was exposed to chloroform vapour for 30 min and the plates open to evaporation of the residual chloroform. Then, semi-solid BHI agar (0.75%) (Difco), inoculated with the indicator bacteria, was poured over the dish and incubated at 37 °C for 24 h. The presence of inhibition halo around the faeces was observed and the diameter of the inhibitory zone determined with a digital calliper (Mitutoyo Digimatic Caliper). The experiments were performed in duplicate.

2.5 Co-aggregation Assay

The method described by Pérez-Sotelo et al. (2005) was used with some modifications to evaluate the ability of the lactobacilli to

aggregate and deposit with *S.* Typhimurium ATCC 6538. Samples of lactobacilli and salmonella were grown twice for 24 h at 37 °C under anaerobic and aerobic conditions, in MRS and BHI broth, respectively. Growth cultures were centrifuged at 5,000 x *g* for 5 min, and after discarding the supernatant, suspended in sterile PBS solution (pH 7.5). Then 500 µl of lactobacilli and 500 µl of salmonella were homogenized in a sterile Eppendorf tube and allowed to deposit at 37 °C. Counting of the salmonella was performed on MacConkey agar (Difco) using the decimal serial dilution of supernatant of the samples 5 h after starting the experiment. As control, culture containing only salmonella was also submitted to the counting process.

2.6 Treatment and Challenge of CV and GF Mice

A single dose of *Lactobacillus* suspension (0.1 ml) containing about 8.0 $\log_{10}$ of CFU was administered to GF mice by intragastric intubation 5–6 days before the faecal enumeration and *ex vivo* antagonism assay. The same dose was administered daily to CV mice, 10 days before the challenge and during all the remaining experimental period. The control CV and GF groups were treated with 0.9% saline according to the same schedule as the corresponding experimental groups. For challenge, *S.* Typhimurium ATCC 6538 was grown in BHI broth (Difco) at 37 °C during 24 h under aerobic conditions. Mice were inoculated through the intragastric route with 0.1 ml of the bacterial suspension containing 5.0 $\log_{10}$ CFU. Cumulative mortality for the CV animals (10 animals in each group) was recorded until 28 days after the challenge. At the end of the experiments, all remaining mice were sacrificed by cervical dislocation. To determine translocation, and histopathological and immunological parameters, CV mice (five animals in each group) were sacrificed by cervical dislocation 8 days after the challenge (corresponding to the beginning of mortality).

2.7 Colonization of GF Gastrointestinal Tract

Freshly collected faeces from three mice monoassociated during 5 days were immediately introduced in the anaerobic chamber (Forma Scientific), diluted 100-fold in saline and vortexed. Serial ten-fold dilutions were performed and 0.1 ml plated onto MRS agar (Difco) for incubation at 37 °C during 48 h for bacterial counts under anaerobic conditions. The experiments were performed in duplicate.

2.8 Translocation Evaluation

After sacrifice, liver and spleen from CV mice were aseptically collected, weighed, and homogenized in sterile PBS (1:10, w/v). Serial decimal dilutions were prepared and 100 µl aliquots were plated onto MacConkey agar (Difco). Colonies were counted after incubation at 37 °C for 24 h.

2.9 Relative mRNA Expression of Cytokine in the Small Intestine

The relative quantitation of mRNA levels of IL-6, IL-10, IL-17 and IFN-γ genes was performed according to Steinberg et al. (2014). Fragments of the small intestine were collected from CV animals immersed in RNAlater (Ambion, Austin, USA) and stored at −20 °C for later extraction of total RNA. Total RNA was isolated using Trizol (Life Technologies Corp., Grand Island, USA) following the manufacturer's recommendations. The isolated RNA was subjected to agarose gel electrophoresis (1% w/v) to assess the integrity and subsequently quantified by NanoDrop (Thermo Scientific, Inc., Bremen, Germany). Only total RNA samples with more than 200 µg/ml and an A_{260}/A_{280} ratio between 1.7 and 2.1 were used. Genomic DNA was removed by the use of Turbo DNase I prior to reverse transcription performed with the kit High Capacity cDNA

Reverse Transcription, both according to manufacturer's instructions (Life Technologies, Carlsbad, USA). The resulting cDNA was amplified by real-time quantitative polymerase chain reaction (RT-qPCR) using the SYBR Green PCR Master Mix 2X kit following the manufacturer's protocol (Applied Biosystems, Foster City, USA). The gene-specific primers for cytokines and for the GAPDH and ACTB genes (used as reference for normalizing expression data) were described by Giulietti et al. (2001).

2.10 Histopathological Analysis

The organs (liver and ileum) were removed from CV mice after opening of the abdominal cavity and washed in PBS. The organs were transferred to Bouin solution with 2% glacial acetic acid for pre-fixation during 10 min, and then fixed by immersion in formaldehyde 4% solution for 24 h. The samples were processed routinely for paraffin embedding and submitted to microtome to obtain histological slides of 4 μm thick. The slides were stained with hematoxylin and eosin (HE), coded and analysed by optical microscopy (BX51 microscope, Olympus, Tokyo, Japan) by a single pathologist who was unaware of the experimental conditions for each group. For morphometric examination of ileum, images were obtained using a micro analyser program and the JVC TK-1270/RGB KS 300 Image Software Kontron Elektronick/Carl Zeiss image analyser (Oberkohen, Germany). At least 20 villi from three different fields from each animal were used to measure villus height.

2.11 Statistical Analysis

Data were expressed as means ± SEM and analyses performed using the statistical software GraphPad Prism 5.00 (GraphPad Software, San Diego, USA). Differences between means were evaluated using analysis of variance (ANOVA test), followed by Newman-Keuls test. Survival data were analysed using the Log Rang survival test. Data were considered significantly different when * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3 Results

Table 1 shows that all the *Lactobacillus* isolates, except *L. plantarum* 96, were quite resistant to the simulated acidic conditions of the gastric environment. On the other hand, only *L. fermentum* 47, *L. plantarum* 81 and *L. plantarum* 90 showed resistance to the simulated intestinal conditions. These results were confirmed when experiments with GF mice showed that *L. plantarum* 81 and *L. plantarum* 90 were both able to colonize the gastrointestinal tract of animals, maintaining high and stable population levels of about 8.0 $\log_{10}$ CFU/g of contents (data not shown).

Table 2 shows that the best results in terms of *in vitro* antagonism assays against the three indicator pathogenic strains were obtained with *L. plantarum* 81 and *L. plantarum* 90. These data were confirmed in Fig. 1, where a significant reduction of *S.* Typhimurium population levels was observed when this pathogenic bacterium

Table 1 *In vitro* resistance to simulated gastrointestinal environment of *Lactobacillus* strains isolated from "cupuaçu" fermentation

			$\log_{10}$ CFU/ml ± SD	
Lactobacillus strains	Control gastric	Pepsin 0.3% + pH 2.5 60 min	Control intestinal	Bile 0.3% + pancreatin 0.1% 180 min
***L. casei* 24**	9.22 ± 0.11	9.05 ± 0.17	8.92 ± 0.09	3.94 ± 3.41
***L. fermentum* 38**	9.40 ± 0.07	9.44 ± 0.08	9.14 ± 0.09	6.22 ± 0.33
***L. fermentum* 47**	9.53 ± 0.04	9.55 ± 0.05	9.24 ± 0.35	8.01 ± 0.22
***L. fermentum* 62**	9.56 ± 0.08	9.50 ± 0.02	8.90 ± 0.36	6.34 ± 0.21
***L. plantarum* 81**	9.63 ± 0.05	8.67 ± 0.17	8.63 ± 0.10	8.64 ± 0.21
***L. plantarum* 90**	9.62 ± 0.07	8.97 ± 0.58	9.10 ± 0.02	9.11 ± 0.10
***L. plantarum* 96**	9.44 ± 0.07	6.79 ± 1.17	6.62 ± 0.88	6.47 ± 0.65
***L. plantarum* 100**	9.07 ± 0.26	8.06 ± 0.85	8.01 ± 0.82	6.88 ± 0.24
***L. plantarum* 105**	9.59 ± 0.14	8.08 ± 1.36	8.00 ± 1.27	7.63 ± 1.03

Table 2 *In vitro* antagonism of *Lactobacillus* strains isolated from "cupuaçu" fermentation against bacterial indicator pathogens

Lactobacillus strains	Inhibition zone		
	S. Typhimurium ATCC 6538	*L. monocytogenes* ATCC 15313	*E. coli* ATCC 11229
***L. casei* 24**	+++	–	+
***L. fermentum* 38**	++	+	–
***L. fermentum* 47**	+	–	–
***L. fermentum* 62**	++	–	++
***L. plantarum* 81**	+++	+	++
***L. plantarum* 90**	+++	++	++
***L. plantarum* 96**	++	+	++
***L. plantarum* 100**	+	+	+
***L. plantarum* 105**	+	++	++

Inhibition zone diameter: +++ More than 4 cm; ++ between 2 and 4 cm; + less than 2 cm; – no inhibition

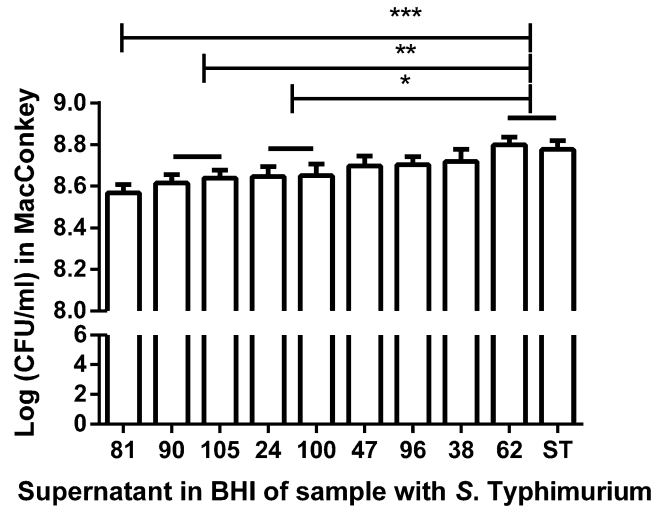

Fig. 1 Supernatant culture inhibition assay of nine *Lactobacillus* strains isolated from "cupuaçu" fermentation against *S.* Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

was grown in culture supernatant of *L. plantarum* 81 and *L. plantarum* 90, but also of *L. plantarum* 100 and *L. plantarum* 105. *Ex vivo* antagonistic assays showed that the production of inhibitory diffusible compounds occurred both *in vitro* and inside the digestive tract of mice since inhibition zone diameters of 15.69 ± 1.12 mm and 17.25 ± 0.4 mm were observed around the faeces of GF animals monoassociated with *L. plantarum* 81 or *L. plantarum* 90, respectively.

In the co-aggregation assays, *L. plantarum* 81 and *L. plantarum* 90, as well as *L. plantarum* 100, *L. casei* 24 and *L. fermentum* 38 showed the best ability to aggregate and deposit with *S.* Typhimurium ATCC 6538 (Fig. 2). Based on the results described above, *L. plantarum* 81 and *L. plantarum* 90 were selected for the next steps of the present study.

When CV mice were previously treated by oral administration of *L. plantarum* 81 or *L. plantarum* 90 and then challenged with *S.* Typhimurium ATCC 6538, a higher survival was observed when compared with mice only challenged with the pathogenic bacterium (Fig. 3). However, this difference was statistically significant only for *L. plantarum* 81 ($p < 0.05$).

Figure 4 suggests that this higher survival in mice treated with the lactobacilli could be due to a lower translocation to the liver and spleen in the animals when compared to the mice only

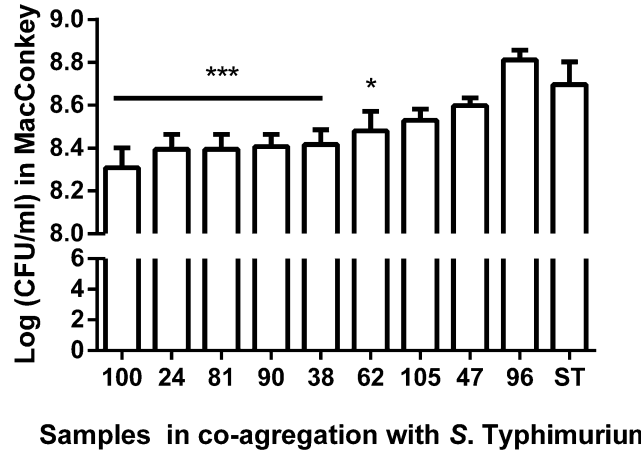

Fig. 2 Co-aggregation assay of nine *Lactobacillus* strains isolated from "cupuaçu" fermentation with *S.* Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$; ***$p < 0.001$)

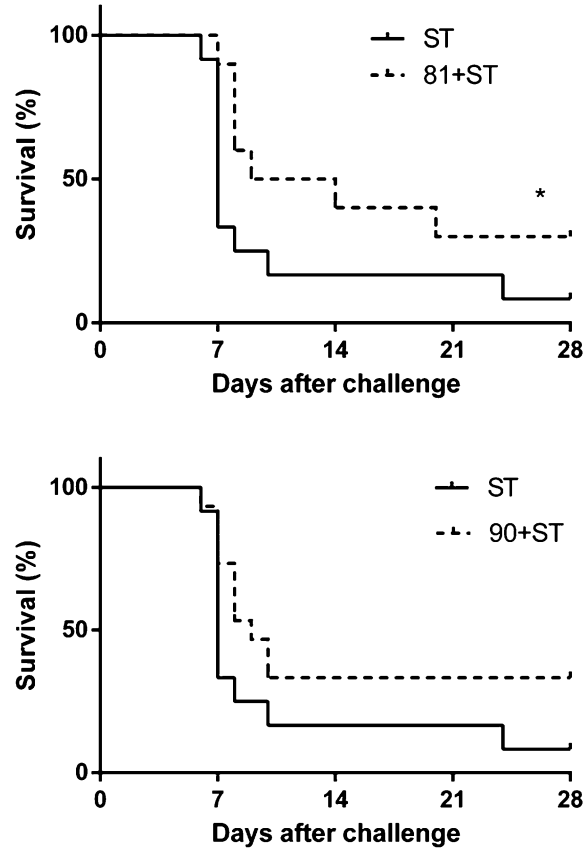

Fig. 3 Survival of CV mice orally treated or not (ST) with *L. plantarum* 81 (81 + ST) or *L. plantarum* 90 (90 + ST) and challenged with *S.* Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (*$p < 0.05$). N = 10

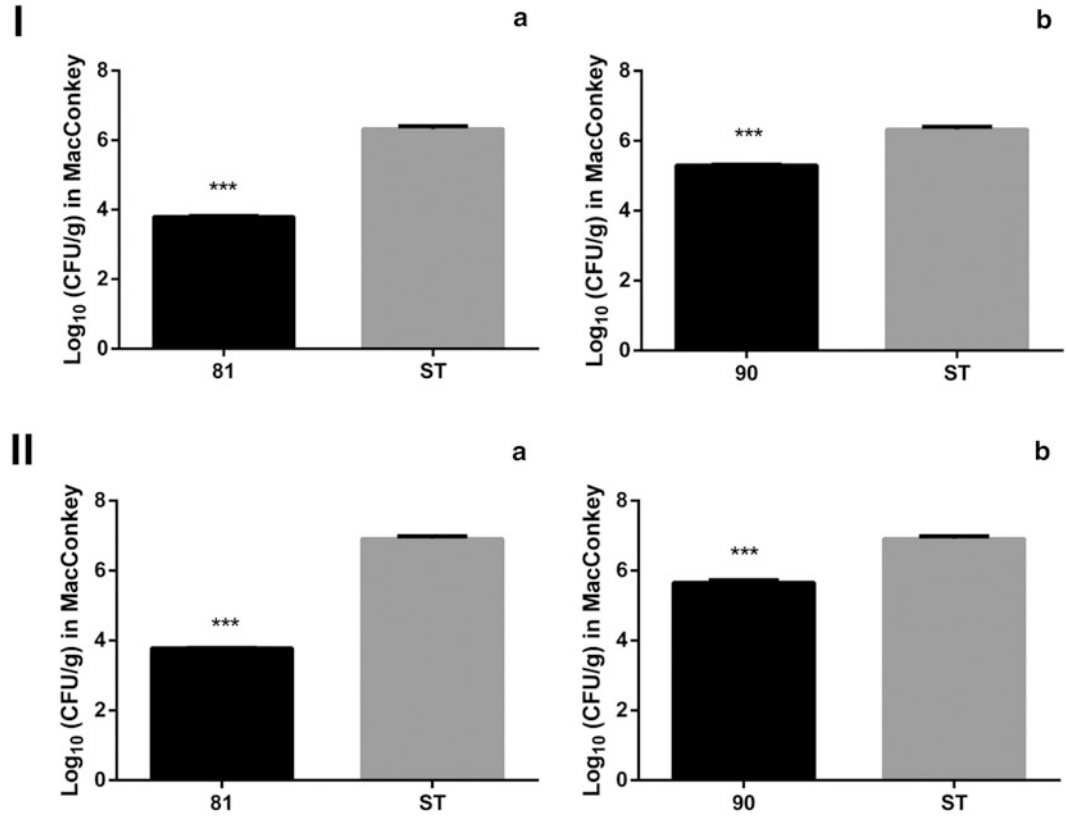

Fig. 4 Translocation of *S*. Typhimurium to the liver (**I**) and spleen (**II**) of CV mice treated with (*A*) *L. plantarum* 81 (81) or (*B*) with *L. plantarum* 90 (90) or not (ST) and challenged with *S*. Typhimurium. *Asterisk* indicates statistically significant difference in relation to the control (ST) (***$p < 0.001$). N = 5

challenged with *S*. Typhimurium ATCC 6538 ($p < 0.001$). This inhibition of the pathogen translocation was higher in CV mice treated with *L. plantarum* 81 than with *L. plantarum* 90 ($p < 0.001$).

The protection observed in CV mice treated with the lactobacilli was confirmed by histopathological examination of liver and ileum tissues (Figs. 5 and 6). Histopathological differences were observed between mice treated with both *L. plantarum* 81 and *L. plantarum* 90 and challenged with *S*. Typhimurium ATCC 6538 and animals only challenged with *S*. Typhimurium ATCC 6538. In the first group of animals, only small inflammatory foci were observed in the liver (Fig. 5A and B), whereas in animals only challenged with *S*. Typhimurium ATCC 6538 hydropic and perivascular degeneration, high number of diffuse and multifocalized (mononuclear and neutrophilic) inflammatory foci, and presence of megakaryocytes were observed (Fig. 5F). In the ileum, mice treated with both *L. plantarum* 81 and *L. plantarum* 90 and challenged with *S*. Typhimurium ATCC 6538 had some foci of inflammatory infiltration and early necrosis (Fig. 6A and B). On the other hand, animals only challenged with *S*. Typhimurium ATCC 6538 showed a profound inflammation process reaching the lamina propria, villus shortening and mucosal necrosis (Fig. 6F). The results of histopathological examination of the ileum were confirmed by morphometric analysis. Treatment with both lactobacilli slightly preserved the villus height (Fig. 6G and H).

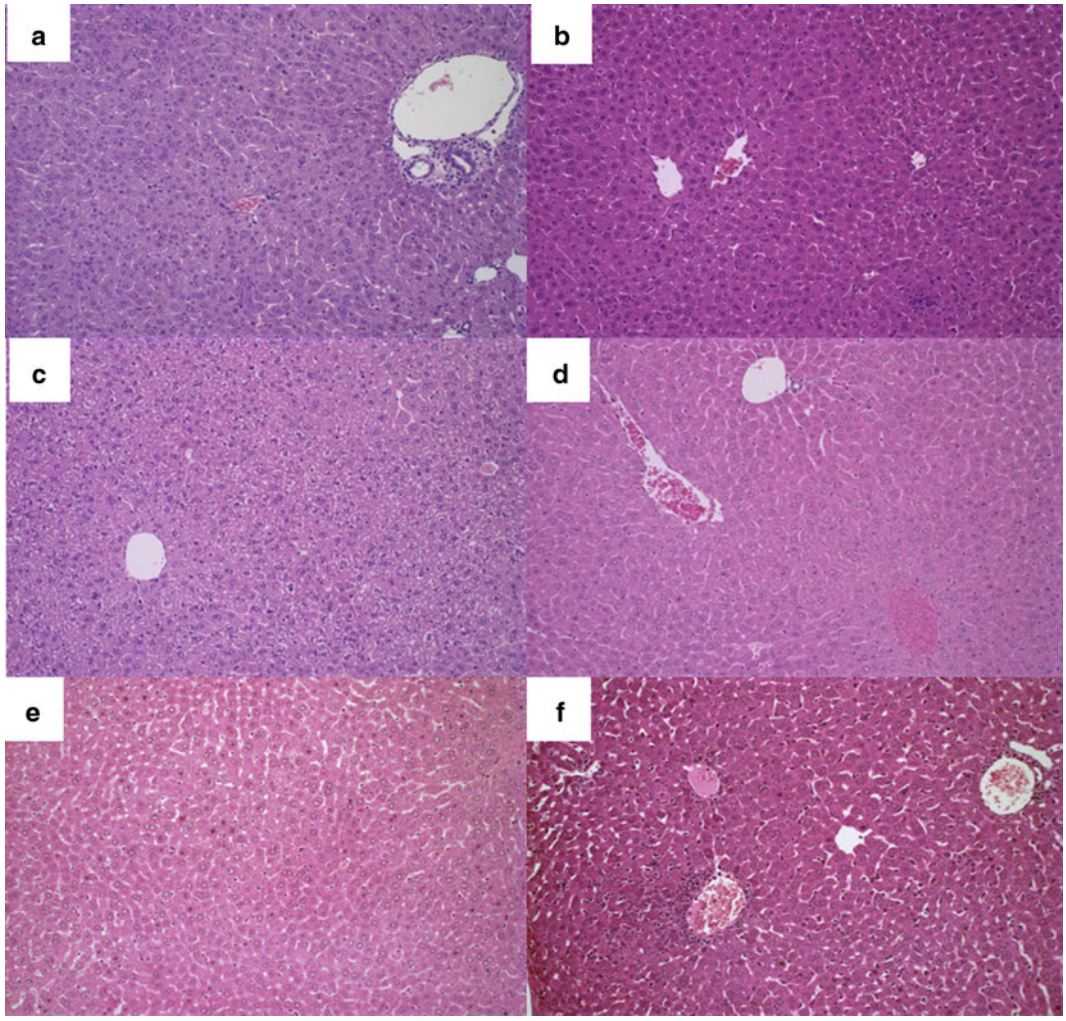

Fig. 5 Histological aspect of liver of CV mice treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (**a**), treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (**b**), only treated with *L. plantarum* 81 (**c**), only treated with *L. plantarum* 90 (**d**), control not treated and not challenged with *S.* Typhimurium (**e**) and only challenged with *S.* Typhimurium (**f**). H & E, 20 X

Figure 7 shows that, as expected, the challenge of CV mice with *S.* Typhimurium ATCC 6538 stimulated the production of the pro-inflammatory cytokines IFN-γ and IL-6 when compared to the control animals. Previous treatment with *L. plantarum* 81 reduced significantly ($p < 0.001$) this increased production, but this was not related with an increase in the production of the regulatory cytokines IL-10 and IL-17.

Figure 8 shows that, similarly to what was observed for *L. plantarum* 81, previous treatment with *L. plantarum* 90 reduced significantly ($p < 0.001$) the increased production of IFN-γ and IL-6 induced by challenge with *S.* Typhimurium ATCC 6538, and here this could be due to an increase in the production of the regulatory cytokine IL-10. Again, there was no difference in the relative expression of the gene mRNA for IL-17.

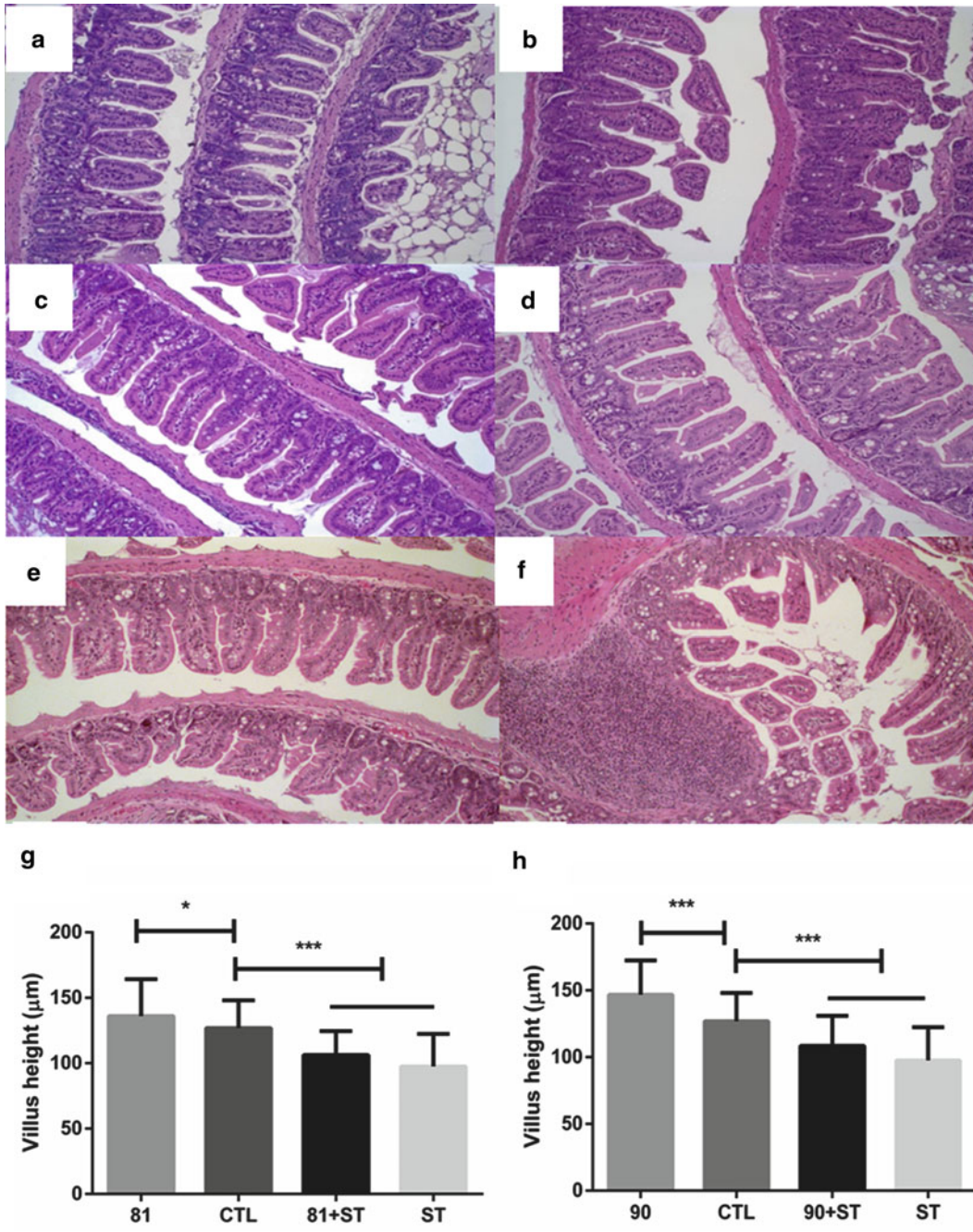

Fig. 6 Histological aspect of ileal mucosa of CV mice treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (**a**), treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (**b**), only treated with *L. plantarum* 81 (**c**), only treated with *L. plantarum* 90 (**d**), control not treated and not challenged with *S.* Typhimurium (**e**) and only challenged with *S.* Typhimurium (**f**). H & E, 20 X. (**g**) (**h**) Villus height in the ileal mucosa of CV mice control (CTL), only treated with *L. plantarum* 81 (81), only challenged with *S.* Typhimurium (ST) and treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (81 + ST). (H) Villus height in the ileal mucosa of CV mice control (CTL), only treated with *L. plantarum* 90 (90), only challenged with *S.* Typhimurium (ST) and treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (90 + ST). N = 5

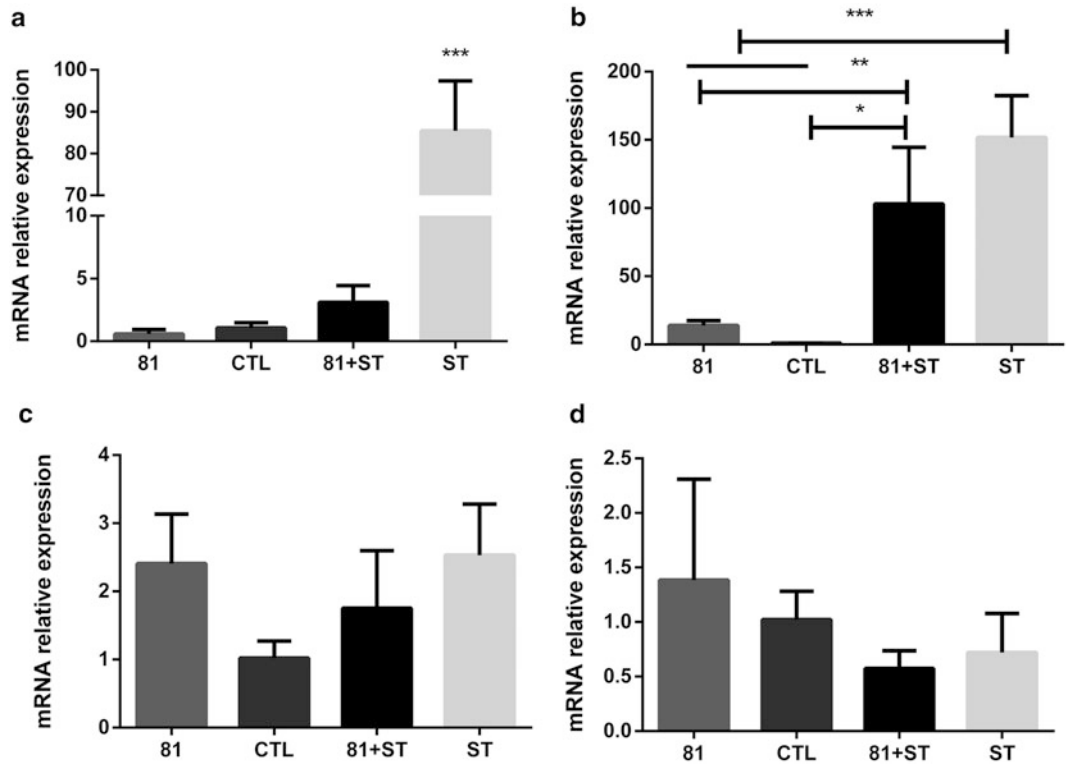

Fig. 7 Relative mRNA expression in the small intestine of INF-γ (**a**), IL-6 (**b**), IL-10 (**c**) and IL-17 (**d**) in CV mice not treated and not challenged (CTL), only treated with *L. plantarum* 81 (81), only challenged *S.* Typhimurium (ST) and treated with *L. plantarum* 81 and challenged with *S.* Typhimurium (81 + ST). *Asterisk* indicates statistically significant difference of ST in relation to 81 + ST (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). $N = 5$

4 Discussion

In the majority of cases, infectious diarrhoea is treated through rehydration or an eventual use of antibiotics. However, the World Health Organization (WHO) has recommended the search for alternative treatments for infection, and probiotics have been proposed for this purpose. Theoretically, any non-pathogenic virus, bacterium, fungus or protozoan is a possible candidate for probiotic use. The probiotics most commonly used in humans belong to genera of the lactic acid bacteria (LAB) group and *Bifidobacterium*, and come from its own body tracts to be thus better adapted when reintroduced. But, the use of selected probiotics from alternative sources known as "unconventional sources" is likely to increase. Unconventional sources of microorganisms were screened for potential probiotics, which have been isolated from non-human body sources, such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juices (Somplang and Piyadeatsoontorn 2016).

As the *Lactobacillus* isolates were foodborne, it was necessary in a first instance to confirm if these bacteria would be able to resist to gastrointestinal environment to be used as probiotics. The results of lactobacillus exposure to simulated conditions found in the human stomach and small intestine, showed a higher resistance of *L. plantarum* 81 and *L. plantarum* 90 when compared to the other lactobacillus isolates.

The production of antimicrobial compounds by candidate to probiotic use is probably one of

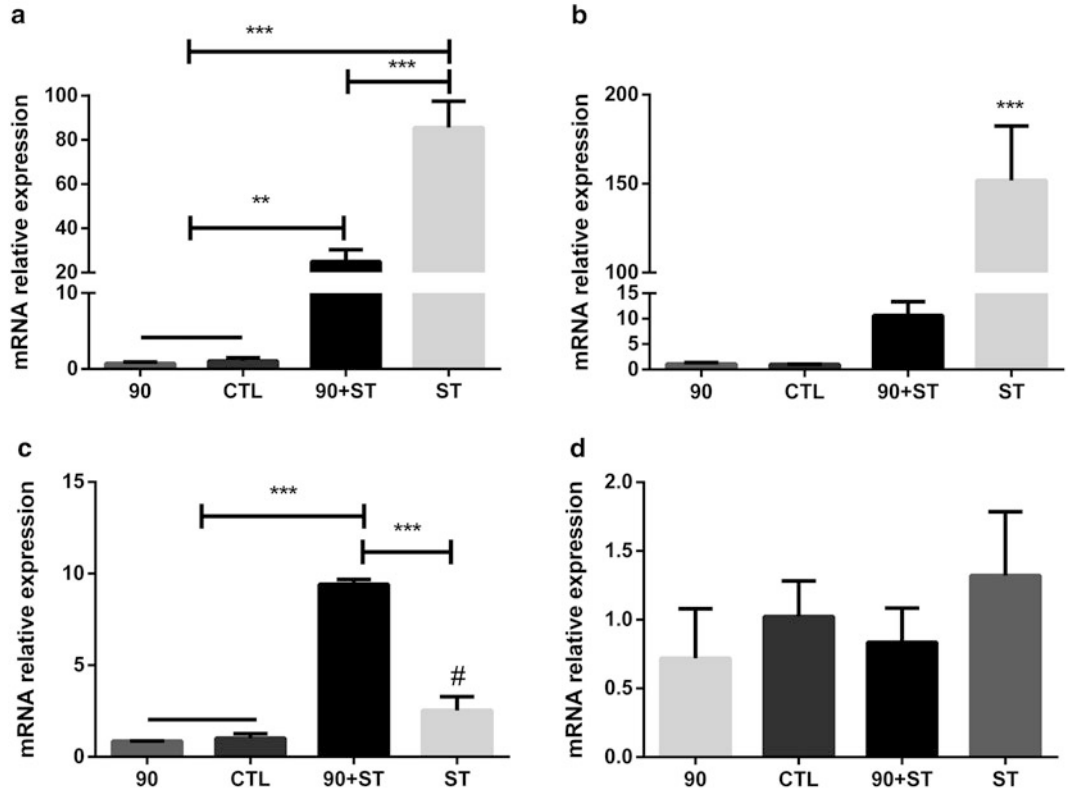

Fig. 8 Relative mRNA expression in the small intestine of INF-γ (**a**), IL-6 (**b**), IL-10 (**c**) and IL-17 (**d**) in CV mice not treated and not challenged (CTL), only treated with *L. plantarum* 90 (90), only challenged *S.* Typhimurium (ST) and treated with *L. plantarum* 90 and challenged with *S.* Typhimurium (90 + ST). *Asterisk* indicates statistically significant difference of ST in relation to 90 + ST (**p < 0.01; ***p < 0.001). N = 5

the most important mechanisms responsible for a protective effect against pathogenic agents. Probiotics can secrete substances such as organic acids and bacteriocins that may have bactericidal or bacteriostatic effects against pathogenic bacteria. Lactic acid is the primary product of LAB metabolism and acts on protein denaturation, altering the permeability of the outer membrane of bacteria. Bacteriocins are inhibitory proteic substances frequently produced by LAB (nisin, diplococcin, lactocidin, bulgaricin, reuterin) which act against pathogens such as pathogenic *E. coli*, *Staphylococcus aureus* and *Salmonella* spp. (Todorov and Dicks 2005). Although the presence of these antimicrobial metabolites has been demonstrated generally *in vitro*, it is unclear whether they are produced in a similar way or have any activity *in vivo*. It is known that the antagonism seen *in vitro* between bacteriocinogenic and sensitive strains is not always expressed *in vivo* in the digestive tract of gnotobiotic mice. Moreover, some data obtained in the gastrointestinal ecosystem of gnotobiotic animals showed that a sensitive strain exerted an *in vivo* barrier effect against its *in vitro* antagonistic strain (Duval-Iflah et al. 1981). On the other hand, Ramaré et al. (1993) demonstrated the presence of an antibacterial substance in the faeces of rats mono-associated with a human *Peptostreptococcus* sp., but which was not produced *in vitro* by this bacterium. This compound appeared to be produced through the concerted action of host's trypsin and a substance produced only *in vivo* by the *Peptostreptococcus* sp. This last example shows the active participation of the host in a bacterial interaction occurring in the

digestive ecosystem and demonstrates the importance of the *in vivo* model, such as the gnotobiotic one, to study diffusible compound protecting against bacterial pathogen, both *in vitro* and inside the digestive tract of gnotobiotic mice (*ex vivo* assays). In the present study, *in vitro* and *ex vivo* antagonisms against *S*. Typhimurium were demonstrated for *L. plantarum* 81 and *L. plantarum* 90, and experiments with culture supernatant suggested that this antagonism was due to the production of organic acids, but also to other inhibitory substances.

The *in vitro* results, suggesting the use of *L. plantarum* 81 and *L. plantarum* 90 as potential probiotics, were reinforced by *in vivo* experiments, as demonstrated by the data of survival, translocation to the liver and spleen and histopathological examination. In relation to translocation, various studies carried out in our laboratory (Silva et al. 1999; Martins et al. 2011, 2013) had similar results in a same experimental model of infection, where cumulative mortality and hepatic translocation of *S*. Typhimurium were attenuated in animals that received a *Bifidobacterium bifidum, Saccharomyces cerevisiae* UFMG 905 or *Saccharomyces boulardii* when compared to the group only challenged with the *Salmonella*.

During an infection by enteropathogens, the first cells encountered by these agents are intestinal epithelial cells, dendritic cells (DCs) and macrophages (Coburn et al. 2007). The interaction with these cells induces the synthesis of pro-inflammatory cytokines such as TNF-α and IFN-γ leading to a massive influx of neutrophils, macrophages and immature DC, which are important for the decrease of bacterial growth in sub-lethal infections by pathogens (Mastroeni and Grant 2011). The TNF-α and IFN-γ cytokines are important in the inflammatory process, having a central role as mediators in the activation and recruitment of neutrophils to the affected region (Dougan et al. 2011). However, in case of exaggerated response and production of these cytokines, an epithelial barrier dysfunction can be caused, thereby contributing to an invasion by the pathogenic bacteria (Castillo et al. 2013). To avoid such problem, regulatory and anti-inflammatory cytokines, such as IL-17, IL-10 and TGF-ß, are produced to down-regulate the expression of pro-inflammatory cytokine genes. The reduction of pro-inflammatory cytokines observed in the present study when mice were pre-treated with lactobacillus before the challenge with *S*. Typhimurium can be explained at least by three mechanisms acting simultaneously or separately. The enteropathogenic bacterium could be killed by inhibitory compounds produced by the lactobacilli and/or its adhesion to the intestinal epithelium prevented by co-aggregation to the lactobacilli. Another explanation could be the production by the lactobacilli of a compound interfering on the pro-inflammatory cellular signalling pathways stimulated by the enteropathogenic adhesion. As an example, supernatant of *S. boulardii* culture exhibits an anti-inflammatory effect suggesting that soluble factor produced by the yeast is implicated. A yeast supernatant fraction containing a small (1 kDa) heat-stable and water soluble anti-inflammatory molecule inhibited NF-κB activation by LPS, IL-1β and TNF-α (Sougioultzis et al. 2006).

Finally, an important aspect in the development of a probiotic product is the safety of its use. Histological aspect of the liver and ileum of mice only treated with *L. plantarum* 81 or *L. plantarum* 90 during 8 days was similar to that observed in control animals, and no clinical signals were noted in these mice. Additionally, results from antimicrobial susceptibility assays (data not shown) showed pattern common to lactobacilli from alimentary origin (Teles et al. 2016).

4.1 Conclusions

Concluding, among nine *Lactobacillus* strains isolated from the "cupuaçu" fermentation, *L. plantarum* 81 and *L. plantarum* 90 were selected as potential probiotics based on better results obtained in *in vitro* evaluations (resistance to simulated gastrointestinal environment, production of diffusible inhibitory compounds and co-aggregation) as well as *in vivo* experiments (intestinal colonization, *ex vivo* antagonism,

higher survival after enteropathogen challenge, lower hepatic translocation of enteropathogen, lower histopathological lesions in ileum and liver and anti-inflammatory pattern of immunological response). The development of a fermented beverage using *L. plantarum* 81 and *L. plantarum* 90 as starter is currently carried out in our laboratory, being evaluated the viability of the lactobacillus cells during storage and a sensory assay.

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Effects of Fibronectin Coating on Bacterial and Osteoblast Progenitor Cells Adherence in a Co-culture Assay

Mathilde Hindié, Dongni Wu, Karine Anselme, Olivier Gallet, and Patrick Di Martino

Abstract

Bacterial adherence to the surface of implants functionalized with cell-adhesive biomolecules is a critical first step of infection development. This study was designed to determine how the immobilization of human plasmatic fibronectin (pFN) could impact bacterial and osteoblast cells interaction with the surface during concomitant exposition to the two cell-types. Calibrated suspensions of *P. aeruginosa* PAOI or *S. aureus* CIP4.83 bacteria and STRO-1^{+}A osteoblast progenitor cells were mixed, co-seeded on glass coverslips coated or not with pFN and incubated at 37 °C. After 3 h of co-culture, the presence of bacteria did not modify the STRO-1^{+}A cells adherence to glass. pFN coating significantly enhanced STRO-1^{+}A cells, CIP4.83 and PAOI adherence to glass and bacterial interaction with STRO-1^{+}A cells. Confocal laser scanning microscopy observations revealed that cells on the pFN-coated substrate exhibited a greater spreading, better organized network of cytoskeletal filaments, and an increased cellular FN expression than cells on the uncoated substrate. The use of fluorescently labeled pFN showed that adherent STRO-1^{+}A cells were able to remodel and to concentrate coated pFN at the cells surface. Thus, the use of FN coating could increase the risk of bacterial adherence to the material surface, acting either directly onto the coating layer or indirectly on adherent osteoblastic cells. This may increase the infection risk in the presence of bacterial contamination.

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Keywords
Staphylococcus aureus • *Pseudomonas aeruginosa* • Osteoblast • Fibronectin • Adherence

Abbreviations

FN	Fibronectin
FnBPs	FN-binding proteins
CIP	Collection de l'Institut Pasteur
FCS	Foetal calf serum
GC	Glass coverslips

1 Introduction

Human cell-biomaterial interactions are decisive in the host response to implanted devices and integration of biomedical prostheses. The implant surface is critical for a good implant biocompatibility since it interacts directly with the host. Indeed, some bio-inspired biomaterial strategies based on presenting Fibronectin (FN) or short bioadhesive motifs derived from FN on implant surfaces have been developed (Bierbaum et al. 2003; Dean et al. 1995; Sousa et al. 2008; Stephansson et al. 2002).

Fibronectin (FN) is a large glycoprotein present in the human body as two forms, the soluble plasmatic FN (pFN) and the less-soluble cellular FN (cFN). pFN is synthesized predominantly in the liver by hepatocytes while cFN is secreted by a wide variety of cell types at the site of matrix assembly. pFN is present in blood plasma as a major component for wound healing and can also shift to fibrils upon binding to integrins at the cells surface (Stathakis et al. 1981). cFN is synthesized at the surface of cells into a fibrillar-type matrix and interacts with cells to control cell adhesion, cytoskeletal organization and cellular signaling (Pankov and Yamada 2002). cFN participates to cell migration, differentiation, cell-cell interactions and deposition of other matrix proteins. pFN and cFN are encoded by a unique gene, the corresponding pre-mRNA being differently spliced between pFN and cFN.

The objective of FN coatings is to stimulate cells in the early stage of implantation and consequently accelerate tissue formation around implant and subsequent rapid implant stabilization. FN used for biomaterial coatings is generally purified from human plasma and has been largely used *in vitro* for increasing the interactions of cells with material surfaces but also *in vivo* for improving integration in tissues (Bierbaum et al. 2003; Dean et al. 1995; Grigorescu et al. 2013; Sima et al. 2011; Sousa et al. 2008; Stephansson et al. 2002). More specifically in the field of bone biomaterials, coated FN has been proposed for enhancing osseointegration (Agarwal and García 2015; Jimbo et al. 2007; Petrie et al. 2009). Nevertheless, many pathogenic bacteria can use cFN at the cells surface or pFN adsorbed on a material surface as a receptor for adherence and subsequent infection (Henderson et al. 2011; Schwarz-Linek et al. 2004; Westerlund and Korhonen 1993). The initial bacterial adhesion to biomaterial surfaces is a critical step in the pathogenesis of foreign body infections and immobilized FN is a major receptor for pathogens adherence to biomaterials (Hudson et al. 1999; Montanaro et al. 2011). The inhibition of bacterial adhesion to the biomaterial surface is considered as the most critical step to prevent implant-associated infection. Moreover, tissue integration occurring before bacterial colonization is also important for an implant to be successful. A post-implantation period of 6 h has been defined to be decisive for implant success and the prevention of bacterial adhesion during this period is critical to the long-term success of an implant (Hetrick and Schoenfisch 2006; Neoh et al. 2012; Poelstra et al. 2002).

Staphylococcus aureus and *Pseudomonas aeruginosa* are two major etiologic agents of infections of prosthetic implants and in particular

infections associated with osteosynthesis materials (Arciola et al. 2005; Fernandes and Dias 2013; Tucaliuc et al. 2014). Most *S. aureus* and *P. aeruginosa* strains express FN-binding proteins (FnBPs) that specifically recognize human FN (Arhin and Boucher 2010; Hudson et al. 1999; Rebière-Huët et al. 1999). Staphylococcal FnBPs are large surface proteins (Jonsson et al. 1991), and *Pseudomonas* FnBPs are outer membrane proteins (de Lima Pimenta et al. 2003; Rebière-Huët et al. 1999; 2002). FnBPs mediate *S. aureus* adherence to and uptake by endothelial and epithelial cells through FN binding (Mongodin et al. 2002; Peacock et al. 1999). FN also acts as a receptor for *P. aeruginosa* adherence to human cells (Gagnière and Di Martino 2004; Rebière-Huët et al. 1999; Roger et al. 1999; Woods et al. 1981). Bacterial adhesion to biomaterials can be significantly influenced by the composition of the adsorbed proteins at the surface of the material. Adsorption of fibronectin promotes adhesion of *Staphylococcus* spp. and *P. aeruginosa* to abiotic materials through specific ligand–receptor interactions (Mohammad et al. 1988). Interestingly, *S. aureus* clinical strains responsible for orthopedic implant-associated infections adhere more efficiently to FN than control strains (Peacock et al. 2000). This increased adherence to FN is associated with an increased prevalence of FnBP.

In the case of an *in vivo* implant infection; bacterial contamination can take place at the moment of implantation. Human cells and contaminating bacteria may be able to simultaneously colonize implanted material. This situation conducts to a competition between human cells and bacteria for adhering to and colonizing the implant material. This competition has been previously qualified as a 'race to the surface' (Gristina 1987). In vitro co-culture models using both eukaryotic and prokaryotic cells are needed to study this phenomenon. Nevertheless, the co-culture of human cells and bacteria is a real challenge because of the highly different growth rates between the two cell types, and the toxicity of different bacterial products against eukaryotic cells. Indeed, only a few human-bacteria co-culture models have been described in the literature (Kim et al. 2010; Subbiahdoss et al. 2009; 2011). In the present study, we realized *in vitro* co-seeding of pathogenic bacteria (*P. aeruginosa* and *S. aureus*) and osteoblast progenitor cells and studied the cells and bacterial adherence to a glass template functionalized or not by human pFN. The goal was to determine the effects of pFN coating on the first hours of the 'race to the surface' between osteoblast progenitor cells and major bacterial pathogens by studying the human and bacterial cells behavior in a co-seeding situation.

2 Materials and Methods

2.1 Bacterial Strains and Cell Culture

The reference *Pseudomonas aeruginosa* PAO1 (CIP104116), and *Staphylococcus aureus* CIP4.83 strains have been purchased from the catalogue of strains, "Collection de l'Institut Pasteur (CIP)", Institut Pasteur CIP, Paris, France. Both strains have been previously shown to adhere efficiently to immobilized FN (Arhin and Boucher 2010; Maddocks et al. 2013). Bacteria were grown in TS broth for 24 h at 37 °C, subsequently plated on TS agar and incubated for 24 h at 37 °C before use in adherence or co-culture experiments.

Immortalized STRO-1^+A osteoblast progenitor cells (Oyajobi et al. 1999) were kindly provided by Dr. P. Marie (U606 Inserm, Université Paris Diderot, Paris, France). They were grown at 37 °C in IMDM medium (Sigma-Aldrich) containing 10 % (v/v) fetal calf serum (FCS), 2 mM Glutamax® (Invitrogen), and antibiotics (200 U of penicillin and 50 mg of streptomycin per liter, respectively), in 25 cm^2 tissue culture flasks (Costar). The culture was incubated in a humidified atmosphere of 5 % CO_2 in air until pre-confluence. The growth medium was renewed every two days.

2.2 FN Coating

FN coating of glass coverslips (GC) was done with either pFN or Alexa fluor 405® labeled pFN. Alexa fluor 405® labeled pFN was prepared from purified pFN as previously described (Hoffmann et al. 2008). pFN was purified from human blood plasma (under an agreement with EFS: French establishment for supplying blood products) by a three-step combination of affinity chromatography according to Poulouin et al. using FN affinity for both gelatin and heparin (Poulouin et al. 1999). Chromatography media with reagents of analytical grade were purchased from GE Healthcare. Purified FN was adjusted to 2.5 $mg.ml^{-1}$ at +10 °C, in the aim to prevent cold-insoluble aggregation. Concentration was determined by absorbance at 280 nm using 1.28 mg^{-1} cm^2 as the extinction coefficient (Mosesson and Umfleet 1970).

The wells of a P24 cell culture plate containing glass coverslips were coated by adding 500 µl per well of pFN diluted to 100 µg/ml in sterile PBS, pH 7.4. The plate was incubated overnight at 9 °C. Before co-culture, the wells were emptied and rinsed twice with 500 µl of sterile PBS buffer.

2.3 Co-Culture of Bacteria and Osteoblast Progenitor Cells

Confluent STRO-1^+A cells cultures were treated with trypsin/EDTA, harvested by centrifugation, and the cell pellet was re-suspended in IMDM or in IMDM containing 10 % FCS and 1 % Glutamax®. The cell suspension was adjusted to 1.1×10^5 cells/ml in IMDM or in IMDM containing 10 % FCS and 1 % Glutamax®. The bacteria were collected at the surface of the agar and suspended in IMDM or IMDM containing 10 % fetal calf serum and 1 % glutamine. The final cell density was adjusted to approximately 10^8 CFU/ml as determined by an optical density (OD) of 0.1 using a visible spectrophotometer (Varian, Inc., CA) at a wavelength of 600 nm. STRO-1^+A and bacterial cells were mixed and co-seeded at a concentration of 10^5 and 10^7 cells per well, respectively, in wells containing glass coverslips with or without coated FN and incubated for 3 h or 6 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air. At the end of incubation, three successive washings were carried out with sterile PBS, the cells were fixed in absolute ethanol, stained with 30 % Giemsa in distilled water (v/v), and examined microscopically under oil immersion as previously described (Di Martino et al. 2002). Three parameters were quantified: number of STRO-1^+A cells adhering to glass per field, number of bacteria interacting with STRO-1^+A cells per cell, number of bacteria adhering to glass per field. Each result represents the average of at least three independent separate experiments, three replicates for each experiment being conducted. The statistical significance of differences was evaluated with equal variance Student' s t -test, following variance test with Fisher F -statistics. P -values below 0.05 were considered significant.

2.4 Cells Staining and Visualization of Cellular Fibronectin

Glass coverslips (GC) were coated by pFN as described above. After growth to confluence, STRO-1^+A cells were detached from the culture flask by trypsinization, and centrifuged for 5 min at 200 g. The resulting pellet was re-suspended in IMDM containing 10 % FCS and 1 % Glutamax®, cells were seeded at a concentration of 50000 cells/cm^2 in wells containing either GC or FN coated GC, and incubated for 3 h at 37 °C. Cells were then fixed in a 3 % paraformaldehyde solution in PBS (Sigma-Aldrich) for 15 min. Non-specific binding sites were blocked by incubating the cells in PBS containing 10 % goat serum (Invitrogen) for 30 min at room temperature. After successive washings in PBS, the cells were incubated for 1 h with mouse monoclonal antibody against extra domain A of cellular FN (DH1 clone; Enzo scientific) dissolved in PBS containing 10 % goat serum at RT. After successive

washings in PBS, bound antibodies were revealed by incubation for 1 h at RT with goat anti mouse Alexa Fluor 488® linked antibody (Invitrogen) diluted in PBS containing 10 % goat serum. Nuclei were stained by 1 μg/ml 4′,6-Diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich), and the cytoskeleton was visualized by 0.02 $U.ml^{-1}$ Alexa-Fluor 532® phalloidin (Invitrogen), both added to secondary antibodies solution. Samples were then washed in PBS, and mounted in Prolong® gold (Invitrogen). Stained cells were examined using a Zeiss LSM 710 confocal light-scanning microscope. A series of random field images, each image corresponding to an area of 353.9 μm × 353.9 μm, were obtained from 3 independent experiments. About 800 cells were analysed for each culture condition. Cell outline, cell surface area and staining intensity were determined using the ImageJ® image analysis software as previously described (Hernandez-Garcia et al. 2010; Hindié et al. 2011). Data were analysed using the Instat3 statistical software (GraphPad Software). The statistical significance of differences was evaluated with Mann–Whitney Test. Experimental results are expressed as means ± standard deviation. *P*-values below 0.05 were considered significant.

To observe remodelling of coated FN by STRO-1^+A cells, GC were coated by Alexa fluor 405® labelled pFN and STRO-1^+A cells suspensions were prepared as described above. Cells were seeded at a concentration of 50,000 cells/cm^2 in wells containing Alexa fluor 405® labelled pFN coated GC, and incubated for 3 h at 37 °C. Cells were then fixed in a 3 % paraformaldehyde solution in PBS (Sigma-Aldrich) for 15 min and permeabilized in a 0.2 % Triton X100 solution in PBS (Sigma-Aldrich) for 5 min. Non-specific binding sites were blocked by incubating the cells in PBS containing 10 % goat serum (Invitrogen) for 30 min at RT. After successive washings in PBS, the cells were incubated for 1 h with rabbit polyclonal antibody against cytoplasmic domain of α5 integrins (AB1928; Millipore) dissolved in PBS containing 10 % goat serum at RT. After successive washings in PBS, bound antibodies were revealed by incubation for 1 h at RT with goat anti rabbit Alexa Fluor 568® linked antibody (Invitrogen) diluted in PBS containing 10 % goat serum. Cytoskeleton was visualized by 0.02 $U.ml^{-1}$ Alexa-Fluor 532® phalloidin in PBS (Invitrogen). Samples were then washed in PBS, and mounted in Prolong® gold (Invitrogen). Stained cells, fluorescent pFN and α5 integrins were examined using a Zeiss LSM 710 confocal laser-scanning microscope.

3 Results

3.1 STRO-1^+A Cells Adherence to Glass

Figure 1 shows the number of STRO-1^+A cells adhering to glass in the presence or absence of bacteria, in the presence or absence of FCS and with or without pFN coating. After an incubation time of 3 h (Fig. 1a), the presence of bacteria (either *P. aeruginosa* or *S. aureus*), did not modify the STRO-1^+A cells adherence to glass that is or not conditioned with pFN, in the presence or absence of FCS ($P > 0.05$). In the presence of FCS, the total number of adherent STRO-1^+A cells after 3 h was significantly increased by a factor 2 ($P < 0.001$). On the pre-conditioned glass surface, i.e. immersed in pFN solution prior to the cells adhesion test, the total number of adherent cells after 3 h was significantly increased by a factor 3 ($P < 0.001$). When the incubation time was increased from 3 h to 6 h (Fig. 1b), the STRO1^+A cells adherence to glass was increased in the absence of bacteria but the presence of either *P. aeruginosa* or *S. aureus* bacteria induced a decrease in STRO1^+A cells adherence.

3.2 Bacterial Adherence

3.2.1 Bacterial Adherence to Flass

Figure 2 shows the number of bacteria adhering to glass in the presence or absence of FCS and with or without pFN coating. The presence of FCS slightly but significantly decreased the *P. aeruginosa* adherence to uncoated glass ($P < 0.01$). In the same conditions, the total

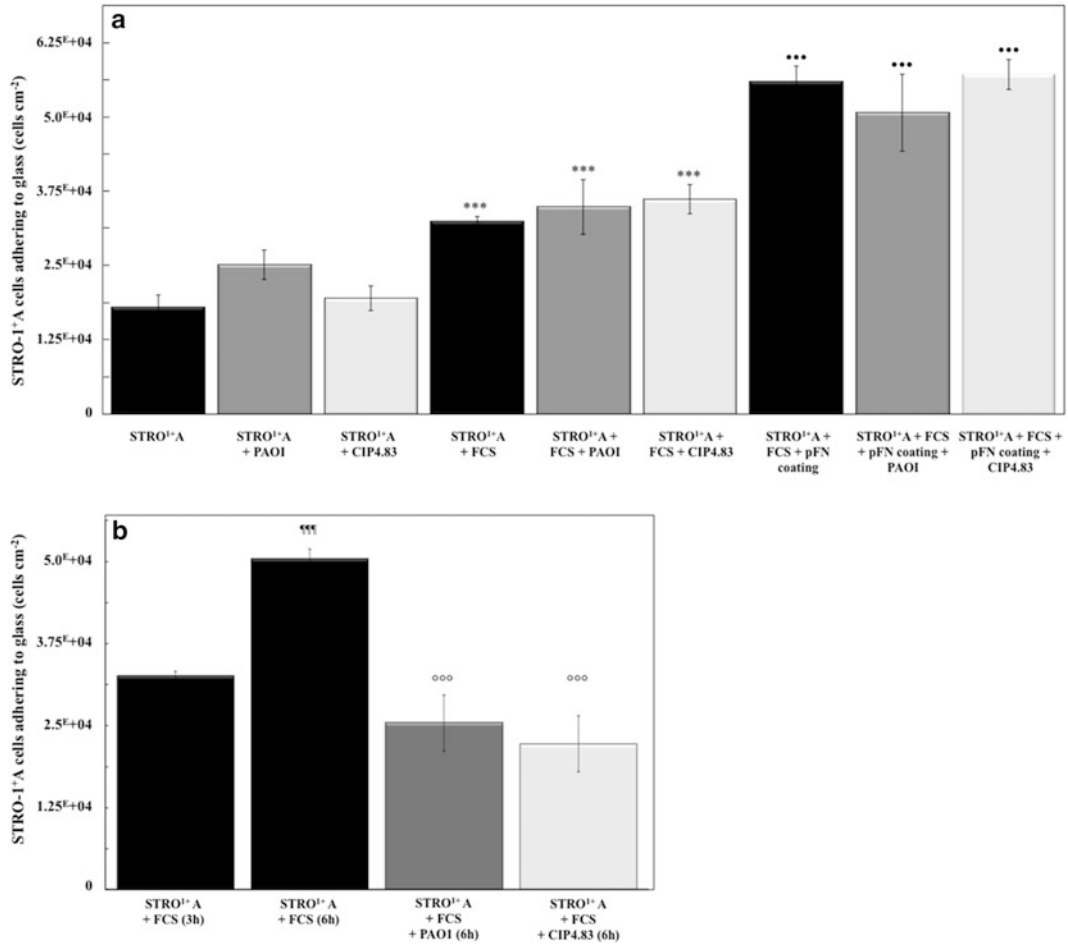

Fig. 1 Number of STRO-1$^+$A cells adhering to glass in the presence or absence of bacteria (*Pseudomonas aeruginosa* PAOI or *Staphylococcus aureus* CIP4.83 strain), in the presence or absence of Fetal Calf Serum (FCS) and with or without plasmatic fibronectin (pFN) coating. (**a**) incubation time of 3 h. (**b**) incubation time of 3 or 6 h as indicated between brackets. $^{***}P < 0.001$ with respect to adhesion of STRO1$^+$A cells to glass without FCS and pFN coating. $^{\bullet\bullet\bullet}P < 0.001$ with respect to adhesion of STRO1$^+$A cells to glass without pFN coating. $^{¶¶¶}P < 0.001$ with respect to adhesion of STRO1$^+$A cells to glass after 3 h without bacteria. $^{°°°}P < 0.001$ with respect to adhesion of STRO1$^+$A cells to glass after 6 h without bacteria

number of adherent *S. aureus* bacteria was significantly decreased by a factor of about 4 ($P < 0.001$). On the FN-coated glass surface, the total number of adherent bacteria was more than doubled for both strains ($P < 0.001$).

3.2.2 Bacterial Interaction with STRO-1$^+$A Cells

Both *P. aeruginosa* and *S. aureus* can adhere to and be internalized by human non-phagocytic cells. Bacteria adhering to and bacteria that entered inside STRO-1$^+$A cells can't be discriminated by the Giemsa-staining procedure used. Giemsa stains the bacteria on the surface or inside the cells in the same way. Thus, we decided to use the word "interaction" to qualify the totality of bacterial adherence to and invasion of STRO-1$^+$A cells. The bacterial interaction with cells was studied by co-seeding the bacteria and STRO-1$^+$A cells. A view of bacterial interaction with STRO-1$^+$A cells is shown in Fig. 3. The *P. aeruginosa* PAO1 bacteria were dispersed

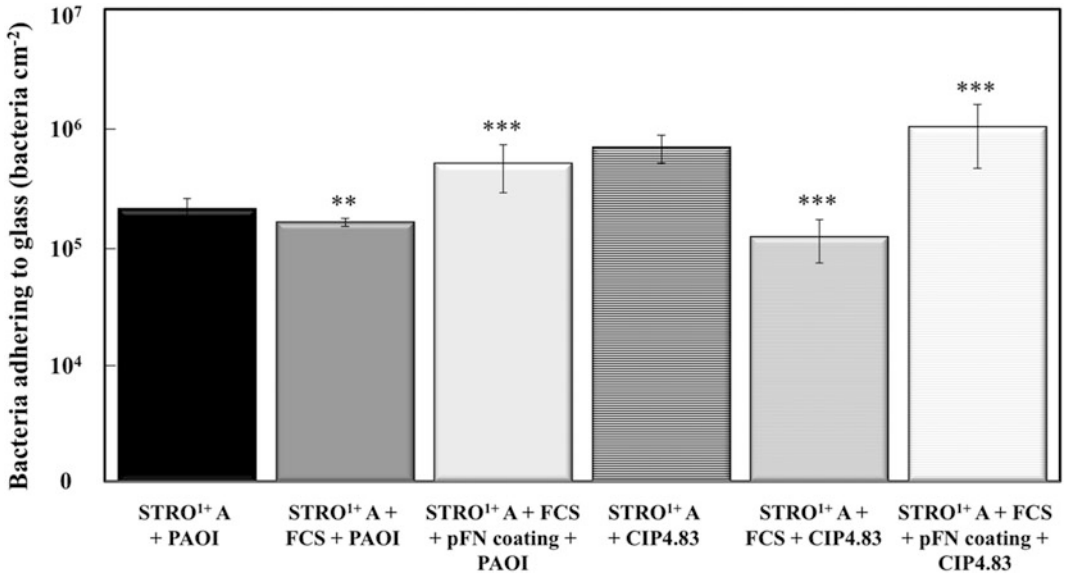

Fig. 2 Number of *Pseudomonas aeruginosa* PAOI and *Staphylococcus aureus* CIP4.83 bacteria adhering to glass in the presence or absence of Fetal Calf Serum (FCS) and with or without plasmatic fibronectin (pFN) coating. The incubation time was 3 h. $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to adhesion of corresponding bacteria to glass without FCS and pFN coating

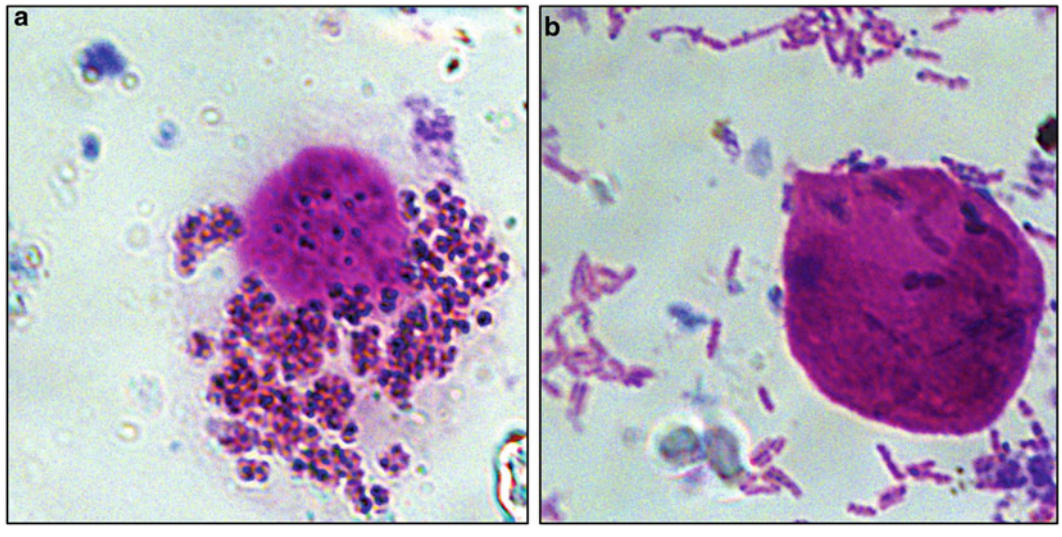

Fig. 3 Light micrographs of Giemsa-stained *P. aeruginosa* PAO1 (**a**) and *S. aureus* CIP4.83 (**b**) strains interacting with STRO-1^{+}A cells during co-culture experiments. Magnification 1000x

individually at the STRO-1^{+}A cell contact whereas *S. aureus* CIP 4.83 bacteria formed variably sized clusters interacting with STRO-1^{+}A cells. Figure 4 shows the number of bacteria interacting with co-seeded STRO-1^{+}A cells in the presence or absence of FCS and with or without pFN coating. The presence of FCS did not modify the *P. aeruginosa* interaction with STRO-1^{+}A cells but slightly decreased the *S. aureus* interaction with cells by approximately

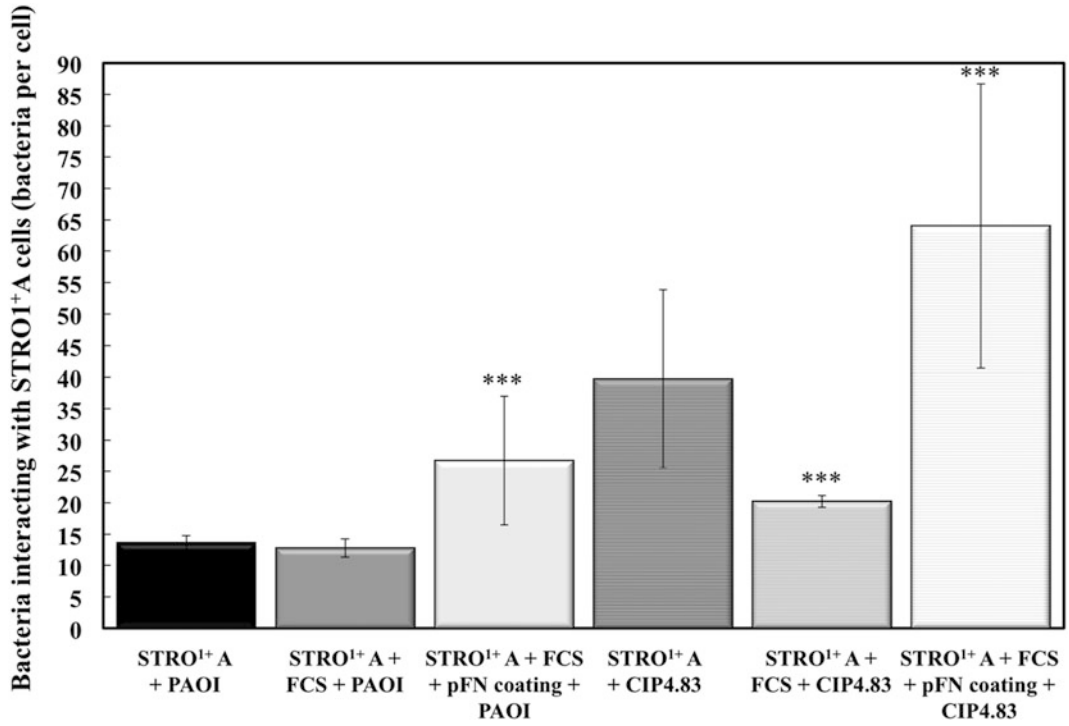

Fig. 4 Interaction indices of *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* CIP4.83 strains with STRO-1$^+$A cells during co-cultures in presence or absence of Fetal Calf Serum (FCS) and with or without plasmatic fibronectin (pFN) coating. The incubation time was 3 h. Each interaction index, representing the average number of bacteria per cell, was determined by examining 100 cells. $^{***}P < 0.001$ with respect to interaction of corresponding bacteria to STRO-1$^+$A cells without FCS and pFN coating

one third ($P < 0.001$). After FN coating, the interaction index to STRO-1$^+$A cells was doubled for *P. aeruginosa* ($P < 0.001$) and multiplied by 3 for *S. aureus* ($P < 0.001$).

3.3 STRO-1$^+$A Cells Spreading and Cellular FN Expression

The STRO-1$^+$A cells spreading and FN expression after adherence to glass were analysed by CLSM observations. Indirect immunofluorescence experiments were performed using monoclonal anti-cFN, and corresponding secondary antibodies linked to Alexa Fluor 488® (purple color), respectively. An example of random field images of CLSM observations is presented in Fig. 5. In the absence of pFN coating (Fig. 5a), adherent and spread STRO-1$^+$A cells were observed. Expression of cFN was detected for some of the cells. In the presence of pFN coating (Fig. 5b), the fibrillar network of the cytoskeleton appeared to be denser and more developed, suggesting an increase in cell spreading. Moreover, the purple signal corresponding to cFN seemed more intense. Cell surface area and cFN staining calculations with the ImageJ® image analysis software confirmed significant increases in the presence of pFN coating. A total of 823 STRO-1$^+$A cells cultured on pFN coating and 808 cells cultured on GC were analysed. Cell surface area was 408.2 ± 22 µm^2 and 606.3 ± 34 µm^2 in the absence and presence of pFN coating, respectively ($P < 0.001$). cFN-positive cells were also counted; 105 cells onto GC and 108 cells onto pFN coating. Then the mean cFn staining intensity was quantified. It was 6.1 ± 0.5 arbitrary fluorescent units (AFU)

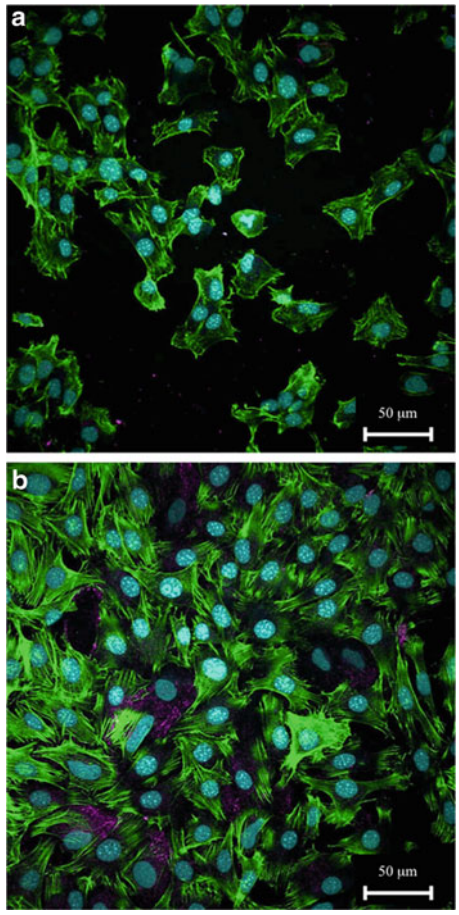

Fig. 5 STRO-1$^+$A cells spreading and cellular FN expression visualization by immunocytochemistry and CLSM observations. Cells were cultured for 3 h on uncoated glass (**a**) or on pFN-coated glass (**b**). FN, F-actin and nuclei were stained in *purple*, *green* and *blue*, respectively. Scale bar: 50 µm

per cell and 8.3 ± 0.5 AFU/cell in the absence and presence of pFN coating, respectively ($P < 0.001$).

Remodelling of coated pFN by STRO-1$^+$A cells after adherence to glass was analysed by CLSM observations. The cells and pFN were stained with Alexa-Fluor 532® phalloidin (green fluorescence) and Alexa fluor 405® (blue fluorescence), respectively. α5 integrins were detected by indirect immunofluorescence experiments using polyclonal anti-α5 integrins, and corresponding secondary antibodies linked to Alexa Fluor 568® (yellow color). Many adherent and spread STRO-1$^+$A cells were observed on the Alexa fluor 405® labeled pFN coated surface. At the cellular level, areas of high blue fluorescence were visible, indicating concentration of labeled pFN (Fig. 6a). These areas of pFN concentration also corresponded to areas of clustering of α5 integrins (Fig. 6b). No areas of concentrated labeled pFN were observed on the coated surface in the absence of STRO-1$^+$A cells (Fig. 6c). Apart from the pFN concentrated areas, the fluorescence of the coating is weakly observed on the photographs presented in Fig, 6 because of fluorescence intensity settings. However, the fluorescence of the coating is visible under direct confocal microscope observation or after changing the settings of acquisition of the fluorescent signal (Data not shown). On uncoated glass, no blue fluorescence corresponding to Alexa fluor 405® labeled pFN was observed but yellow fluorescence corresponding to α5 integrins expression by adherent STRO-1$^+$A cells was detected (Fig. 6d).

4 Discussion

FN coating of a biomaterial aims to stimulate cell adhesion in the early stage of implantation and consequently accelerate tissue formation around implant and subsequent rapid implant stabilization and/or osseointegration (Bierbaum et al. 2003; Dean et al. 1995; Jimbo et al. 2007; Sousa et al. 2008; Stephansson et al. 2002). When bacterial contamination takes place at the moment of implantation of the material, human cells and bacteria may be able to interact independently or together with the coated surface. It is known that FN coating can influence either human or bacterial cells adherence to the surface (Dean et al. 1995; Rochford et al. 2014). A simultaneous effect of FN coating on both eukaryotic and prokaryotic cell types and the influence of the two cell types upon one another are largely unknown. In the current investigation, the affinity of osteoblast progenitor cells and contaminating bacteria for an implant surface was investigated using a co-culture test where

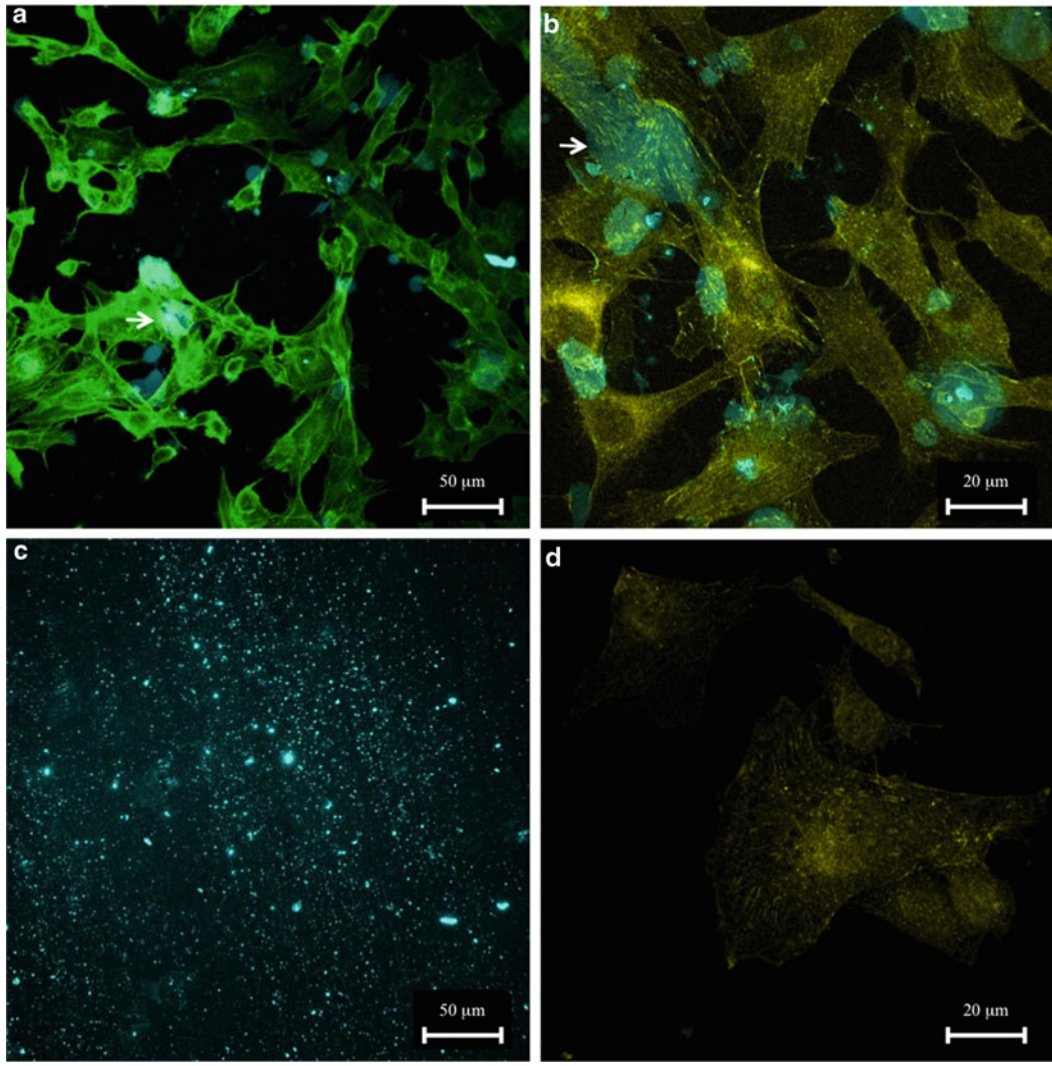

Fig. 6 CLSM observations of the remodelling of coated pFN and α5 integrins expression by STRO-1$^+$A cells after adherence to glass. The incubation time was 3 h. Cell morphology and α5 integrins visualization on pFN coated substrate (**a**, **b**, respectively); Alexa fluor® 450 labeled pFN control coating (**c**); α5 integrins visualization on uncoated glass (**d**). Alexa fluor coupled pFN is stained in *blue*, cytoskeleton is stained in *green*, and α5 integrins are stained in *yellow*, respectively. *Arrows* indicate examples of high *blue* fluorescence regions. Scale bar: 20 µm

eukaryotic and prokaryotic cells were simultaneously seeded on a glass surface functionalized or not by human pFN. This protocol was designed to provide a simple controlled method for assessing bacterial and osteoblast progenitor cells simultaneous adhesion to an implant surface.

FN coating strongly increased both STRO-1$^+$ A cells and bacterial adherence to glass. The increased adherence of *S. aureus* and *P. aeruginosa* to the material after pFN coating can be explained by the use of immobilized FN as a receptor for bacterial attachment (Henderson et al. 2011). The increased adherence of osteoblast progenitor cells to the material after pFN coating is consistent with the observation of α5 integrins expression by adherent cells. The STRO-1$^+$A cells adherence increase was not modified in the presence of co-seeded *P. aeruginosa* or *S. aureus* cells. This means

that, at the beginning of the infection, contaminated bacteria do not interfere with human cells adherence to the FN coated surface. An *in vitro* method has been previously described to investigate the competition between osteoblastic cells and contaminating bacteria for colonizing the surface of a material *in vitro* for incubation times varying from 18 to 48 h (Saldarriaga et al. 2011; Subbiahdoss et al. 2009; 2011). In this model, there is no co-seeding of eukaryotic and prokaryotic cells, but the surface of the material is first exposed to bacteria, and osteoblastic cells are subsequently added after planktonic bacteria removal. Using this co-culture model, the presence of a *Staphylococcus epidermidis* strain has been shown to induce a decrease in U2OS osteoblastic cells colonization of glass and poly(ethylene glycol)-based polymer coatings after 48 h (Saldarriaga et al. 2011; Subbiahdoss et al. 2009). In another study, *S. aureus* and *P. aeruginosa* strains have been shown to stimulate U2OS cell death within 18 h of simultaneous growth on a poly (methylmethacrylate) surface (Subbiahdoss et al. 2011). In addition to the infection duration, another major difference with the experiments described here is the successive addition of bacteria and cells on the surface whereas we realized co-seeding experiments, osteoblastic cells and bacteria being added together to the material. In the model of co-seeding, we also observed a decrease in STRO1$^+$A adherence to glass in the presence of either *P. aeruginosa* or *S. aureus* when we increased the incubation time from 3–6 h. This may be due to the induction of cells death by bacterial endotoxins like LPS or teichoic acid and by the numerous virulence factors secreted by *P. aeruginosa* and *S. aureus* such as exotoxins (Nau and Eiffert 2005; Subbiahdoss et al. 2009).

In the present experiments, pFN coating not only induced an increase in adherence of osteoblastic cells to the material but also resulted in increased STRO-1$^+$A cells spreading and cFN expression at the cell surface. Increased adhesion and cell spreading are known to be associated with increased cell survival and cell cycling (Chen et al. 1997; Frisch et al. 1996), and cell adhesion to FN is important to osteoblast function (Stephansson et al. 2002). The coating of FN or RGD containing peptides is known to enhance osteoblast adhesion to the surface and to increase the mRNA levels of many genes including important markers in osteoblast differentiation but not mRNA of FN gene (Rapuano et al. 2012; Yamamichi et al. 2008). Concerning increased cFN expression, immunocytochemistry experiments were performed in nonpermeabilized STRO-1$^+$A cells to target extracellular molecules. Thus, the cFN increased expression after pFN coating may be the result of increased extracellular FN assembly. The use of fluorescently labelled pFN confirmed that STRO-1$^+$A cells remodel exogenous coated pFN. This pFN remodelling was associated with α5 integrins clustering at the cell membrane level in areas of high FN concentration. Osteoblastic cells interact with FN through α5β1 integrin mobilization and this α5β1 integrin mediated interaction is critical for osteoblast differentiation and bone morphogenesis (Moursi et al. 1997). Recent data indicate that coated fibronectin presents specific active conformations in response to different pre-coated surface functional groups. Those conformations of FN enhance osteoblast adhesion largely via α5β1 integrin mobilization and in some cases via αvβ3 integrin cell expression depending on the nature of surrounding functional groups. This integrin specificity modulates osteoblast differentiation and can affect osseointegration (Agarwal and García 2015; Stephansson et al. 2002). Whether any indicators of osseointegration such as mineralization or relevant gene expression changes are affected by the pFN coating with or without bacteria remains to be determined.

pFN coating not only influenced the osteoblastic cells behavior but also had a significant impact on the bacterial interaction with osteoblastic cells *in vitro*. Indeed, pFN coating resulted in increased *P. aeruginosa* and *S. aureus* interaction with STRO-1$^+$A cells. This increased interaction may be at least in part the result of the increase in cFN expression by human cells. *S. aureus* clinical strains are

known to adhere efficiently and invade human osteoblasts in primary cultures (Ahmed et al. 2001; Kalinka et al. 2014; Sinha et al. 1999). *S. aureus* binds to fibronectin via surface exposed FnBPs, and fibronectin serves as a bridging molecule to the integrin α5β1 which acts as a "phagocytic" receptor (Sinha et al. 1999). *P. aeruginosa* LPS is known to induce robust osteolysis *in vivo* and to stimulate osteoclastogenesis *in vitro* in bone marrow monocytes-osteoblast co-cultures (Zhuang et al. 2007). *P. aeruginosa* adheres to immobilized FN thanks to outer membrane proteins and can target cFN and its natural ligand α5β1 integrins for adherence to human pneumocyte cells (Gagnière and Di Martino 2004; Rebière-Huët et al. 1999) but interaction of *P. aeruginosa* with osteoblasts was unknown until now. Our present study demonstrates that, like *S. aureus*, *P. aeruginosa* can efficiently interact with osteoblast progenitor cells. This interaction of pathogenic bacteria with osteoblasts is set up very early, as soon as the osteoblast cells adhere to the material.

All the experiments done in our study used glass as a convenient and reference test material. Other materials such as titanium and its alloys have been extensively used to support coatings designed to enhance osseointegration (Feller et al. 2015). The co-seeding model described in our study may be a useful and easy to implement tool to develop and test new implant coatings that may promote osteoblast colonization and functions but also concomitantly limit bacterial colonization.

In conclusion, a co-culture of pathogenic bacteria and osteoblast progenitor cells, based on co-seeding experiments, was used to study the effects of pFN coating on the concomitant short-term colonization of a material by the two cell-types. In this model, pFN coating has a great and diverse impact on the osteoblastic cells behavior: increase of cell spreading, increase of cells adherence to the surface, increase of cFN expression. pFN coating increases the target number for bacterial adherence to the material surface i.e., coated pFN molecules directly on the material and cFN expressed by osteoblastic cells adhering to the material. Experiments with a longer incubation time requiring a renewal of the culture medium should be conducted to determine if fibronectin coating helps bacteria to win the 'race to the surface' of the implant and to determine the conditions of this aid. In such a situation, osteoblastic cells would not be able to counter the bacterial colonizers, and biofilm formation may occur, leading to infection and implant failure.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Improving the Bacterial Recovery by Using Dithiothreitol with Aerobic and Anaerobic Broth in Biofilm-Related Prosthetic and Joint Infections

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Abstract

Biofilm-related infections are serious complications in the orthopaedic prosthetic field and an accurate, quick microbiological diagnosis is required to set up a specific antimicrobial therapy. It is well known that the diagnosis of these infections remains difficult due to the bacterial embedding within the biofilm matrix on the implant surfaces. Recently, the use of DL-dithiothreitol (DTT) has been proved effective in biofilm detachment from orthopaedic devices.

The purpose of the study is to evaluate the efficacy of two DTT solutions enriched with specific broths for aerobic or anaerobic bacteria to dislodge pathogens from the biofilm, while supporting the bacterial recovery and viability. To do this, different experimental solutions were tested for efficacy and stability on strong biofilm producers: *S. aureus* and *P. acnes*. Mainly, we evaluate the capability of DTT dissolved in saline solution, brain heart infusion or thioglycollate broth to support the bacterial detachment from prosthetic materials and bacterial growth at different time points and storage conditions.

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We demonstrated that the use of DTT enriched with specific bacterial broths could be a suitable approach to optimize the bacterial detachment, recovery, growth and viability in the diagnosis of biofilm-related infections developed on orthopaedic prosthetic devices.

Keywords
DL-dithiothreitol • Biofilm • S. aureus • P. acnes • Prosthetic infections

Abbreviations

DTT	DL-dithiothreitol
BHI	brain heart infusion
TH	thioglycollate
SS	saline solution
RT	room temperature
TSA	Trypticase Soy Agar
SCH	Schaedler Blood Agar

1 Introduction

Biofilm-related infections are among the most challenging complications in patients receiving prosthesis, implantable medical devices and long-term catheterization (Matthews et al. 2009; Veerachamy et al. 2014; Ryder 2005). Orthopaedic biofilm-related infections could lead to severe complications such as prolonged hospitalization, antimicrobial treatments or revision surgeries bearing on the National Health Care System and affecting patients' lifestyle. Thus, this requires a fast and accurate microbiological diagnosis in order to identify the proper approach and therapy.

It is well known that aerobic bacteria are the most involved microorganisms in biofilm formation on prosthetic implants (Arciola et al. 2015) and, despite poorly investigated, also anaerobic bacteria could induce the biofilm formation on metal implants used in orthopaedics (Portillo et al. 2013; Zimmerli and Moser 2012). The microbiological diagnosis of these infections remains difficult because the bacteria are strictly embedded within the biofilm and they are undetectable with conventional culture (Kobayashi et al. 2009). Several techniques have been developed to detach adherent bacteria from the biofilm of explanted prosthetic or medical devices, including mechanical and chemical treatments (Weber and Legge 2010; Vassena et al. 2014; Kashef et al. 2015). Nowadays, sonication still represents the golden standard to detach and isolate bacteria involved in biofilm-related infections. However, this procedure retains some drawbacks in terms of laboratory equipment, risk of cross-contamination and handling of large implants (Drago et al. 2012). Recently, the use of chemical substances to dislodge the biofilm matrix has been proposed as a valid alternative to overcome these limitations maintaining a high sensitivity and specificity. DL-dithiothreitol (DTT) is a sulfhydryl compound that acts both as a reagent reducing disulfide bonds and as a protein denaturant on staphylococcal biofilm (Wu et al. 2011). Thanks to these properties, DTT is routinely used in clinical microbiology to liquefy respiratory specimens, but it has also been proven effective to detach biofilm from orthopaedic prosthesis (Drago et al. 2012, 2013). Since it is crucial to discriminate implant-related infections from aseptic loosening in orthopaedics, and because of the difficulty to diagnose subclinical infections, a prompt diagnosis of indwelling device-related infection is important for successful treatments (Borens et al. 2013). A fast microbiological diagnosis and bacterial identification should be recommended in order to set up a specific antimicrobial therapy. According to this strategy, the bacterial detachment from biofilm on explants should be accelerated by supporting the pathogen viability.

In the present study, we propose to dissolve DTT in broths specific for aerobic or anaerobic bacteria, instead of sterile saline solution in order to dislodge pathogens from the biofilm, while supporting the bacterial recovery and viability. This innovative approach aims also to improve microbiological culture conditions and to reduce false negative results related to inappropriate sample storage in terms of temperature and time of preservation.

2 Methods

2.1 Bacterial Strains

One strain of *Staphylococcus aureus* and one of *Propionibacterium acnes,* both isolated from patients with prosthetic joint infections, were used in this study. Strains were characterized by the strong biofilm production determined according to a spectrophotometric assay as described elsewhere (Drago et al. 2013; Christensen et al. 1985). Briefly, after development for 72 h, biofilm was washed two times with sterile saline and air-dried. Dried biofilms were stained by adding a 5 % crystal violet solution for 10 min. After staining, biofilms were washed with sterile saline to remove excess dye and left to air-dry completely. Finally, 96 % ethanol was added to solubilize the dye included into the biofilm. The optical density was measured at 595 nm using a microplate photometer. Measurements were carried out in triplicate for each strain. Both strains were classified as strong producers ($4 \times ODc < ODs$) by comparing the optical density of samples (ODs) with the absorbance of non-inoculated wells a negative control (ODc) (Stepanović et al. 2000).

2.2 Efficacy Test

The microbial efficacy test was performed to evaluate the DTT capability in supporting the bacterial growth and viability when dissolved in sterile saline solution or in enrichment broths for aerobic and anaerobic bacteria.

In order to perform this test, 0.1 % w/v DTT (6.5 mM, Sigma Aldrich, Italy) was dissolved in brain heart infusion broth (BHI, BioMériux, France) (DTT + BHI) or thioglycollate broth (TH, Thermo Fisher Scientific, Italy) (DTT + TH) and compared to the control consisting in DTT dissolved in sterile saline solution (DTT + SS). We inoculated *S. aureus* and *P. acnes* directly in each of the aforementioned solutions at a load of about 1×10^3 and 1×10^7 CFU/ml (T0). Then, aliquots from each suspension were stored at room temperature (RT) or at 4 °C to assess the influence of different storage temperatures. At different time points (3, 6, 24 and 48 h), 10 µl of each suspension were seeded after proper tenfold dilution on Trypticase Soy Agar (TSA) or Schaedler Blood Agar (SCH) plates (BioMériux, France). TSA was incubated in aerobiosis at 37 °C for 24 h, while SCH was incubated in anaerobiosis at 37 °C for 48 h. The anaerobic atmosphere was created in jars by anaerobic gas generating sachets (Oxoid™ AnaeroGen™ 2.5 L, Thermo Scientific). After incubation, bacterial colonies were counted for each sample, compared to the initial inoculum (T0) and reported as Log (CFU/ml). The experiment was repeated in triplicate.

2.3 Stability Test

The stability test was performed to investigate the efficacy of the experimental solutions (DTT + SS, DTT + BHI and DTT + TH) to disrupt the biofilm formed by *S. aureus* or *P. acnes* on sandblasted titanium discs resembling an infected prosthetic sample. We also verified the stability of the experimental solutions in a time- and temperature-dependent storage conditions.

Briefly, 50 ml aliquots of 0.1 % w/v DTT in BHI or TH and in sterile saline solution were prepared as previously described, stored at RT or at 4 °C in darkness, and tested at day 0, 3, 7, and 15 to assess the stability of the DTT in different conditions.

To obtain bacterial biofilm, sterile sandblasted titanium discs (Ø 25 mm; thickness 5 mm) (Adler Ortho, Italy; batch J04051) were incubated in six-well polystyrene plates (CELLSTAR® Multiwell Plates, VWR International, Italy) containing 5 ml of fresh BHI or TH broth and

Table 1 Experimental design

Experimental solutions	Storage temperature	Time points (days)
DTT + SS	4 °C	0, 3, 7, 15
DTT + BHI		
DTT + TH		
DTT + SS	RT	
DTT + BHI		
DTT + TH		

inoculated with *S. aureus* or *P. acnes* at a final density of approximately 1×10^3 CFU/ml, as reported in another study (Drago et al. 2012).

After incubation at 37 °C overnight, the exhausted growth media containing non-adherent bacteria were removed and replaced with 5 ml of fresh broths. After 48 h, titanium discs were washed three times with sterile saline to remove non-adherent bacteria. The titanium discs covered with visible mature biofilm were transferred in a new six-well plate and treated with 8 ml of the experimental solutions as reported in Table 1. The experiment was performed in triplicate for each condition and at different time points (0, 3, 7, 15 days).

Then, after 15 min under agitation at RT (Drago et al. 2013), 10 µl of each suspension were seeded on TSA or SCH plates and incubated at 37 °C for 24 and 48 h, respectively. After incubation, bacterial colonies were counted for each sample and reported as Log CFU/ml.

2.4 Statistical Analysis

The normal distribution of data was assessed with the Shapiro-Wilk test. Comparisons among tested solutions and time points were analysed with two way analysis of variance (ANOVA) (Graph Pad Prism v5.00 Software, USA) coupled with Bonferroni's post-hoc test. Values of $p < 0.05$ were considered statistically significant.

3 Results

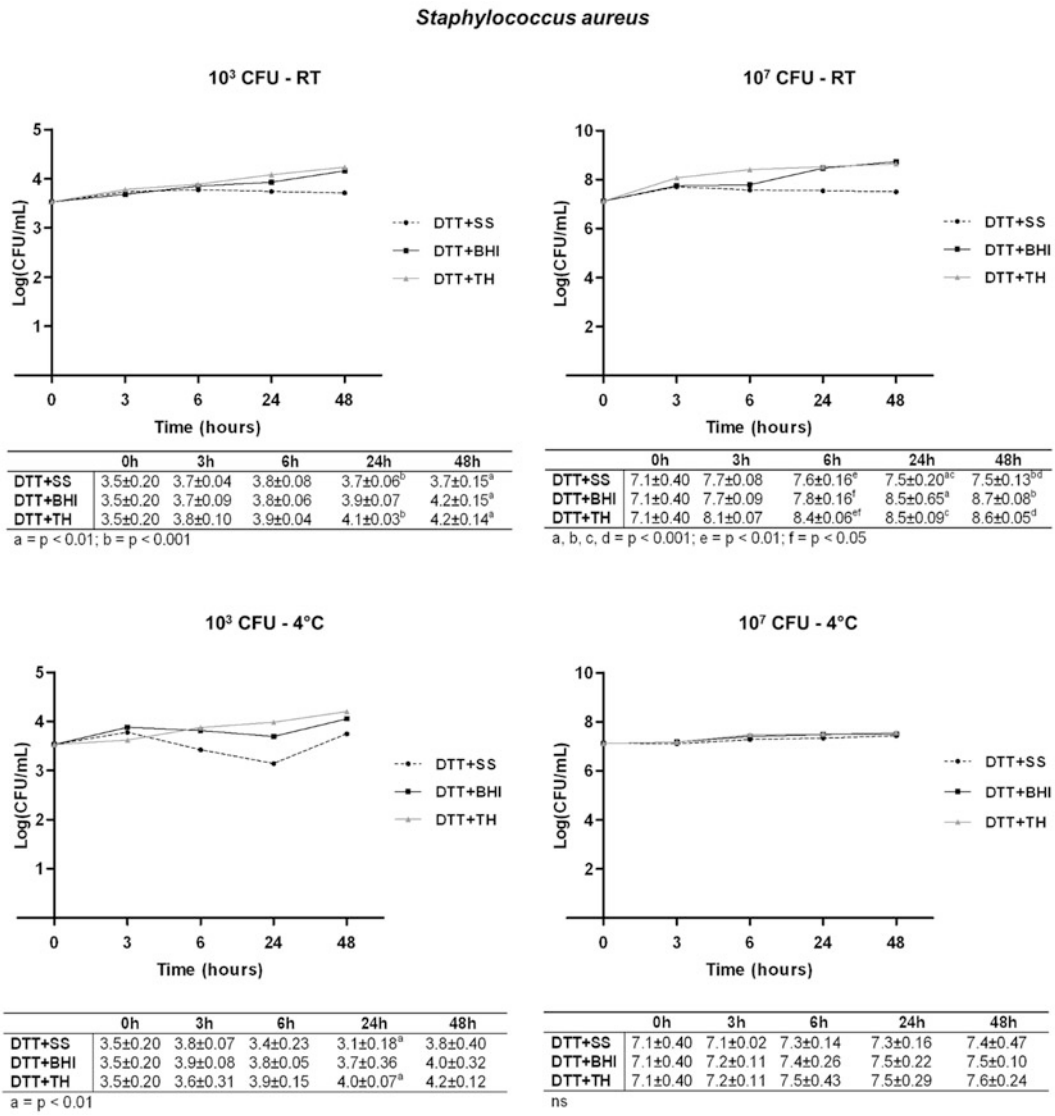
3.1 Efficacy Test

We determined whether exposing *S. aureus* and *P. acnes* to different experimental solutions would influence the bacterial growth capability. The DTT + SS supported a good *S. aureus* growth and viability until 3 h of culture, at low and high loads at both RT and 4 °C. Similarly, the DTT + BHI and DTT + TH promoted the bacterial recovery, significantly maintaining a long-term bacterial growth. In particular, after 24 h of culture, we appreciated a significant decrease of bacterial number when cultured in DTT + SS compared to the other experimental solutions both at RT ($p < 0.001$) and 4 °C ($p < 0.01$), though no differences were observed in respect to the initial inoculum. No significant differences were detected at high *S. aureus* loads at 4 °C at any time point. More importantly, after 6 h of culture in DTT + TH at RT, a greater bacterial growth was found in high load *S. aureus* inocula compared to DTT + BHI ($p < 0.05$) and DTT + SS ($p < 0.01$) (Fig. 1).

Comparable results in terms of the bacterial growth over time were found in *P. acnes* cultured in DTT + TH at low and high loads both at RT or 4 °C when compared to DTT + SS ($p < 0.001$), suggesting that DTT + TH significantly fostered the pathogen growth. In particular, since early time points, the DTT + SS weighed negatively on *P. acnes* growth when cultured at RT ($p < 0.001$) (Fig. 2).

3.2 Stability Test

By means of the stability test, we investigated whether the storage of the experimental solutions at RT and 4 °C for long periods could affect their efficacy in terms of biofilm detachment. Overall, no visible alterations of colour and turbidity of the experimental solutions were recorded during the storage period. The *S. aureus* biofilm detachment



	0h	3h	6h	24h	48h
DTT+SS	3.5±0.20	3.7±0.04	3.8±0.08	3.7±0.06[b]	3.7±0.15[a]
DTT+BHI	3.5±0.20	3.7±0.09	3.8±0.06	3.9±0.07	4.2±0.15[a]
DTT+TH	3.5±0.20	3.8±0.10	3.9±0.04	4.1±0.03[b]	4.2±0.14[a]

a = p < 0.01; b = p < 0.001

	0h	3h	6h	24h	48h
DTT+SS	7.1±0.40	7.7±0.08	7.6±0.16[e]	7.5±0.20[ac]	7.5±0.13[bd]
DTT+BHI	7.1±0.40	7.7±0.09	7.8±0.16[f]	8.5±0.65[a]	8.7±0.08[b]
DTT+TH	7.1±0.40	8.1±0.07	8.4±0.06[ef]	8.5±0.09[c]	8.6±0.05[d]

a, b, c, d = p < 0.001; e = p < 0.01; f = p < 0.05

	0h	3h	6h	24h	48h
DTT+SS	3.5±0.20	3.8±0.07	3.4±0.23	3.1±0.18[a]	3.8±0.40
DTT+BHI	3.5±0.20	3.9±0.08	3.8±0.05	3.7±0.36	4.0±0.32
DTT+TH	3.5±0.20	3.6±0.31	3.9±0.15	4.0±0.07[a]	4.2±0.12

a = p < 0.01

	0h	3h	6h	24h	48h
DTT+SS	7.1±0.40	7.1±0.02	7.3±0.14	7.3±0.16	7.4±0.47
DTT+BHI	7.1±0.40	7.2±0.11	7.4±0.26	7.5±0.22	7.5±0.10
DTT+TH	7.1±0.40	7.2±0.11	7.5±0.43	7.5±0.29	7.6±0.24

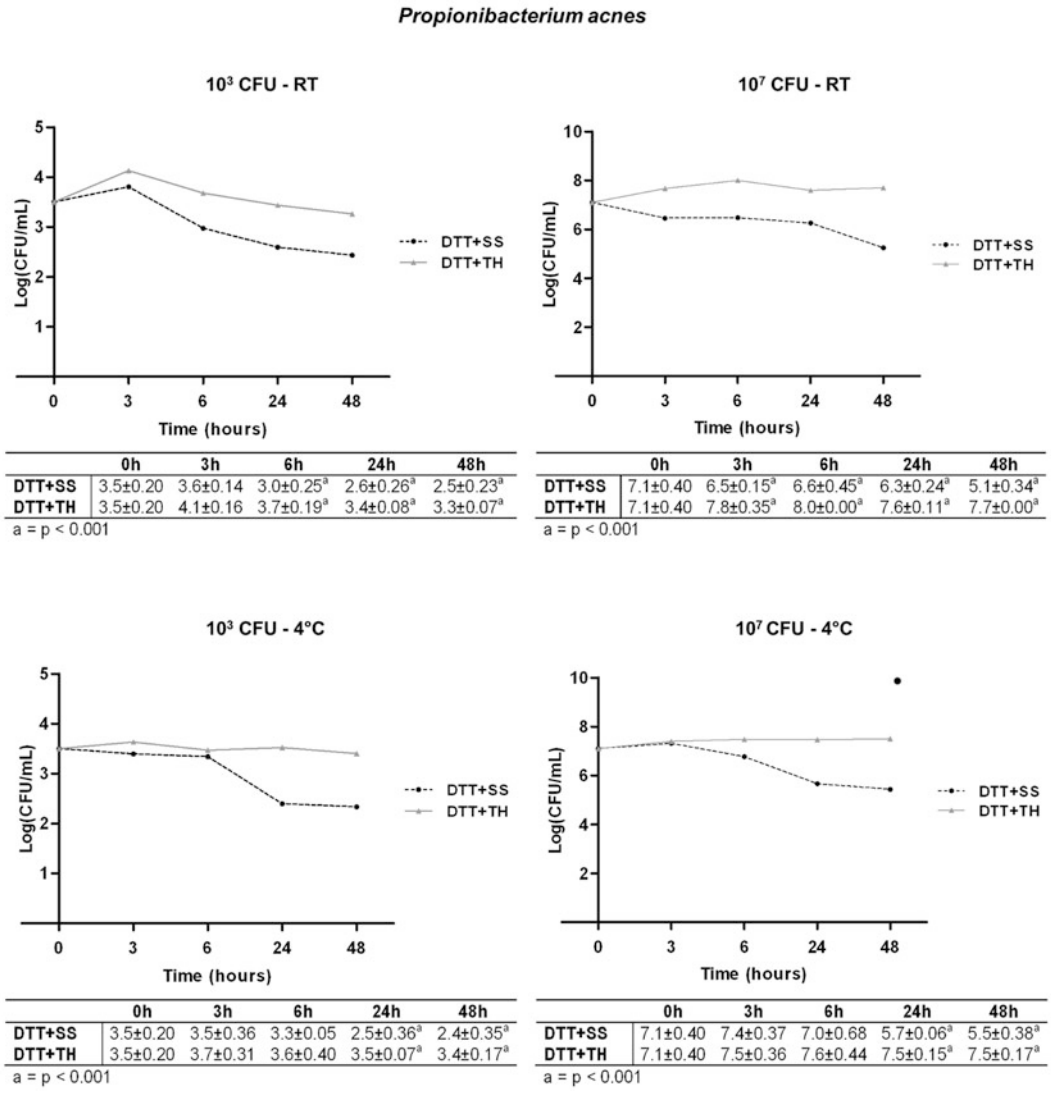
ns

Fig. 1 Efficacy test of *Staphylococcus aureus*. Bacterial count at low (10^3 CFU/ml) and high (10^7 CFU/ml) load of *S. aureus* at RT and 4 °C analysed at different time points (Data are reported as Log(CFU/ml) and considered statistically significant for $p < 0.05$)

capability of the experimental solutions was not affected until 15 days either at RT or at 4 °C, as shown in Fig. 3, in which no significant differences are detected. Similar data were found for the *P. acnes* biofilm detachment at RT and 4 °C until 15 days, only a significant difference was present in the DTT + SS efficacy after 7 days of storage at RT ($p < 0.05$).

4 Discussion

Due to the ageing population, orthopaedic prosthetic surgery is constantly increasing. Implant-related infections are the most devastating complication of joint replacement surgery, specifically when performed in patients with comorbidities,



	0h	3h	6h	24h	48h
DTT+SS	3.5±0.20	3.6±0.14	3.0±0.25[a]	2.6±0.26[a]	2.5±0.23[a]
DTT+TH	3.5±0.20	4.1±0.16	3.7±0.19[a]	3.4±0.08[a]	3.3±0.07[a]

a = p < 0.001

	0h	3h	6h	24h	48h
DTT+SS	7.1±0.40	6.5±0.15[a]	6.6±0.45[a]	6.3±0.24[a]	5.1±0.34[a]
DTT+TH	7.1±0.40	7.8±0.35[a]	8.0±0.00[a]	7.6±0.11[a]	7.7±0.00[a]

a = p < 0.001

	0h	3h	6h	24h	48h
DTT+SS	3.5±0.20	3.5±0.36	3.3±0.05	2.5±0.36[a]	2.4±0.35[a]
DTT+TH	3.5±0.20	3.7±0.31	3.6±0.40	3.5±0.07[a]	3.4±0.17[a]

a = p < 0.001

	0h	3h	6h	24h	48h
DTT+SS	7.1±0.40	7.4±0.37	7.0±0.68	5.7±0.06[a]	5.5±0.38[a]
DTT+TH	7.1±0.40	7.5±0.36	7.6±0.44	7.5±0.15[a]	7.5±0.17[a]

a = p < 0.001

Fig. 2 Efficacy test of *Propionibacterium acnes*. Bacterial count at low (10^3 CFU/ml) and high (10^7 CFU/ml) load of *P. acnes* at RT and 4 °C analysed at different time points (Data are reported as Log(CFU/ml) and considered statistically significant for $p < 0.05$)

such as obesity, diabetes and immunosuppression (Dryden 2014).

The therapeutic success rate of prosthetic infections can be tremendously improved with an early diagnosis (Saeed 2014). Conventional microbiological culture still represents the approved method to detect and identify bacteria, despite the sensitivity ratio can be lower than 20 % due to the inability to detect bacteria embedded in biofilm (Gbejuade et al. 2015). In the past decades, the sonication of prosthetic devices has improved the diagnosis of biofilm-related infections, presenting a higher sensitivity in comparison with conventional culture techniques (Esteban et al. 2008), but it is associated with several drawbacks.

Recently, the use of DTT has been proposed as a valid technique in place of sonication, thanks to its higher positive and negative predictive values and rapidity to process samples (Drago et al. 2013).

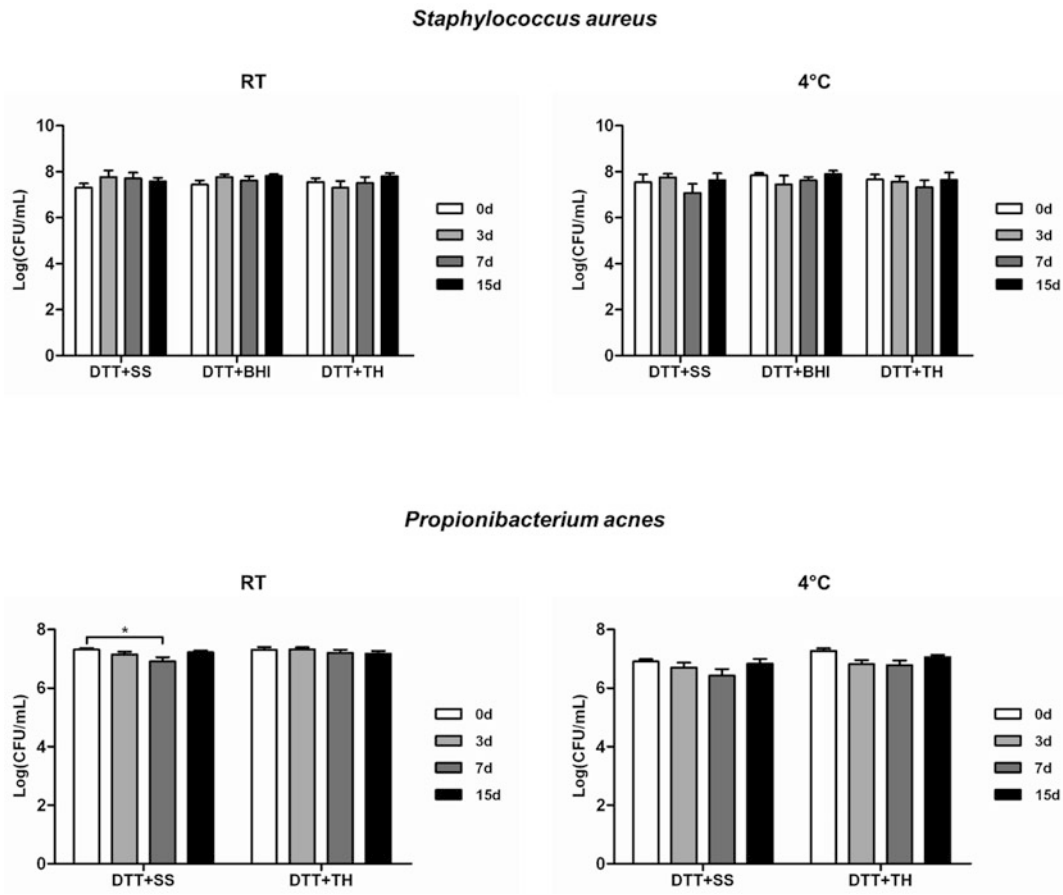


Fig. 3 Stability test data. The histograms report the capability of the experimental solutions to detach *Staphylococcus aureus* and *s* embedded in the biofilm from day 0 to day 15 at RT and 4 °C (Data are reported as Log (CFU/ml) and considered statistically significant for $p < 0.05$)

In 2013, this method has been finally approved and published in the guidelines of the Italian Association of Clinical Microbiologists (AMCLI) (http://www.amcli.it/documenti/percorsi/percorsi-2013/). In our previous studies, we demonstrated the high sensitivity of the DTT when dissolved in sterile saline solution to detach bacteria from prosthetic biofilm after 15 min of processing (Drago et al. 2012, 2013).

In the current study, we want to assess the efficacy of DTT dissolved in specific bacterial broths (BHI and TH) to detach bacteria from the biofilm in order to obtain a higher and faster bacterial growth compared to DTT + SS.

Here, we evaluated two common species related to prosthetic infections such as *S. aureus* (Saadatian-Elahi et al. 2008) and *P. acnes* (Shah et al. 2015), as representative aerobic and anaerobic bacteria able to form the biofilm, respectively.

In order to standardize our results, we performed this study on an *in vitro* model of bacterial biofilm that it is not affected by the potential antibiotic treatment of commonly retrieved clinical specimens. Moreover, thanks to this approach, we were able to test a low bacterial load (1×10^3 CFU/ml) to evaluate the viability and growth capability of pathogens, resembling the most common scenario in microbiological laboratories, in which the diagnosis of low-grade prosthetic infections is often complex.

According to our results, the use of DTT dissolved in specific broths can detach pathogens from the biofilm and can ameliorate the bacterial recovery, particularly referred to long-term storage before microbiological analysis. It is well-known that exists an extreme need to shorten the time for microbiological diagnosis, thus the use of DTT enriched with either BHI or TH could be an interesting approach to start the biofilm detachment from explanted prostheses directly in the operating room before reaching the microbiological laboratory maintaining bacterial viability. Since diagnosis is essential for effective infection control, prolonged periods of storage and delay in the transport of specimens to the laboratory might affect the bacterial recovery and viability. Thus, the main advantage of our approach in using DTT + BHI or TH is to permit an efficacious retrieval of viable bacteria embedded in biofilm until 48 h of storage at both RT and 4 °C.

Overall, data obtained from the stability test of the experimental solutions at RT or at 4 °C demonstrated high efficacy in *S. aureus* and *P. acnes* biofilm detachment until 15 days. This result suggests that it is possible to storage DTT + BHI or TH without decreasing any efficacy of the experimental solutions.

Based on our findings, we asked which is the proper bacterial broth combined with DTT to be used for the diagnosis of orthopaedic implant-related infections. As well recorded in literature, the most frequently involved pathogens in prosthetic joint infections are aerobic and facultative anaerobic, like *S. aureus* (Tande and Patel 2014). Hence, our data suggest the use of DTT + TH as a suitable solution to dislodge the bacteria from biofilm in periprosthetic tissues and orthopaedic devices more than DTT + SS and DTT + BHI.

Despite the numerous advantages in testing *in vitro,* the efficacy of DTT in association with culture broths, in the next future further investigations need to be performed on explanted orthopaedic devices in order to confirm our data in a clinical scenario. Testing the experimental solutions only on titanium discs represents the main limitation of this study. In fact, additional analyses should be carried out on different implant devices, such as various metals, polyethylene and bone cements which are currently used in orthopaedic surgery.

5 Conclusions

This study demonstrated that the use of DTT enriched with specific bacterial broths could be a suitable approach to optimize the bacterial recovery, growth and viability in the retrieval and diagnoses of biofilm-related infections developed on orthopaedic prosthetic devices.

Competing Interests The authors declare that they have not any competing interests.

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High Prevalence of Human Herpesvirus 8 Infection in Diabetes Type 2 Patients and Detection of a New Virus Subtype

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Abstract

The prevalence of Human Herpesvirus 8 (HHV8) DNA and antiviral antibodies in Diabetes type 2 (DM2) and control subjects was studied, in order to confirm a possible link between DM2 and HHV8 infection. The HHV8-DNA from diabetic patients was typed for detecting possible genomic differences with known HHV8 reference viruses.

DM2 patients and healthy controls were examined for the presence of HHV8 DNA into the peripheral blood lymphocytes. Both anti-lytic and latent phase antibodies were detected in HHV8 positive and negative diabetic patients, as well in a number of controls. The HHV8 *ORF K1* and *ORF 26* genes from DM2 patients were typed and matched to reference strains.

A significant prevalence of HHV8 DNA in DM2 subjects versus healthy controls was detected (about 58 % against 27 %). Anti-lytic phase, but not anti-latent phase antibodies, were significantly increased in DM2 patients versus controls. In addition, about 30 % of HHV8 strains isolated from DM2 lymphocytes showed consistent differences in the *ORF 26* gene sequence, so that a new HHV8 subtype was proposed. These findings give additional support to the hypothesis that HHV8 could be considered an additional risk factor for DM2 onset.

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Keywords
Human Herpesvirus 8 • Latent viruses • Diabetes type 2 • Viral DNA typing • Antiviral antibodies

1 Introduction

The Human Herpesvirus 8 (HHV8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), belongs to the Herpesviridae family, subfamily Gamma-herpesvirinae, genus Rhadinovirus. HHV8 is etiologically associated to all the clinical forms of Kaposi's sarcoma (KS), and is correlated to other disorders developed in immunosuppressed individuals, such as multicentric Castleman's disease, primary effusion lymphoma (PEL) and body cavity-based lymphoma (BCBL) (Ablashi et al. 2002). HHV8 has a specific tropism to lymphocytes and endothelial cells and is highly oncogenic in immunosuppressed adults and HIV infected patients (Uldrick and Whitby 2011). To date, both the method of virus transmission and the typical mechanism of viral switching from the latent to the lytic phase, which leads to severe clinical symptoms, have not been completely clarified, although sexual transmission and saliva seem to be the most important routes of infection (Uldrick and Whitby 2011).

Unlike other herpesviruses, HHV8 infection is not ubiquitous in the general population (Adjei et al. 2008; de Souza et al. 2007; Rohner et al. 2014). This fact underscores the potential involvement of host-specific membrane receptors for HHV8 (Chakraborty et al. 2012). In addition, HHV8 seroprevalence varies in different geographic regions. In sub-Saharan countries HHV8 seroprevalence can reach values as high as 40–50 %, compared to rates of 11–19 % in some Italian regions and 6–8 % in the USA and in most north-European countries (Anderson et al. 2008; Engels et al. 2007; Kedes et al. 1996; Tornesello et al. 2010; Whitby et al. 1998). However, even in these countries, HHV8 prevalence is progressively increasing (Geddes et al. 1994; Ingianni et al. 2009; Pfeiffer et al. 2010; Serraino et al. 2006; Sobngwi et al. 2008; Valdarchi et al. 2007).

HHV8 is divided into 8 subtypes based on the *ORF K*1 gene polymorphism. Subtypes A and C predominate in Europe and the USA, whilst the B subtype is prevalent in Africa and D is diffused in the Pacific islands (Cordiali-Fei et al. 2015; Endo et al. 2003; Leao et al. 2013; Mancuso et al. 2008; Zhu et al. 2005; Zong et al. 2007). Recently, HHV8's *ORF 26* gene has also been found to have polymorphisms linked to *ORF K1* and has been considered an even easier and more reliable target for gene amplification than K1 (Endo et al. 2003; Zong et al. 2007). In the last few years, we and others have observed an increased frequency of HHV8 infection in cardiopathic (Ingianni et al. 2009) and diabetes mellitus type 2 (DM2) patients, in the sub-Saharan African regions and in the Sardinian population, respectively (Sobngwi et al. 2008; Ingianni et al. 2007). According to the World Health Organization (WHO), there are over 347 million people suffering from DM2 in the world, doomed to become an increasing global epidemic (Chen et al. 2011; Nolan et al. 2011; Pories and Dohm 2012; Whiting et al. 2011; WHO 2015; Wild et al. 2004). Prospective studies suggest that by 2030 diabetes will have become the seventh leading cause of death worldwide, the vast majority (>90 %) being caused by DM2. Susceptibility to DM2 is thought to be related to both genetic and environmental factors, possibly including infection by chronic stressors for the immune system such as herpesviruses (Ingianni et al. 2007). In the present study the analysis of viral infections in DM2 patients was also extended to the other lymphotropic herpesviruses as well as HHV8. In addition to episomal viral DNA, herpes seroprevalence was also detected in DM2 subjects from our study. In this work we also identified a

novel viral subtype, that, at the best of our knowledge, was found only in DM2 affected patients.

2 Materials and Methods

2.1 Subjects Recruited in the Study

DM2 patients were diagnosed at the Diabetes and Metabolic Disease Service (Hospital S. Giovanni di Dio, AOU Cagliari), accordingly with criteria of the World Health Organization for classification of diabetes (WHO 2006). The methods used for experiments involving human subjects were carried out in accordance with the guidelines approved from the Local Ethical Committee (AOU-EC). Blood samples were collected after receiving informed consent from all the subjects, and were anonymized before being used. HHV8 screening was performed on 646 DM2 patients (360 males and 286 females, range 50–75 years) and on 363 healthy subjects (187 males and 148 females, range 50–70 years), who were blood donors at the immuno-hematology service at Cagliari's "Brotzu" Hospital. All samples were collected in the period between 2008 and 2014.

2.2 HHV8-DNA Detection

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 ml of heparinized blood by the lymphoprep technique with Lympholyte - ® H (Cedarlane ®). Total cellular DNA was extracted using an Easy-DNA™ kit from Invitrogen (San Diego, CA) and suspended in 40 μl of TE buffer (10 mMTris-HCl, 1 mM EDTA). The 178-bp Orf-50 coding region amplification from nucleotide positions 209–386 took place in 200 ng of DNA with primers ORF50 f. 5′-CATGCAGCGGGGTGAGCCTG-3′ and ORF50 rev 5′- AGCAGCCCGGCGGTATCGTA-3′ for 40 cycles (15″ at 95 °C, 1′ at 60 °C and 15′ at 72 °C). The expected amplified segments of 178 bp were detected with an electrophoretic assay on 2 % agarose gel (Invitrogen, Groningen, Netherlands). Acquisition and image processing were performed with a PhotoDoc-It Imaging System Digital (UVP, Cambridge, MA) and PhotoPaint expression (Corel, Ottawa, Canada). For each experiment, a positive control DNA extracted from human B lymphocytes containing the HHV8 genome (BC3 cells, ATCC, Manassas, Va.) was employed and a negative control without a DNA template was also performed.

2.3 Multiplex PCR for Other Lymphotropic Herpesvirus DNA Detection

For the detection of other lymphotropic herpesviruses, as well as HHV8, a multiplex PCR was performed with a nested multiplex PCR for the amplification of the polymerase gene *ORF59*. Forward and reverse primer sequences in the 1st PCR amplification of HHVs DNA polymerase gene were aligned. A pair of degenerate primers were then designed and synthesized as HHV/1+ (5′ GTCATTTATGGBGAYACKGA 3′) and HHV/1- (5′ ATCCCCATGTATCKYTTYTT 3′), when B = C or G or T, Y = C or T, K = G or T, as previously described (Caselli et al. 2014). A 1st PCR reaction mixture of 50 μl in total volume was prepared containing 5 μl of viral DNA extraction, 20 pM of degenerate primers (Life Technologies, ML, USA), 200 μM of each dNTPs (Life Technologies), 1.25 U of Platinum® Taq DNA Polymerase (Life Technologies), 4 mM of MgCl2 and 2X PCR buffer. An initial denaturation step at 94 °C for 4 min was followed by 30 cycles consisting of 30 s at 94 °C, 1 min at 45 °C and 30 s at 72 °C. A final extension step at 72 °C was carried out for 10 min. For the 2nd PCR reaction, the following specific primers were used for the amplification of different HHV fragments (EBV = 54 bp, HHV-6 = 68 bp, CMV = 78 bp, HHV-8 = 97 bp and HHV-7 = 122 bp): *ORF59* CMV fw inner 5′-GAG TTT GAA AAG GTG TTC GTC-3′ and ORF 59 CMV rev inner 3′-GGG CCC AGC CTG GCG CAC TA-5′; *ORF59* EBV fw inner 5′-ACC CGG AGC CTG TTT GTA GC-3′ and *ORF59* EBV rev inner 3′-

GAA GCC GAG AAG ACC TTC TCC-5′; *ORF59* HHV6 f. inner 5′-GCC AAA CAT ATC ACA GAT CG-3′ and *ORF59* HHV6 rev inner 3′-GAG TTY GAR AAG ATT TTA TGT CC-5′; *ORF59* HHV7 f. inner 5′-GTT ACT AAA AAT GTT TGT CCC-3′ and *ORF59* HHV7 rev inner 3′-GAA TTT GAA AAG ATC CTA TTT CC-5′; *ORF59* HHV8 f. inner HHV8/2+ GGA CAG CGT GTC AGA CTT CG and HHV8/2+ rev inner CTT GAA GAT CTT TTC AGC CTC. In the 2nd PCR reaction 3 µl of the 1st PCR product were added to 47 µl of reaction mixture, consisting of 10 pM of each specific primer, 200 µM of each dNTPs, 1.25 U of Taq DNA polymerase, 2 mM of MgCl2 and 2X PCR buffer. Amplification was performed by applying the same conditions used in the 1st PCR step, except the annealing temperature that was 47 °C.

2.4 Serological Analysis

Sera from the DM2 (No. 74) and control subjects (No. 36) were tested for the presence of antibodies to EBV (ELISA classic EB Virus IgG, Serion Wurzburg, Germany), CMV (ELISA classic CMV IgG, Serion). HHV8 (IFA-IgG against both lytic K8.1 and latent LANA HHV8 proteins, Scimedx C. Denville, USA), HHV7 (IFA-IgG against HHV7, Scimedx) and HHV6 (IFA-IgG against HHV6, Scimedx).

2.5 HHV8 ORFK1 and ORF26 Genotyping

Genotype characterization of HHV8 strains was based on sequencing the viral region *ORFK1* coding for a trans-membrane protein (Endo et al. 2003; Zong et al. 2007). The 338-bp *ORFK1* was amplified from 100 ng of DNA of patients and donors collected over 6 years by nested PCR with an outside primer pair (5′-GTTCTGCCAGGCATAGTC -3′ and 5′-GTAA-CATGCTGACCACAAG-3′ at 30 cycles of 94 °C for 30″, 53 °C for 30″ and 72 °C for 60″) and an inside primer pair (5′-GTGGCGGCCCTTGTGTAAAC -3′ and 5′-GGATCCGTGTTGTCTACG -3′ at 40 cycles of 94 °C for 30″, 58 °C for 45″ and 72 °C for 60″). The expected amplified segments of 178 bp were detected with an electrophoretic assay on 2 % agarose gel (Invitrogen). The expected bands were excised, purified with a Mini Elute Purification Kit® (Quiagen) and with a QuickClean II PCR Extraction Kit (GeneScript) and subjected to molecular cloning with a TOPO® TA Cloning® Kit for Sequencing (Life Technologies). The 336-bp ORF26 coding region from nucleotide positions 890–1226 was amplified by nested PCR with an outside primer pair (Endo et al. 2003; Zong et al. 2007). (5′-ATCTATCCAAGTGC-ACACTCG-3′ [826–846] and 5′-CTGG-GAACCAAGGCTGATAGG-3′ [1291–1270]) and an inside primer pair (5′-GATGGATCC-CTCTGACAACCT-3′ [890–910] and 5′-GGAT-CCGTGTTGTCTACG-3′ [1226–1204]). The expected amplified segments were detected with an electrophoretic assay on 2 % agarose gel (Invitrogen) and then excised, purified, and subjected to molecular cloning as indicated before.

2.6 Molecular Cloning

The Topo Cloning reaction was set up with excised bands of *ORFK1* and *ORF26* PCR products, which were used for the transformation of DH5α™ competent cells. 50 µl from each transformation reaction were dispensed on a pre-warmed selective LB plate containing 50–100 µg/ml ampicillin and then the plasmid DNA was isolated with a GFX Micro Plasmid Prep Kit (Amersham Biosciences, Pittsburgh, USA) and analyzed by PCR Screening for M13 Plasmid primers: fwd:5′-TGTAAAACGACG-GCCAGT-3′ and rev 5′-CAGGAAACAGCT-ATGACC -3′. Gene sequencing was performed by BMR GENOMICS srl (Padua, Italy) and nucleotide sequences were analyzed with a multiple-alignment system by using CLUSTAL W (DNA Data Bank of Japan, Mishima, Japan;

http://www.ddbj.nig.ac.jp/Welcome-j.html), in order to produce the phylogenetic trees.

2.7 Statistics

All data were analyzed by unpaired two-tailed t-student test, or by one-/two-way ANOVA when required. Differences were considered to be statistically significant when p values were <0.05. Microsoft Excel software was used for designing the linear trend-line.

3 Results

3.1 Detection of Lymphotropic Herpesvirus DNA and Serum Antibodies in DM2 Patients and in Healthy Controls

The lymphotropic herpesvirus DNA and antibody screening study included a group of 74 patients with DM2 (40 males and 34 females, range 60–75 years) and 36 control subjects (15 males and 21 females, range 50–70 years). The DM2 patients were recruited on the basis of the following criteria: (i) they had been diagnosed with type 2 diabetes for at least 3 years; (ii) they were all under treatment with anti-diabetic drugs; (iii) they had no other diagnosed pathologies.

A putative relationship between HHV8 and DM2 has been previously postulated (Ingianni et al. 2007; Sobngwi et al. 2008; Caselli et al. 2014); to confirm this hypothesis we devised a set of experiments to analyze the prevalence of several lymphotropic herpesviruses in diabetic subjects and healthy controls. A positive association between HHV8 and DM2, but not with other herpesviruses, would support this hypothesis. For this purpose, we analyzed both the herpesvirus DNA in the peripheral lymphocytes (Table 1) and the anti-herpes antibodies in the blood sera (Table 2). We confirmed a significant prevalence of HHV8 DNA in DM2 subjects (58.1 % in DM2 versus 27.7 % in controls, $p < 0.01$), whilst no significant differences were observed for all the other herpesvirus species tested.

There was no relevant difference between DM2 and controls for antibody titres against CMV, EBV, HHV6 and HHV7, whilst the difference was highly significant for the presence of anti-HHV8 lytic antibodies (55.4 % in DM2 and 16.6 % in controls, $p < 0.001$), but not for the anti-HHV8 latent antibodies. Overall our results are consistent with a possible association between HHV8 and DM2.

3.2 Construction of the Phylogenetic Tree of DM2 Patients and Control Subjects Based on the ORFK1 DNA Sequencing

We hypothesized that the two groups (DM2 patients and healthy individuals) may have different HHV8 variants. Since *ORFK1* is the most frequently used gene for the identification of HHV8 subtypes (Mancuso et al. 2008; Zhu et al. 2005), we first examined the variability of this gene in the DM2 and control individuals enrolled in this study. *ORFK1* genotyping

Table 1 Detection of lymphotropic Herpesvirus DNA in BC3 cells

Number of positive subjects for the following viruses						
	Subjects	EBV	CMV	HHV6	HHV7	HHV8
Test performed	(No.)	No. ± SE[§§] (%)	No. ± SE (%)	No. ± SE (%)	No. ± SE (%)	No. ± SE (%)
PCR for	DM2[§] (74)	42 ± 5 (56.7)	56 ± 6 (75.6)	42 ± 6 (56.7)	48 ± 5 (64.8)	**43** ± 4 **(58.1)***
ORF59 DNA	Controls (36)	20 ± 3 (55.5)	34 ± 3 (94.4)	15 ± 4 (41.6)	26 ± 4 (72.2)	**10** ± 3 **(27.7)***

Note: [§]*DM2* diabetes type 2 patients. [§§]*SE* standard error. *The difference between these values was statistically highly significant ($p < 0.01$). The test was repeated at least three times

Table 2 Titration of antiviral antibodies to lymphotropic Herpesviruses

Detection of anti-viral antibodies for the following viruses						
Subjects (No)	Antibody titres	EBV % ± SE[§§]	CMV % ± SE	HHV6 % ± SE	HHV7 % ± SE	HHV8 % ± SE
DM2[§]	<1:64	35.2 ± 2	4.2 ± 2	25.7 ± 3	28.4 ± 2	Lytic 44.6 ± 3
						Latent 63.5 ± 5
(No. 74)	≥1:64 ≤ 1:512	64.8 ± 4	95.9 ± 4	74.3 ± 6	71.6 ± 5	**Lytic: 55.4** ± 4*
						Latent: 36.5 ± 3
Controls (No. 36)	<1:64	41.7 ± 4	2.2 ± 1	33.4 ± 2	8.4 ± 2	Lytic 83.4 ± 4
						Latent 66.7 ± 5
	≥1:64 ≤ 1:512	58.3 ± 4	97.2 ± 3	66.6 ± 5	91.6 ± 6	**Lytic: 16.6** ± 3*
						Latent: 33.3 ± 5

Note: [§]*DM2* diabetes type 2 patients. [§§]*SE* standard error. *The difference between these values was statistically highly significant ($p < 0.01$). Each assay was repeated at least three times

showed that most samples were attributable to genotypes C1 and C3 (Endo et al. 2003) or A/C (Zong et al. 2007), as expected for the general geographic distribution in Europe (Fig. 1 and Table 3). Only one strain (DM192) was attributable to the B subtype, but this resulted as being from a Philippine immigrant. Our analysis did not detect significant differences among the two groups. Therefore to tease apart potential marker we examined another ORF region (*ORF26*), since this is another described region for potential polymorphisms (Tornesello et al. 2010; Endo et al. 2003; Zong et al. 2007).

3.3 ORF26 Gene Sequencing and Identification of a New Subtype Among the DM2 Subjects

The HHV8 *ORF26* gene, which codes for a capsidic TR2 protein, was typed. The experiment was run according to the method described by Endo et al. (2003), as revised by Zong et al. (2007) (Table 3). All the controls fell into subtype C3 (subtype J for Zong). As regards the DNA of the DM2 subjects, 2 belonged to the A1 type, as in the BCBLR cells, 16 had sequences comparable to subtype C3 (J for Zong), but 10 were different for at least 2 aminoacids in positions 1032 (nucleotide T instead of A) and 1055 (C instead of T). On the basis of these findings we propose that these strains could segregate into a new original subtype that, at the best of our knowledge, so far has been described only in DM2 patients.

3.4 Progressive Increase of HHV8 Infection in Both DM2 Patients and Healthy Controls in South Sardinia in the Period Between 2008 and 2014

Since, as compared to our recent papers (Ingianni et al. 2007; 2009; Caselli et al. 2014), the data acquired in the present study showed an increased prevalence of HHV8 infection in both the general population and the DM2 subjects, we then conducted a retrospective analysis of all our previous data on HHV8 infection in both DM2 (No. 646) and healthy controls (No. 363) in the South-Sardinian region in the period between 2008 and 2014 (Fig. 2a–c). As expected, we found a higher increase of HHV8 infection than the rest of Italy (Tornesello et al. 2010; Whitby et al. 1998; Geddes et al. 1994; Valdarchi et al. 2007), with an average of 23 % of positive cases. In diabetic patients, HHV8 infection was more than doubled with respect to the controls, reaching an average value of 49 %. From 2008 to 2014 we found an increase of HHV8 infection in the general population from about 11 % to nearly 30 % (+272 %); moreover, in the DM2 subjects, HHV8 infection showed a rapid and constant

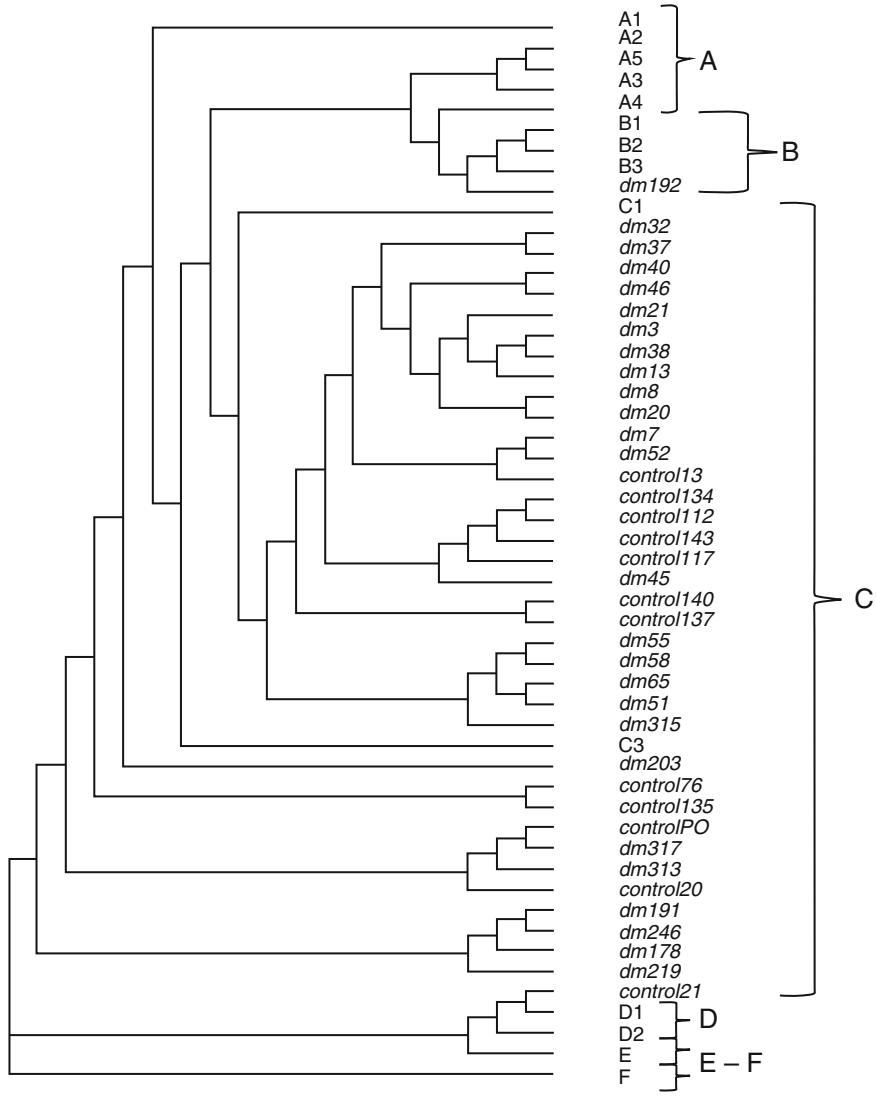

Fig. 1 The phylogram originated by the 52 HHV8 strain DNAs typed in this work. As expected for the European regions, all the ORF K1 genomes fell into groups C1 or C3 (Endo et al. 2003), (subtype A/C according to Zong et al. 2007). Only 1 strain (DM192) fell into group B, but this originated from an immigrant from Philippines

increase from about 19 % to as high as 58 % (+294 %).

4 Discussion

HHV8 is the latest herpesvirus to have been discovered and characterized. Unlike other herpesviruses it is not highly diffused in the general population, scoring values between 6 and 8 % in most American and European countries. However, this virus is found with a higher prevalence in some regions, such as sub-Saharan countries and on some Italian islands, and also in patients with some widely diffused diseases, such as cardiovascular diseases and type 2 diabetes (Ingianni et al. 2007; Sobngwi et al. 2008; Ingianni et al. 2009; Caselli et al. 2014). Genetic conditions can explain this finding and particular

Table 3 DNA typing of HHV8 *ORF26*-935-1139 bp gene segment

SNP[§] and the following nucleotide position in *ORF26*											
Subject	935	981	1032	1055	1086	1094	1103	1122	1132	1139	Genotype[§§]
BCBLR	C	T	C	G	C	G	C	G	A	A	A1 (A/C)
Dm6	–	–	–	–	–	–	–	–	–	–	A1 (A/C)
Dm283	–	–	–	–	–	–	–	–	–	–	A1 (A/C)
BC3	–	C	A	T	–	–	–	–	G	C	C3 (J)
Controls (strains No. 12)	–	C	A	T	–	–	–	–	G	C	C3 (J)
Dm2 (strains No. 15)	–	C	A	T	–	–	–	–	G	C	C3 (J)
Dm141	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm154	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm158	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm35	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm74	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm96	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm200	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm231	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm278	–	C	**T**	**C**	–	–	–	–	G	C	**NS**
Dm337	–	C	**T**	**C**	–	–	–	–	G	C	**NS**

Notes: [§]*SNPs* single nucleotide positions of *ORF26*. [§§]In *brackets* the ORF 26 genotypes according to Zong et al. (2007). The sign (–) indicates no polymorphism. 2 strains were found to belong to the wild type strain A1 as described for the BCBLR cells (Endo et al. 2003). 24 DM2 strains fell into subtype C3 (Endo et al. 2003) or J (Zong et al. 2007). 10 strains (**NS**) belonged to a subtype not yet described by others and have been included in a new specific subtype for DM2 patients; the DM2 patients with the new DNA nucleotide variants are indicated in bold characters

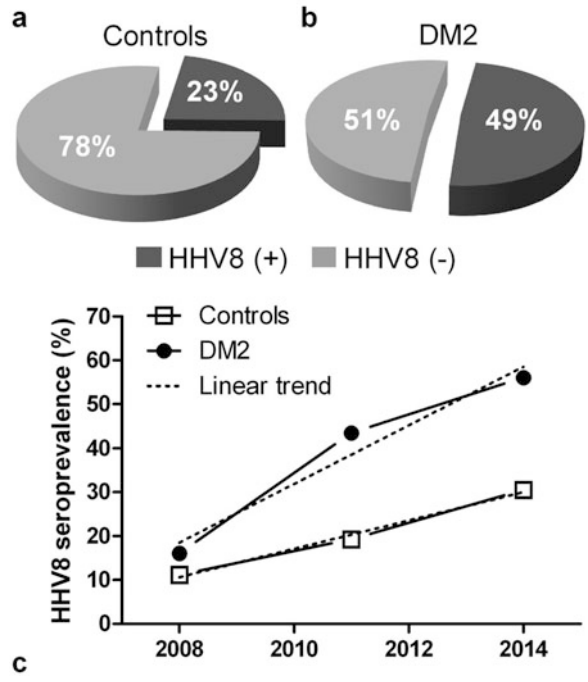

Fig. 2 Survey of HHV8 DNA detection in peripheral lymphocytes of persons with DM2 and healthy controls in the period between 2008 and 2014. A total of 646 DM2 patients and 363 healthy subjects were examined. *Panel a* and *b*: general prevalence of HHV8 infection in either DM2 patients or healthy controls. *Panel c*: progressive increase of HHV8 infection in both the general population and DM2 subjects scored in South Sardinia in the period 2008–2014. The *thin grey line* represents the expected trend of HHV8 infection in both controls and DM2 subjects

specific cell receptors could be responsible for the different susceptibility of some populations to this viral infection (Chakraborty et al. 2012). This fact led us to question whether diabetes is a pathology that favours HHV8 infection (e.g. by decreasing immune system efficiency), or, on the

contrary, whether HHV8 infection creates the conditions for the metabolic modifications that lead to DM2 (Angius et al. 2015; Ingianni et al. 2013). A possible link between HHV8 infection and DM2 has been one of the aims of our recent research (Ingianni et al. 2007; Caselli et al. 2014); a collaborative study by French and Senegalese researchers on sub-Saharan immigrants also suggested a possible onset of DM2 caused by HHV8 (Sobngwi et al. 2008). The present epidemiological study supports these observations, since in these last 7 years HHV8-DNA in Sardinia was isolated from as much as 58 % of the DM2 patients examined, against 27 % of controls. It is also important to consider that it is only HHV8 infection which significantly increases in DM2 patients, whereas all the other lymphotropic Herpesviruses demonstrate an equal diffusion in both DM2 and healthy individuals. In addition, testing for the presence of anti-HHV8 antibodies led to the interesting finding that only antibodies against the lytic phase of HHV8 infection are significantly increased in DM2, whilst antibodies against the latent phase are not. This suggests that, after a first acute infection, HHV8 enters a state of latency that can last for several years and causes a slow and progressive decrease in immune system efficiency, which leads to the triggering of a new lytic phase of HHV8 infection, with possible metabolic alterations compatible with diabetes onset (Ingianni et al. 2013; Angius et al. 2015). Therefore, the findings reported in this work strengthen the hypothesis that HHV8 infection can trigger the physiological and metabolic modifications that lead to DM2 onset (Pompei 2016).

HHV8 is known as the agent of KS. This is a very rare disease that normally affects less than 20 persons per million inhabitants in most regions (Ablashi et al. 2002; Uldrick and Whitby 2011). Its incidence increases dramatically in immune-suppressed subjects, such as AIDS and transplant patients. If we consider HHV8 prevalence in the general population, the question arises as to why KS disease is so rare if HHV8 diffusion covers at least 6–8 % or more of the total individuals. Immune system efficiency is clearly an important factor, but viral specific determinants may also play a significant role in KS pathology. It has been demonstrated that HHV8 subtype A is more frequently isolated from severe clinical KS (Endo et al. 2003; Mancuso et al. 2008). Thus, HHV8 typing does not only have importance for epidemiological purposes, but is also a useful tool for the identification of particular subtypes of viruses, which can be more harmful for some human pathologies. The present work has revealed the presence of a novel subtype of HHV8 in DM2 patients. Is this subtype of HHV8 only specific to DM2? The data so far are suggestive, but more robust analysis is required for stronger claims. Studies from other regions and with more cases of DM2 are needed to increase our confidence in the association of this viral subtype with DM2. In addition, viral factors other than *ORFK1* and *ORF26*, that could be involved in HHV8 replication and metabolism, such as LANA (*K73*) and *vGPCR*, should be included for detecting DM2 specific strains. The rapidly increasing prevalence of both HHV8 infection and DM2 diffusion in Sardinia should be closely monitored. Recent studies have reported an increasing presence of HHV8 infection in the general population and in particular in some regions, such as Southern Italy (Sicily and Sardinia), and the Po river valley (northern Italy) (Geddes et al. 1994; Whitby et al. 1998; Valdarchi et al. 2007; Ingianni et al. 2009; Pfeiffer et al. 2010) and confirmed the high values in the sub-Saharan countries (Sobngwi et al. 2008). Genetic pressure, caused by endemic diseases (malaria, thalassemia, G6PD deficiency), has been claimed by some authors as being the possible cause of the selection of a population with an increased sensitivity to HHV8 infection (Serraino et al. 2006). In our almost 7 years of experience from 2008 to 2014, we observed an increase in HHV8 infection in the general population from about 11 to 27 %. In the last year of our study, we scored percentages close to 30 % in controls and 58 % in DM2. At the same time DM2 was found to have risen from 5–6 % in 2004 to about 10 % in the final year. These values do not only apply to Sardinia, but similar percentages of DM2 have been detected

in many other countries, and within the next 15 years, it is estimated that DM2 will involve at least 10 % of the total world population (Chen et al. 2011; Whiting et al. 2011). In conclusion, if the HHV8 infection were to be proven and confirmed as an additional risk factor for DM2, then anti-HHV8 drugs (Chen et al. 2012) and specific vaccines (Wu et al. 2012) would potentially be used to support the conventional therapeutic treatments for DM2.

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Activity of Norspermidine on Bacterial Biofilms of Multidrug-Resistant Clinical Isolates Associated with Persistent Extremity Wound Infections

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Abstract

Biofilm formation is a major virulence factor for numerous pathogenic bacteria and is cited as a central event in the pathogenesis of chronic human infections, which is in large part due to excessive extracellular matrix secretion and metabolic changes that occur within the biofilm rendering them highly tolerant to antimicrobial treatments. Polyamines, including norspermidine, play central roles in bacterial biofilm development, but have also recently been shown to inhibit biofilm formation in select strains of various pathogenic bacteria. The aim of this study was to evaluate *in vitro* the biofilm dispersive and inhibitory activities of norspermidine

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against multidrug-resistant clinical isolates of *Acinetobacter baumannii* (n = 4), *Klebsiella pneumoniae* (n = 3), *Pseudomonas aeruginosa* (n = 5) and *Staphylococcus aureus* (n = 4) associated with chronic extremity wound infections using the semi-quantitative 96-well plate method and confocal laser microscopy. In addition to the antibiofilm activity, biocompatibility of norspermidine was also evaluated by measuring toxicity *in vitro* to human cell lines and whole porcine tissue explants using MTT viability assay and histological analysis. Norspermidine (5–20 mM) had variable dispersive and inhibitory activity on biofilms which was dependent on both the strain and species. Of the clinical bacterial species evaluated herein, *A. baumannii* isolates were the most sensitive to the effect of norspermidine, which was in part due to the inhibitory effects of norspermidine on bacterial motility and expression of genes involved in the production of homoserine lactones and quorum sensing molecules both essential for biofilm formation. Importantly, exposure of cell lines and whole tissues to norspermidine for prolonged periods of time (≥24 h) was observed to reduce viability and alter tissue histology in a time and concentration dependent manner, with 20 mM exposure having the greatest negative effects on both tissues and individual cell lines. Collectively our findings demonstrate that, similar to other polyamines, norspermidine displays both inhibitory and dispersive activities on biofilms of clinical multidrug-resistant bacterial isolates, in particular for strains of *A. baumannii*. Additionally our findings suggest that direct application may be considered on tissues, albeit for limited exposure times.

Keywords

Norspermidine • Polyamine • Biofilm dispersal • Biofilm inhibition • Wound infection

1 Introduction

Bacteria can exist in either a planktonic, free-living individual state, or as a part of a surface-attached multicellular community embedded within a self-produced extracellular polymeric matrix, known as a biofilm (Costerton 1999; Costerton et al. 1999; Hall-Stoodley et al. 2004). To date numerous studies have demonstrated that bacterial biofilms can develop within host tissues and are cited as a significant bacterial virulence factor contributing to chronic wound infections (Bjarnsholt et al. 2008; Costerton et al. 1999; Hall-Stoodley et al. 2004; James et al. 2008). In contrast to planktonic bacteria, the excessive extracellular matrix secretion and concurrent metabolic changes that bacteria undergo while in a biofilm render them highly tolerant to antimicrobial treatments, often up to 1000-fold compared to their planktonic counterparts (Anderson and O'Toole 2008; Mah and O'Toole 2001; Kouidhi et al. 2015). Consequently, the use of conventional antimicrobials for the treatment of biofilm-related infections, including chronic wounds, has limited efficacy as they fail to eradicate bacteria within the biofilms. Given the limitations of antimicrobials against biofilm bacteria, efforts by many groups have been directed towards identifying substances as well as developing

strategies to inhibit and/or trigger dispersal of biofilms (Romling and Balsalobre 2012).

Biofilm development involves an elaborate life cycle, initiated by the attachment of microorganisms to the surface, followed by continued growth and eventual maturation. As the biofilm matures, resources become limited and waste products accumulate, initiating a cascade of events that lead to the disassembly of extracellular matrix and dispersal of bacteria from the biofilm. Studies evaluating the signals and cues utilized by bacteria to initiate dispersal have led to the identification of a diverse group of secreted diffusible molecules including polyamines (Kaplan 2010; Kostakioti et al. 2013). Polyamines are small molecular weight, cationic molecules that have been broadly implicated in bacterial growth due to their ability to interact with nucleic acids and protein translation machinery, to exert antibacterial action against a number of various human pathogens, and contribute to disease pathogenesis by enhancing the expression of bacterial virulence factors (Karatan and Michael 2013). Polyamines have also been shown to play an essential role in biofilm formation (Wortham et al. 2007; Kolodkin-Gal et al. 2012). For example, putrescine is essential for biofilm formation in *Yersinia pestis* and *Escherichia coli* (Patel et al. 2006; Wortham et al. 2010). Similarly, spermidine and its shorter structural analog, norspermidine, have been reported to enhance biofilm formation in *Bacillus subtilis* and *Vibrio cholera* (Lee et al. 2009) respectively. Interestingly, polyamines have also been reported to inhibit biofilm formation of *B. subtilis*, *Staphylococcus aureus* and *E. coli*, and promote the disassembly of *B. subtilis* biofilms (Kolodkin-Gal et al. 2012; Karatan and Michael 2013). Based on the chemical nature of polyamines and the likely interaction with polyanions, interactions between polyamines with exopolysaccharides within the biofilm matrix were presumed to destabilize the matrix promoting disassembly and dispersal of biofilms (Kolodkin-Gal et al. 2012; Wortham et al. 2007). However, recent reports have proposed that norspermidine may inhibit biofilm formation through polysaccharide-independent mechanism of bacterial growth inhibition, in part explaining the inhibitory activity on biofilms of *B. subtilis* (Hobley et al. 2014). While these studies have provided preliminary evidence supporting the antibiofilm activities of norspermidine *in vitro*, these original studies were limited to only a few bacterial species and did not evaluate the biocompatibility of norspermidine, which is of particular importance given its suggested clinical use as a topical agent within studies. Therefore, the aim of the present study was to evaluate the inhibitory and dispersive activities of norspermidine on biofilms of several clinical isolates of multidrug-resistant organisms (MDRO) associated with chronic wound infections, as well as the potential clinical use as a topical agent by assessing biocompatibility using cell lines and whole tissues *in vitro*.

2 Results

2.1 Preliminary Screening of the Inhibitory and Dispersive Activity of Norspermidine Against Bacterial Biofilms

Exposure of preformed biofilms to norspermidine, at concentrations between 0.1 and 20 mM, had only modest dispersive activity against preformed biofilms of representative strains of *K. pneumoniae* and *S. aureus,* reducing biomass between 20 and 38 % of the untreated control group (Fig. 1a). In contrast, significant dispersal activity of norspermidine was observed against preformed biofilms of isolates of *A. baumannii* and *P. aeruginosa* following overnight exposure to norspermidine. For *A. baumannii* and *P. aeruginosa*, dispersive activity was concentration-dependent, with significant activity at 20 mM, and between 5 and 20 mM, against *A. baumannii* and *P. aeruginosa* biofilms, respectively. In contrast to the dispersive activity against preformed biofilms, norspermidine had a much greater effect on inhibiting biofilm formation. The activity, as with dispersal, was also dose-dependent and

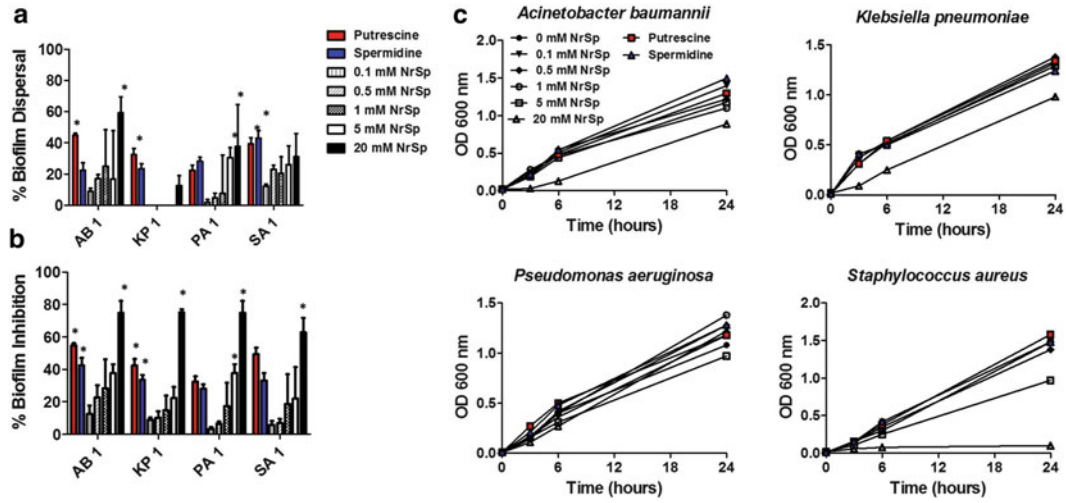

Fig. 1 Screening of the antibiofilm and antimicrobial activity of norspermidine on bacterial isolates. Screening of the dispersive (**a**) and inhibitory (**b**) activity of norspermidine, at concentrations ranging from 0.1 to 20 mM in HEPES buffer (pH ~7.4), putrescine (0.1 mM) and spermidine (0.1 mM) against preformed biofilms (**a**) or following overnight incubation in the presence of norspermidine (**b**) against four representative clinical isolates of *A. baumannii* (AB1), *K. pneumoniae* (KP1), *P. aeruginosa* (PA1), and *S. aureus* (SA1) (described in Table 1). Biomass was determined using the crystal violet method and reported as the percentage of biofilm dispersal and inhibition to untreated controls. (**c**) Planktonic bacterial growth up to 24 h in liquid media supplemented with norspermidine (0.1–20 mM), putrescine (0.1 mM) and spermidine (0.1 mM), as determined by measuring the optical density at 600 nm and plotted over time. *Bars/lines* represent the average ± SD of three independent experiments. Statistical analysis was performed using a student's *t* test; $p < 0.05$ was considered to be statistically different from the untreated control group

most effective at concentrations between 5 and 20 mM. Notably, while the dispersive activity of norspermidine was observed primarily against *A. baumannii* and *P. aeruginosa*, significant inhibitory activity was observed against the majority of the representative clinical isolates tested (Fig. 1b). Importantly, while norspermidine was observed to have dispersive and inhibitory activity herein, this activity was only observed at much higher concentrations compared to other classes of polyamines previously shown to have antibiofilm activity, including putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014) (Fig, 1a, b).

2.2 Effect of Norspermidine on Bacterial Growth

In light of previous reports suggesting that the antibiofilm activity of norspermidine may occur as a consequence of inhibition of bacterial growth, we also evaluated the effect of exposure to norspermidine on bacterial growth using the representative isolates evaluated above. With the exception of *S. aureus*, exposure to concentrations of norspermidine up to 20 mM had minor, albeit insignificant, activity on planktonic bacterial growth (Fig. 1c). Increasing concentrations of norspermidine had the least effect on the growth of *P. aeruginosa*, whereas the 20 mM concentration resulted in only minor and insignificant impairments on the growth of *A. baumannii* and *K. pneumoniae*. In contrast to most of the other bacterial species evaluated, exposure of *S. aureus* to norspermidine at 20 mM essentially abolished the growth of the strain. Consistent with the growth curves, enumeration of each bacterial strain at similar time points at each tested concentration in a planktonic growth assay confirmed only moderate reductions of bacterial numbers in the presence of norspermidine up to 20 mM. *S. aureus*

viability was significantly reduced following exposure to norspermidine at 20 mM. These findings suggest that the inhibitory and dispersive activity of norspermidine is, with the exception of *S. aureus*, largely independent of planktonic growth inhibition, and is exclusive to the biofilm phenotype.

2.3 Norspermidine Inhibits and Disperses Biofilms of Clinical Isolates Associated with Chronic Human Infections

Consistent with preliminary screening results above, norspermidine at 5 and 20 mM demonstrated both dispersive and inhibitory activity against a panel of genetically diverse clinical isolates associated with chronic human infections (Fig. 2a, b). The activities of norspermidine against the biofilms of clinical isolates were both species- and strain-dependent. Notably, clinical isolates of *A. baumannii* were the most sensitive to the effects of norspermidine. Although the activity was variable amongst the strains, exposure to norspermidine on average resulted in $\geq$ 60 % dispersion and $\geq$ 80 % inhibition of *A. baumannii* biomass. In contrast, the dispersive activity of norspermidine was variable against isolates of *P. aeruginosa* and *S. aureus*. For isolates of *K. pneumoniae*, dispersive activity tended to increase with rising norspermidine concentration, with the greatest effect on biofilm inhibition following exposure to 20 mM. Importantly, for *P. aeruginosa*, exposure to norspermidine in some cases significantly enhanced biofilm formation (Fig. 2a, b). As with the results in the preliminary screenings, the activity of norspermidine required much higher concentrations to achieve a significant effect compared to other previously characterized polyamines, putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014). As a qualitative confirmation of the above findings, visualization of bacterial biofilms by confocal laser microscopy (CLSM) showed similar effects of norspermidine on biofilm inhibition and dispersion, with *A. baumannii* and *K. pneumoniae* having the most visually significant changes in the biofilm phenotype (Fig. 2c).

2.4 Norspermidine Inhibits Motility and Quorum Sensing in *A. baumannii*

Among the isolates tested, *A. baumannii* biofilms were more sensitive to the activity of norspermidine. Given the propensity for multidrug resistance among *A. baumannii* isolates, as well as reports indicating biofilm formation by this pathogen may be a risk factor for the development of persistent infections associated with traumatic wounds (Akers et al. 2014), we further evaluated the effects of norspermidine on aspects crucial for *A. baumannii* biofilm formation including bacterial motility and quorum sensing.

In the presence of norspermidine at 5 and 20 mM, migration (i.e. bacterial motility) of all three clinical isolates of *A. baumannii* on 0.3 % TSA plates was significantly reduced (Fig. 3a, b). For *A. baumannii* isolates 2 and 4, supplementation of TSA plates with 5 and 20 mM norspermidine significantly reduced bacterial motility compared to control groups; whereas for isolate 3, a significant effect on bacterial migration was only observed following exposure to 20 mM norspermidine. In addition to bacterial motility, expression of a gene operon identified in *A. baumannii* strain 17978 known to be related to quorum sensing production in *A. baumannii* (A1S_0109, A1S_0112, A1S_0113, A1S_0114) was slightly decreased ($\leq$1 fold) compared to untreated controls (Fig. 3c). This effect of norspermidine on the expression of genes involved in quorum sensing was also observed in two of the *A. baumannii* clinical isolates (Fig. 3d).

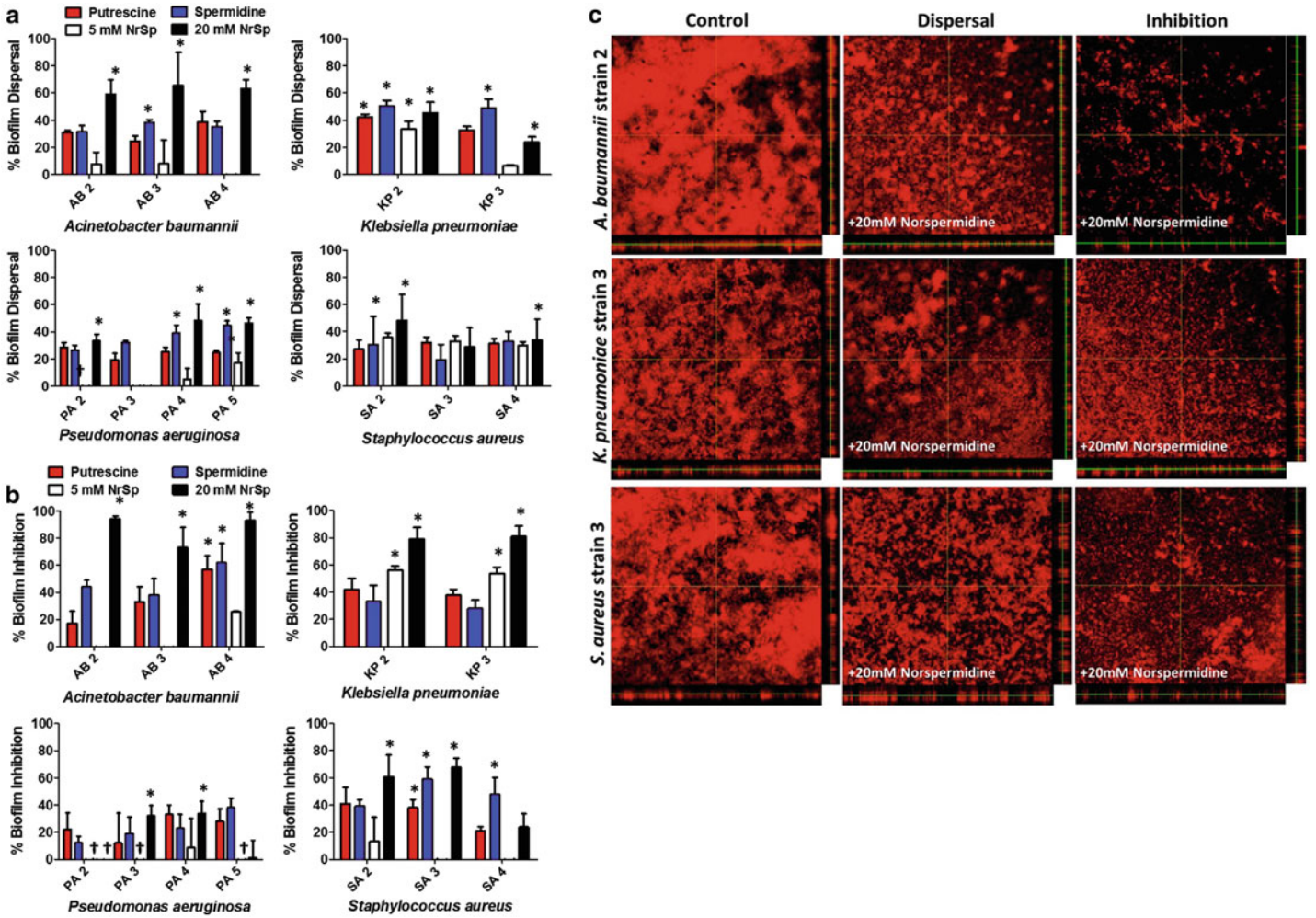

Fig. 2 Norspermidine disperses biofilms and prevents biofilm formation in clinical isolates associated with persistent infections. Dispersive (**a**) and inhibitory (**b**) activity of norspermidine at 5 and 20 mM in HEPES buffer (pH ~7.4) against preformed biofilms or following overnight incubation in the presence of norspermidine (NrSp), 5 and 20 mM, putrescine (0.1 mM) or spermidine (0.1 mM) against clinical isolates of bacteria associated with persistent infections (described in Table 1). Biomass was determined using the crystal violet method, and reported as the percentage of biofilm dispersal and inhibition to untreated controls. (**c**) Representative CLSM images of the biofilm dispersive and inhibitory activity of norspermidine at 20 mM on the various bacterial species. *Bars* represent the average ± SD of three independent experiments. Statistical analysis was performed using a student's *t* test; $p < 0.05$ was considered to be statistically different from the untreated control group

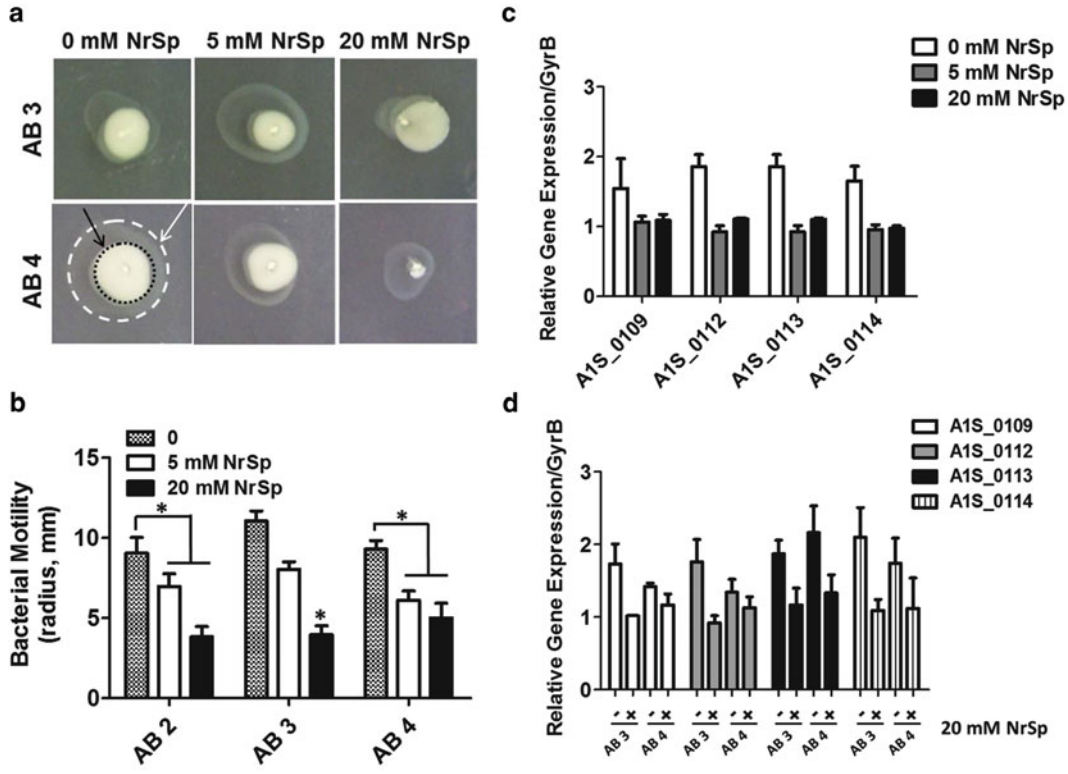

Fig. 3 Effect of norspermidine on bacterial motility and quorum sensing in *Acinetobacter baumannii*. (**a**) Representative images of semi-solid 0.3 % TSA plates with or without norspermidine (NrSP) (5 and 20 mM) after 24 h growth at 37 °C demonstrating the motility of *A. baumannii* strains. (**b**) bacterial motility, as determined by measuring the diameter of the motility zone (the difference between *white* and *black dashed lines* in (**a**)) in mm. (**c**, **d**) comparison of the expression levels of genes related to homoserine lactone synthesis (see Table 2) in *A. baumannii* reference strain 17978 (**c**) and clinical strains (**d**) as determined by qRT-PCR assays following exposure to norspermidine. *Bars* represent the average ± SD of three independent experiments. Statistical analysis was performed using an ANOVA One-Way; $p < 0.05$ was considered to be statistically different from the untreated control group

2.5 Assessment of Biocompatibility of Norspermidine on Whole Cell Tissues and Human Cell Lines *in vitro*

As an initial assessment for the potential clinical application of norspermidine as a topical agent for wounds, we evaluated the effect of norspermidine on porcine whole tissues. Direct application of norspermidine to whole tissues was observed to have a time- and concentration-dependent detrimental effect on tissue viability. In particular, exposure of tissues to concentrations of norspermidine exceeding 5 mM had the greatest effect, significantly altering tissue histology and reducing tissue viability after 6 and 24 h of exposure (Fig. 4a–c). In contrast, concentrations at ≤ 5 mM did not impair tissue viability even following exposures up to 24 h. Having observed the effect of norspermidine on whole tissues, we further evaluated its effect on individual cell lines. Consistent with the experiments performed in whole tissues, both a concentration- and time-dependent effect of norspermidine was observed on the individual cell lines (Fig. 4d, e). As with the whole tissues, exposure of cell lines, human keratinocytes and fibroblasts to concentrations

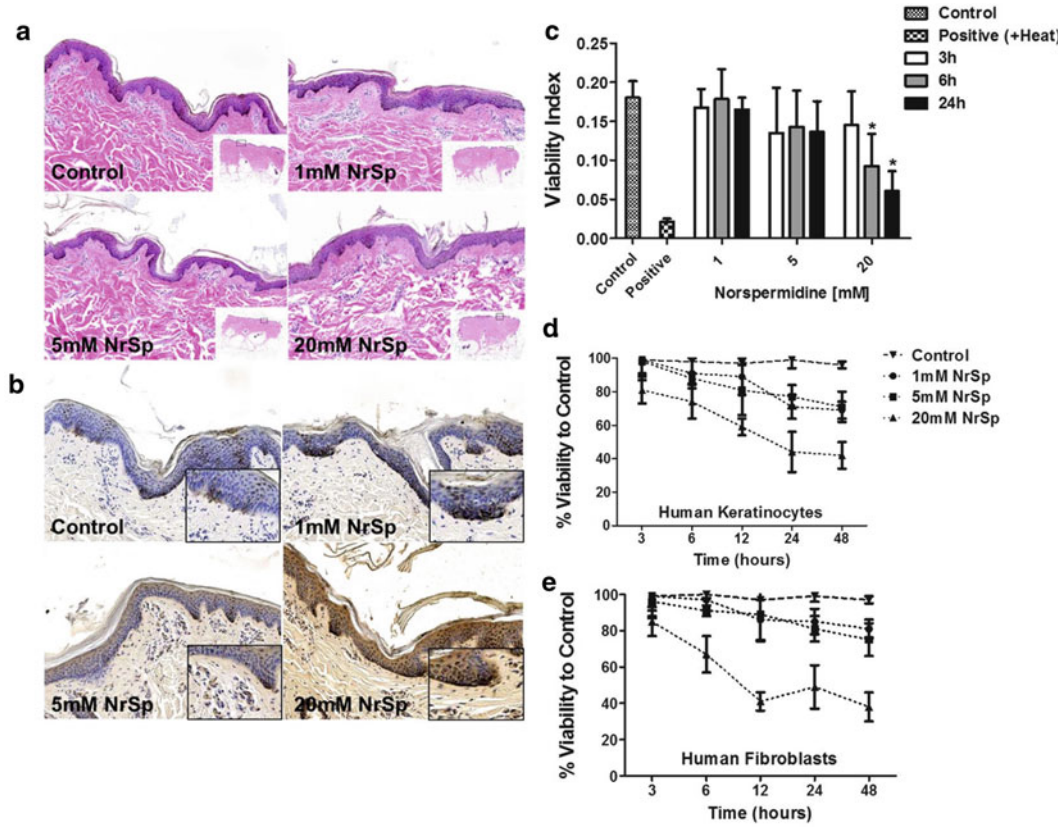

Fig. 4 Assessment of the biocompatibility of norspermidine *in vitro*. *In vitro* evaluation of the effect of norspermidine, 1, 5, and 20 mM, on whole tissue porcine explants following overnight exposure. (**a**, **b**) Representative images (10X; 20X inset) of norspermidine-treated porcine tissues as evaluated by H&E staining (**a**) and immunohistochemistry using an antibody to activated caspase-3 (**b**). (**c**) Cell viability of tissues as determined using the MTT viability assays and reported as an index of viability relative to the control (media only) group. A heat-treated group was included as a negative control. (**d**, **e**) *Bars* represent the average ± SD of three independent experiments. Values above the bars represent the % viability loss for each group. Statistical analysis was performed using an ANOVA One-Way; $p < 0.05$ was considered to be statistically different from the untreated control group

exceeding 5 mM were associated with significant losses in cell viability, evident at 6 h and increasing up to 48 h. Consistent with the whole tissue experiments, exposure to concentrations below 5 mM were associated with minor reductions in cell viability.

3 Discussion

Polyamines are small molecular weight, cationic molecules that have been broadly implicated in a number of bacterial processes including biofilm formation. In a recent study, Kolodkin-Gal et al. speculated that the polyamine norspermidine inhibited biofilm formation by *B. subtilis* (NCB13610), *S. aureus* (SC01), and *E. coli* (MC4100), and dispersed mature biofilms of *B. subtilis* via destabilizing interactions with exopolysaccharide polymers within the biofilm matrix (Kolodkin-Gal et al. 2012). Recent findings have challenged this proposed mechanism, however, instead suggesting a polysaccharide-independent mechanism of action (Hobley et al. 2014). While these studies provide insight into the potential antibiofilm

activities of norspermidine, they are limited to evaluating a select few bacterial species; moreover, there has been discourse surrounding variability in concentrations required to exert this effect on bacterial biofilms. To address this, herein we evaluated the inhibitory/dispersive activity of norspermidine using a panel of genetically distinct multidrug-resistant clinical strains of Gram-negative and Gram-positive organisms associated with chronic wound infections. Additionally, we evaluated the biocompatibility of norspermidine using whole tissues and cell lines to determine the potential application of norspermidine as a strategy to control bacterial biofilms. Collectively, we demonstrated that 5–20 mM norspermidine has inhibitory and disruptive activity against biofilms of clinical isolates of various bacterial species, including *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, with the greatest effect on clinical strains of *A. baumannii*. Importantly, exposure of whole tissues and cell lines revealed a concentration- and time-dependent cytotoxic effect, potentially delineating limits to concentration and/or duration of application for clinical use. To our knowledge, this is one of the few studies evaluating the activity of norspermidine against clinical isolates of Gram-negative and Gram-positive bacteria associated with chronic human infections and is the first to evaluate the biocompatibility as an initial assessment of its potential clinical utility.

Norspermidine was observed to have anti-biofilm, and in some instances antimicrobial, activity against the bacterial species we evaluated herein. The activity of norspermidine was observed to be both species- and strain-dependent, with *A. baumannii* isolates being the most sensitive to these effects. Strain- and species-dependent differences in the response of isolates to norspermidine are consistent with previous studies and have been previously documented (Nesse et al. 2015; Ramon-Perez et al. 2014). This may be due differences between strains in their ability to develop biofilms on abiotic surfaces, differences in the composition of polymeric matrix, and undefined mechanisms impeding biofilm formation or dispersal initiation. Notably, while anti-biofilm activity was observed with norspermidine, this activity required much higher concentrations compared to putrescine and spermidine (Goytia et al. 2013; Nesse et al. 2015; Ramon-Perez et al. 2014). Because other polyamines have similar chemical properties and are suggested to have similar mechanisms of biofilm disruption to norspermidine, given the specific activity on biofilm but not planktonic growth, it is possible that relative differences may arise from differences in solubility, and/or other undefined mechanisms of activity, for the species evaluated herein.

With the exception of *S. aureus*, the antimicrobial activity of norspermidine was largely independent of planktonic bacterial growth inhibition, and was specific to biofilms. Our observations of a limited effect of norspermidine on bacterial cell growth for most species contrast with previous findings reported by Hobley et al., who noted significantly impaired growth of *B. subtilis* at concentrations $\geq$ 1 mM (Hobley et al. 2014). Our findings showing minimal effect of norspermidine on planktonic growth of the Gram-negative species evaluated herein are consistent previous studies evaluating the effect of norspermidine at concentrations up to 5 mM on the planktonic growth of *E. coli* and *Salmonella enterica* (Nesse et al. 2015). Similarly, studies evaluating the activity of the related polyamine, spermine, on biofilms of *Neisseria gonorrhoeae* have reported that concentrations as high as 4 mM inhibited biofilm formation but had no notable effect on bacterial growth (Goytia et al. 2013). In contrast, exposure of *S. aureus* to norspermidine at 20 mM was associated with a negative effect on cell growth. While the antimicrobial mechanism is not currently known, it is possible that susceptibility to norspermidine of Gram-positive organisms, such as *B. subtilis* and *S. aureus*, would differ from Gram-negative organisms.

The concentrations of norspermidine required for activity observed in our study are much higher than those reported by *Kolodkin-Gal* et al., who reported activity with concentrations as low as 25 μM for *B. subtilis*, and between

100 and 500 μM for *S. aureus* and *E. coli* for biofilm inhibition (Kolodkin-Gal et al. 2012). Although we did not evaluate their strains, for *S. aureus* we observed activity only at norspermidine concentrations $\geq$ 5 mM (i.e., a ~10–40 fold difference). Recent studies evaluating the antibiofilm activity of norspermidine and other polyamines against clinical isolates have demonstrated that higher concentrations are required for biofilm inhibition. In one such study, concentrations of norspermidine between 500 and 5000 μM were required to inhibit biofilms of clinical isolates of *E. coli*, (Nesse et al. 2015) whereas the inhibitory activity of spermine on *N. gonorrhoeae* biofilm formation required concentrations as high as 4 mM (Goytia et al. 2013). Aside from inhibition of biofilm formation, dispersal of biofilms was also observed. Although *Kolodkin-Gal* et al. showed that norspermidine was capable of dispersing biofilms of *B. subtilis*, their study did not evaluate its dispersive activity against *S. aureus* or *E. coli* (Kolodkin-Gal et al. 2012).

Importantly, we are not the first to observe discrepancies in potency compared to the report of Kolodkin-Gal et al. (Hobley et al. 2014). These differences between studies have been attributed to differences in strain and/or species; however they may also relate to the use of strongly alkaline (high pH) commercial preparations of norspermidine. To eliminate the antimicrobial effect of pH, we neutralized norspermidine solutions with HEPES buffer to near neutral pH (7–7.4) before experimental use. This important step was not mentioned in the methods of the original study by Kolodkin-Gal et al., and subsequent studies using neutral preparations have demonstrated the need for higher concentrations of norspermidine (Goytia et al. 2013; Hobley et al. 2014; Nesse et al. 2015; Ramon-Perez et al. 2014). Despite methodological differences making comparisons difficult, numerous reports indicate that norspermidine and other polyamines do have antibiofilm activity, and may ultimately prove to be clinically useful to regulate biofilm formation.

A. baumannii isolates were most susceptible to the effect of norspermidine among the various species tested. Recently, *A. baumannii* has received significant attention for its propensity to acquire resistance to multiple antibiotics and its ability to cause a spectrum of severe infections (Corbella et al. 2000; Dijkshoorn et al. 2007; Gordon and Wareham 2010; Munoz-Price and Weinstein 2008; Peleg et al. 2008). Additionally, the ability of *A. baumannii* to develop biofilms has been implicated as an important pathogenic mechanism (Gurung et al. 2013; Lee et al. 2008; Rodriguez-Bano et al. 2008; Sanchez et al. 2013; Wand et al. 2012), and identified as a potential risk factor for the development of persistent wound infections (Akers et al. 2014). A major component of *A. baumannii* biofilm matrices, similar to that described for the staphylococcal species, are polysaccharides, in particular poly-β-1,6-N-acetylglucosamine (Bentancor et al. 2012; Choi et al. 2009). Because the main mode of action described for norspermidine was the interference of interactions between polysaccharides central to the development of a stable biofilm matrix (Kolodkin-Gal et al. 2012), interface with the polysaccharide components could in part explain the potent activity observed herein. This is consistent with previous reports involving *S. epidermidis* biofilms (Ramon-Perez et al. 2014). However, exposure of *A. baumannii* isolates to norspermidine also reduced bacterial motility and the expression of genes involved in the synthesis of the quorum sensing signal, acyl-homoserine lactone (ACH), potentially contributing to the observed potency of norspermidine in these isolates (Rumbo-Feal et al. 2013; Saroj and Rather 2013).

Pili-like structures have been shown to contribute to the initial steps of bacterial attachment and formation of biofilms on abiotic surfaces (Gaddy and Actis 2009; Longo et al. 2014). The pili-like structures, including fimbriae encoded by the *csuA/BABCDE* gene and the type IV pilus, are involved in different stages of *Acinetobacter* biofilm development, and are widely distributed among clinical strains (Nait Chabane et al. 2014; Tomaras et al. 2003). The general loss of bacterial motility observed herein, which is largely dependent on these structures,

are similar to findings in a previous study evaluating the effect of virstatin, a small organic molecule capable of targeting virulence factors including the type I and type IV pili (Cegelski et al. 2009; Clemmer et al. 2011; Hung et al. 2005). In this study, virstatin was shown to inhibit bacterial motility and consequently decrease in biofilm formation by clinical isolates of *A. baumannii*, in part through the activities on pili biogenesis (Nait Chabane et al. 2014). Although we did not directly evaluate the effect of norspermidine on pili biogenesis, our findings do suggest a potential role of norspermidine on motility.

Interestingly, the expression of genes involved in the synthesis of quorum signaling molecules was also diminished following exposure to norspermidine. Biofilm formation for many bacteria, including *A. baumannii*, is mediated through quorum-sensing pathways (Gaddy and Actis 2009; Longo et al. 2014; Rodriguez-Bano et al. 2008). Surface motility for *A. baumannii* is regulated by diverse mechanisms, including quorum sensing (Clemmer et al. 2011; Saroj and Rather 2013). Previous studies evaluating this relationship have shown that strains of *A. baumannii* deficient in the genes responsible for quorum signal production were also hindered in motility as well as biofilm formation (Clemmer et al. 2011). Collectively, our findings indicate that norspermidine has multiple effects on biofilms independent of interactions with polysaccharides. For *A. baumannii*, the loss in motility and subsequently biofilm formation could in part have been due to interference with quorum sensing. Future studies evaluating the effects of similar aspects of biofilm formation for other bacterial species are necessary.

As a preliminary evaluation of the potential clinical application of norspermidine, we evaluated the associated toxicity on whole tissues as well as individual cell lines following exposure to norspermidine for specified periods of time. Both a concentration- and time-dependent cytotoxic effect of norspermidine was observed within tissues as well as on individual cells lines following exposure to concentrations of norspermidine of 20 mM at times exceeding 6 h. Exposure of tissues beyond 6 h was associated with significant changes in tissue histology, as well as a significant increase in the number of apoptotic cells. Likewise, when evaluated using individual cell lines of keratinocytes and fibroblasts, we observed similar negative effects on viability. Given that the optimal biofilm dispersive and inhibitory activities of norspermidine were observed at higher concentrations, our findings indicate that direct application of norspermidine may incur a risk of local tissue toxicity, particularly when the higher concentrations are used for extended periods. However, it is important to note that while these *in vitro* findings suggest high tissue toxicity, similar findings have been reported for other commonly used wound irrigants, such as Dakin's Solution (buffered sodium hypochlorite) for which clinical use is well accepted (Siala et al. 2014). While host tissue toxicity may limit prolonged, direct application of norspermidine, further studies evaluating brief exposure to higher concentrations, and/or prolonged exposures (>24 h) to lower biocompatible concentrations, are warranted. Additionally, further studies examining the potentiating effect of topical norspermidine on systemic antimicrobials (Siala et al. 2014) may be helpful to inform the potential clinical utility of norspermidine as an adjunctive treatment for wound infections.

4 Conclusions

Biofilm formation is recognized as a significant pathogenic mechanism contributing to the development of chronic human infections. Herein, we demonstrate that the polyamine norspermidine has inhibitory and dispersive activity against clinical isolates of Gram-positive and Gram-negative multidrug-resistant bacteria obtained from persistent human infections. Norspermidine

appears to be particularly potent against biofilms of *A. baumannii*, presumably due to multiple mechanisms of biofilm inhibition. Our in vitro assessment suggests a potential risk for tissue toxicity from norspermidine at higher concentrations (5–20 mM), with more favorable biocompatibility at concentrations below 5 mM. While this may limit its suitability as a topical anti-biofilm agent, further studies should explore treatment strategies using norspermidine concentrations below 5 mM, with or without conventional antibiotics, to optimize the treatment of biofilm-related bacterial wound infections.

5 Methods

5.1 Bacterial Strains and Culture Conditions

Bacterial isolates used in this study were prospectively collected from wound infections, as defined by criteria of the U.S. National Healthcare Safety Network, of the upper and lower extremities of injured U.S. military personnel as part of the Trauma Infectious Disease Outcomes Study (Table 1) (Akers et al. 2014; Tribble et al. 2011). All isolates were originally collected from patients as a part of treatment and not related to research. All isolates were previously determined to be phenotypically positive for biofilm formation according to the crystal violet assay (Akers et al. 2014; Sanchez et al. 2013). Isolates were considered to be associated with persistent infections if they were recovered from the same anatomic site $\geq$14 days after the initial isolate of the same species. For this study, only isolates associated with persistent wound infections were selected to ensure that strains evaluated were likely to be biofilm producers *in vivo*, as well as *in vitro*, given the chronicity of infection. Organisms were defined as multidrug-resistant if they exhibited resistance to at least three of the major antibiotic classes (aminoglycosides, β-lactams, carbapenems, and fluoroquinolones) or produced either extended spectrum β-lactamases or *K. pneumoniae* carbapenemases. Bacterial isolates were recovered from frozen storage at −80 °C and sub-cultured on blood agar plates (Remel, Lenexa, KS) overnight at 37 °C prior to each experimental assay. Bacterial cultures were grown in cation adjusted Mueller-Hinton Broth (MHB-II) with agitation at 37 °C.

5.2 Reagents and Preparation of Norspermidine

Norspermidine (bis-(3- aminopropyl)-amine), putrescine, and spermidine were purchased from Sigma-Aldrich (St. Louis, MO). For use in experimental assays, norspermidine was diluted into MHB-II containing 100 mM HEPES buffer, to maintain a near-neutral pH, at the tested concentrations. Putrescine and spermidine were diluted in sterile water per the recommendations of the manufacturer.

5.3 Biofilm Formation and Dispersal/Inhibition Assays

Biofilm formation was examined under static conditions using the semi-quantitative 96-well plate (Corning, Inc., Corning, NY, USA) biofilm model as previously described (Christensen et al. 1985). Briefly, overnight bacterial cultures were diluted 1:100 in fresh MHB-II and individual wells were filled with 180 μL and incubated at 37 °C for 24 h. Following overnight incubation, plates were gently washed with 1X phosphate buffered saline (PBS; pH 7.4) to remove non-adherent bacteria and stained with 100 μL of 0.1 % (w/v) crystal violet (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Excess crystal violet was removed by washing with PBS, and resulting biofilm was quantified by measuring the corresponding OD_{570} nm of the dye following solubilization in 70 % ethanol (v/v). Biofilm biomass was determined by measuring the absorbance of solubilized stain at 570 nm using a microtiter plate reader.

To assess the biofilm dispersal activity of norspermidine, culture medium from biofilms

Table 1 Characteristics of bacterial strains used in this study

Bacterial species	Isolate[a]	Source	Specific site	Phenotype[b]	Pulsed-field Type (PFT)	Days from initial isolate	Biofilm former
Acinetobacter baumannii							
	AB 1	Wound	Lower leg	MDR		0	Y
	AB 2	Wound	Forearm	MDR	7	20	Y
	AB 3	Wound	Lower leg	MDR	50	21	Y
	AB 4	Wound	Upper arm	MDR	44	24	Y
Klebsiella pneumoniae							
	KP 1	Wound	Thigh	MDR		0	Y
	KP 2	Wound	Thigh	MDR	68	44	Y
	KP 3	Wound	Upper arm	MDR	67	15	Y
Pseudomonas aeruginosa							
	PA 1			MDR			Y
	PA 2	Wound	Forearm	MDR	26	14	Y
	PA 3	Wound	Thigh	MDR	25	47	Y
	PA 4	Wound	Thigh	MDR	29	16	Y
	PA 5	Wound	Thigh	MDR	32	17	Y
Staphylococcus aureus							
	SA 1	Wound	Bone	MSSA	USA 200	–	Y
	SA 2	Wound	Foot	MRSA	USA 100	206	Y
	SA 3	Wound	Foot	MRSA	17	20	Y
	SA 4	Wound	Knee	MRSA	16	91	Y

[a]Isolates collected from wound infections of the upper and lower extremities of injured U.S. military personnel as part of the Trauma Infectious Disease Outcomes Study (Tribble et al. 2011; Akers et al. 2014)
[b]A multidrug-resistant (*MDR*) organism was defined as an organism resistant to antimicrobials in 3 or more classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides, and quinolones) not including tetracyclines or colistin. *MSSA/MRSA*: methicillin-susceptible/-resistant *S. aureus*

was removed after 24 h, and 200 μL of culture medium supplemented with norspermidine in 100 mM HEPES buffer at the indicated concentrations was added into individual wells with the pre-established biofilm. After overnight treatment, plates were gently washed with PBS to remove unattached cells, stained with crystal violet, rinsed, and then solubilized with ethanol as described above. For assays evaluating the ability of norspermidine to inhibit biofilm formation, cells were grown as described above in the presence of media containing norspermidine at the indicated concentrations. Dispersive and inhibitory activity of norspermidine was reported as a percentage dispersal (or inhibition) relative to the untreated control group, exposed to media containing 100 mM HEPES buffer. For each clinical strain tested, biofilm assays were performed in triplicate with a minimum of three replicates per experiment, and the mean biofilm absorbance value of the replicates was determined.

5.4 Confocal Scanning Laser Microscopy (CLSM)

Bacterial biofilms were visualized using an Olympus FluoView confocal laser scanning microscope (Olympus, Pittsburgh, PA). Briefly, biofilms were grown as described above using glass chamber slides, as previously described

(Cardile et al. 2014). Following overnight treatment (inhibition or dispersal), biofilms were gently washed with sterile PBS, fixed for 10 min at room temperature with 4 % paraformaldehyde in PBS, and stained using Film Tracer ™ SYPRO® Ruby Biofilm Matrix Stain, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). CLSM images were acquired at 20X magnification using an argon laser (488 nm). Image analysis and Z-stacks were acquired using the Olympus FluoView software. Images were taken from three distinct regions on the slide, and representative images were selected for each treatment group.

5.5 Motility Assays

Bacterial motility was analyzed as previously described on 0.5 % agar TSB plates supplemented with 5 g/L tryptone, 2.5 g/L NaCl and 100 mM HEPES, pH 7 (Nait Chabane et al. 2014; Skiebe et al. 2012). Briefly, single bacterial colonies cultured on blood agar plates were selected with a sterile pipet tip. This tip was then used to puncture the agar to inoculate bacteria directly into the motility plate. Motility was analyzed after 24 h of growth at 37 °C, by measuring the radius, in millimeters (mm), of the motility zone of bacteria growing between the bottom of the agar layer and the polystyrene petri dish. Assays were repeated in triplicate.

5.6 RNA Isolation and Quantitative Real Time Reverse Transcription PCR (qRT-PCR)

To evaluate the effect of norspermidine on the expression of genes related to homoserine lactone synthesis (A1S_0109–A1S_0114) in *A. baumannii*, including a reference strain ATCC 17978 as well as three representative clinical isolates, biofilms were formed in 6-well tissue culture plates using previously described methods in the presence of 5 and 20 mM norspermidine (in 100 mM HEPES) for 24 h at 37 °C (Cardile et al. 2014; Malachowa et al. 2011). Following overnight incubation, supernatants were removed, individual wells were gently washed with PBS and attached bacteria (i.e. biofilm bacteria) were detached from wells by scraping with pipette tips and resuspended in 500 µL of PBS. Bacteria were harvested by centrifugation, washed, lysed, and the RNA was isolated using PureLink RNA Mini Kit (Life Technologies, Grand Island, NY) following treatment of samples with Bacterial-RNA Protect (Qiagen, Valencia, CA) as recommended by the manufacturer. Isolated RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Life Technologies), per the manufacturer's protocol. Quantitation of gene expression was via TaqMan (Life Technologies) methodology using the relative standard curve method. The gene-specific PCR primers (Table 2) were developed with Primer Express software (Life Technologies). Real-time quantitative PCR reactions consisted of the cDNA template (1.5 ng), 1X Universal PCR Master Mix for Gene Expression (Life Technologies), gene specific primers (900 nM) and probe (250 nM) in a total volume of 20 µL. Standard curves consisted of ten-fold dilutions of a positive control sample. PCR reactions were performed in triplicate and cycled in a 7900HT Sequence Detection System using standard protocols (Applied Biosystems, Grand Island, NY). Transcript levels were normalized to the expression levels of the internal control mRNA, Gyrase B (*gyrB*) and fold-regulation changes was calculated using $2^{-\Delta\Delta Ct}$ method.

5.7 Tissue Viability Evaluation

Tissues used in this study were harvested from the dermis of Sinclair Miniature pigs (*Sus scrofa*) which was provided post-mortem as part of a tissue sharing agreement and in compliance with an approved animal study protocol through the Animal Welfare Act and the implementing Animal Welfare Regulations. For the experiments in this study, no direct animal experimentation was involved. Briefly, porcine skin

Table 2 Quantitative real-time PCR (qRT-PCR) primers and probes used in this study

Gene	Primer/Probe name	Sequence
DNA gyrase	A1S_004-F	5′-GATGATGCGCGTGAAGGTTT-3′
	A1S_004-R	5′-GACGAGAATTTCGGATCAGGAA-3′
	A1S_004 PROBE	5′-ACAGCCATTATTTCTG-3′
Homoserine lactone synthase	A1S_0109-F	5′-GATTTTTCAAATCCGCCTTCCT-3′
	A1S_0109-R	5′-TGCAATTGAGACCGGTGATG-3′
	A1S_0109 PROBE	5′-AGCAGTCAGGCTGTG-3′
Acyl-CoA synthetase	A1S_0112-F	5′-GATCGGCTCGGTTTTGCA-3′
	A1S_0112-R	5′-GCGGCGACATGACATAACAA-3′
	A1S_0112 PROBE	5′-ACCGTATATGTGGGACTGA-3′
Acyl-CoA dehydrogenase	A1S_0113-F	5′-TCGCTACAGCTTATGCCTACCTT-3′
	A1S_0113-R	5′-TCGCGCACCAACAAGTGA-3′
	A1S_0113 PROBE	5′-TAGCGGCTGAGGTGTT-3′
Acyl-carrier protein	A1S_0114-F	5′-AAGCTTACTGGAGCGCAATCA-3′
	A1S_0114-R	5′-CAGGCTCTACGCGCATCTCT-3′
	A1S_0114 PROBE	5′-TCGTACGCTAGTCGCT-3′

explants were extracted from tissues using a 6 mm biopsy punch. Tissues were washed with PBS and then sterilized by 15 min exposures to 70 % ethanol solution followed by 0.615 % sodium hypochlorite solution prepared in PBS (Yang et al. 2013). Biopsied tissues (n = 6) were then exposed to norspermidine at the designated concentrations diluted in saline with 100 mM HEPES buffer for 24 h. Following exposure, tissues were washed twice with PBS, transferred to a 96-well plate, and incubated with MTT cell viability reagent (ATCC, Manassas, VA) for 2 h at 37 °C. Tissues were then transferred to individual wells of a 96-well plate containing dimethyl sulfoxide (0.07 mL) and incubated for an additional 1 h at 37 °C to extract the MTT reagent. Solubilized formazan was quantified by measuring the absorbance at 540 nm. As a positive control for the MTT assay, a group of tissue samples were heated to 200 °C for 5 min. The average of optical density values of the positive control were then subtracted from the absorbance values obtained for all other samples. Tissue viability of explants was expressed as the ratio between the observed OD_{540nm} and the weight of the explant in grams, and reported as a percentage to non-treated control as previously described (Castagnoli et al. 2003).

5.8 Histological Analysis and Immunohistochemistry

Excised tissue sections were fixed in 10 % neutral buffered formalin for 48 h, embedded in paraffin, and then cut into 7 μm cross-sectional slices. Slides were deparaffinized in xylene, rehydrated in water and stained with Weigert's iron hematoxylin solution kit (Sigma Aldrich, St. Louis, MO). Immunohistochemistry was performed as follows: heat-mediated antigen retrieval with 0.01 M citrate buffer at 95–98 °C for 15 min, blocking with 10 % horse serum in Hanks' balanced salt solution (HBSS) for 30 min at room temperature, followed by incubation with a 1:300 dilution of rabbit polyclonal antibody to caspase-3 (Abcam, Cambridge, MA) overnight at 4 °C. Following overnight incubation, slides were washed with HBSS and exposed to horse anti-rabbit biotinylated secondary antibody for 60 min at room temperature (Vector Labs, Burlingame, CA). Tissue sections were then incubated with Vectastain-RTU Kit solution and ImmPACT DAB Diaminobenzidine (Vector Laboratories) followed by counterstaining with hematoxylin and dehydration prior to cover slipping. Representative images of H&E and caspase-3 stained tissue sections were captured at 10 and 20 X magnifications using a Zeiss AxioScan Z1 slide scanner (Munich, Germany).

5.9 Cell Lines and Viability Assays

Human epidermal keratinocytes (HEK001; ATCC CRL-2404; ATCC, Manassas, VA, USA) were grown in keratinocyte serum-free medium (GIBCO, Grand Island, NY) supplemented with 5 ng/ml of human recombinant epidermal growth factor (EGF) and 2 mm of L-glutamine. Human dermal fibroblasts (PromoCell, Heidelberg, Germany) were grown in Dulbecco Modified Eagle Medium supplemented with 10 % fetal bovine serum, 10 U/mL of penicillin, and 10 μg/mL of streptomycin. Cell lines were grown and maintained at 37 °C in 5 % carbon dioxide. Cellular viability assays were performed as previously described (Barsoumian et al. 2013), which in brief consisted of exposing confluent monolayers of cells to norspermidine (1, 5, 25 mM in 100 mM HEPES buffer (pH 7)) diluted into the appropriate cell medias for up to 24 h at 37 °C in 5 % CO_2. Following exposure, cells were washed, resuspended in phosphate buffered saline (pH 7.4), and cell viability measured using Cell Titer-Fluor assay (Promega, Madison, WI, USA) as recommended by the manufacturer. As a negative control, cells were exposed to media containing only 100 mM HEPES buffer and viability reported as a percentage of the non-treated control group. Assays were performed at least twice with a minimum of three technical replicates per test condition.

5.10 Statistical Analysis

Where appropriate, statistical analysis was performed using an unpaired, two-tail student's *t* test or a One-way ANOVA with a Dunnett's post-hoc evaluation for comparison of the control group between multiple treatment groups. *P* values of <0.05 were considered to be statistically significant.

Authors' Contributions APC, RLW and CJS conceived and designed the experiments. RLW, SCB, RAG and CJS performed the experiments. APC and CJS analyzed the data. APC, KM, KSA, JCW and CJS contributed to preparation and review of the final manuscript. All authors read and approved the final manuscript.

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Competing Interests The authors declare that they have no competing interests.

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Factors Affecting Outcome of Tuberculosis in Children in Italy: An Ecological Study

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Abstract

INTRODUCTION: Tuberculosis is a major problem in children depending on their families for management and a re-emerging disease in low incidence countries, where foreign-born cases account for a large proportion of cases.

METHODS: We investigated socioeconomic features of families and their impact on management and outcome of children with tuberculosis disease seen at a tertiary care centre for paediatric infectious diseases in Italy.

RESULTS: Forty-nine Italian and 30 foreign-origin children were included. Children from foreign families had more complicated diseases (20 % vs 0 %; $P = 0.002$), harbored more drug resistant strains (20 % vs 2 %; $P = 0.011$), showed longer hospital stay (12 ± 13.1 vs 5.1 ± 6.5 days; $P = 0.012$) and higher proportion of missed medical visits (15.7 ± 16 vs 8.6 ± 9.6; $P \leq 0.042$) than those from Italian families. Harboring drug resistant strains was an independent risk factor for complicated disease course (OR: 72.98; 95 %CI: 1.54–3468.58; $P = 0.029$), and this risk is higher in children from Eastern Europe (OR: 10.16; 95 %CI: 1.7–61.9; $P = 0.012$).

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CONCLUSIONS: Children from immigrant families showed an increased risk of complicated course of tuberculosis due to a higher rate of resistant strains and raise problems in clinical management. Specific protocols are needed to support these populations ensuring easy access to health services and monitoring.

Keywords
Tuberculosis • Children • Foreign • Multiresistant

1 Introduction

Tuberculosis (TB) is the leading curable cause of death worldwide with a disproportionate burden between low- and high-income countries. In 2013, 9 million subjects developed TB disease and 1.5 million died worldwide (WHO/HTM/TB 2013). In Europe there is a long-term decreasing trend and 72,334 cases were reported in 2011 (Ducomble et al. 2013). Childhood TB has a relatively low burden, with approximately 40,000 notified pediatric cases between 2000 and 2009 (Sandgren et al. 2011). However, similar to other low incidence countries, in Italy an increase in the incidence of TB has been observed in children and adolescents in the last 10 years (Istituto superiore di sanità 2008). Social and economic factors may play a major role in the disease (Lin and Melendez-Torres 2016). Low income, ethnic factors and education have been associated with TB incidence (Harling et al. 2008; Mahomed et al. 2011), as lower education and unemployment are associated with delayed access to care and poorer outcome in adults with TB in developing countries (Obuku et al. 2012; Xu et al. 2005; Duarte et al. 2009). However, in European countries, little is known on the social, economic and ecological factors that may affect the risk of infection as well as the disease course, and even less is known about TB in children. Pediatric TB provides a reliable measure of the epidemiology and of local risk factors due to the shorter lag time between infection and clinical disease. In an ecological study in California, pediatric cases were selected and linked to census data, showing that lower incomes, higher ethnic heterogeneity, and more immigrants, had higher rates of tuberculosis (Myers et al. 2006). In low prevalence countries, TB is closely linked to immigration: about 30 % of children with TB living in the US are foreign-born, but two thirds of the US-born children have at least one foreign-born relative (Menzies et al. 2010). The impact of familial socioeconomic profile is virtually unknown in this setting but it is likely to have an important role in a disease where long-term therapy and a close follow up are needed.

Aim of our study was to compare the social and economic profile in two populations of immigrant and native children with TB disease seen at our pediatric reference Centre in Italy in order to identify possible factors affecting the course of the diseases.

2 Methods

We retrospectively reviewed the clinical records of consecutive children and adolescents with TB disease referred at our tertiary care centre for pediatric infectious diseases, between January 2009 and May 2015. The centre sees all children below 14 years and most of those below 18 years of a region with a resident population of five million people with a 15.5 % of people between 0 and 14 years in 2015 (Istituto nazionale di statistica 2013). All patients with a diagnosis of active TB disease were included in the analysis. Patients with comorbidities and those with latent TB infection were excluded. Latent TB infection is defined as a state of persistent immune

response to stimulation by *Mycobacterium tuberculosis* antigens, as proved by a positive skin test or by interferon-gamma release assays, without clinical evidence of clinically manifested TB (Mack et al. 2009). About one-third of global population is estimated to be infected with *M. tuberculosis* (Dye et al. 1999) and the majority has no signs or symptoms of TB, but has 5–10 % risk of TB diseases in the lifetime (Comstock et al. 1974). The minimum follow up was 12 months. Written consent to use information for a clinical study was asked and obtained for most patients enrolled in the study. For less than 15 % patients this was not possible since they were not available for contact or lost to follow up. However, all patients seen at our teaching/university hospital are asked to give their informed consent for use of personal and clinical data and this consent is routinely enclosed in the clinical records. Patient records were anonymised and all items were recorded by a medical student preparing his medical thesis. On the form that was examined there was no reference to personal or family identity.

The following social and economic data were extracted: parental origin and education, family income and parental employment, number of household residents, presence of elderly or immunosuppressed household residents or household residents with chronic cough. A clinical record attachment form was included in all clinical records of patients with suspected or confirmed TB. This form had to be filled out by clinicians at first admission. A score from 1 (very poor) to 5 (excellent) was given to estimate knowledge of Italian or English language. Foreign origin children were defined as those with at least one parent born in a country other than Italy.

The primary outcome was the presence of complicated TB disease. According to the WHO definitions (WHO/HTM/TB 2013) and to previous studies (Falzon et al. 2005; Yen et al. 2012), TB can be defined as complicated when culture positivity or clinical and/or radiologic findings of active TB are: *(i)* persistently present after 3 months of therapy or *(ii)* relapsed within 6 months after the end of the treatment or *(iii)* therapy was not administered for at least 2 months.

Secondary endpoints were: adherence to TB treatment, length of the hospital stay, and compliance to the scheduled visits during the follow up.

Adherence to TB treatment was assessed by the 4-question Morisky scale (Morisky et al. 1986). According to this scale, parents or adolescents were defined non adherent if they responded "yes" to at least one of the following questions in three repeated tests: (1) Do you ever forget to take your medicine? (2) Are you careless at times about taking your medicine? (3) When you feel better do you sometimes stop taking your medicine? (4) Sometimes if you feel worse when you take the medicine, do you stop taking it?

Drug resistance was assessed by standard drug susceptibility tests or, in case this was unavailable, by evaluating the clinical response and the detection of mutation in the rpoB gene by PCR, which is a reliable surrogate marker of multidrug resistance (MDR)(Caws and Drobniewski 2001).

The Student *t*-test, the χ^2 method, or exact Fisher's test were performed when appropriate. Univariate logistic regression was performed to analyze risk factors related to complicated disease course; statistically significant variables were then included in a multivariate model in order to identify independent risk factors. A *P*-value <0.05 was considered as statistically significant. Data were analyzed with IBM SPSS package version 20.

3 Results

Seventy-nine children and adolescents (41 boys; median age 72.3 months; range 2.2–17.8 months) with active disease were diagnosed in 6 years. The pediatric TB incidence normalized for total admissions at our Pediatric Department showed a progressive increase, from 74 (2009) to 180/100,000 hospital referrals (2014) (Fig. 1). Fifty (63.3 %) cases were symptomatic at the admission and the remaining children had positive chest radiography. There was no difference

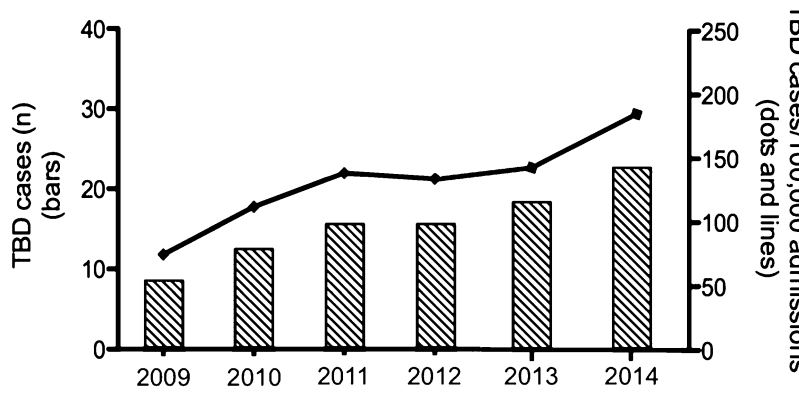

Fig. 1 Incidence of pediatric cases of tuberculosis disease at our tertiary care centre over time, expressed as cases per year (*bars*, left axis) and rate of TB disease cases per 100,000 hospital referrals (*dots* and *lines*, right axis)

Table 1 Symptoms reported and localization of tuberculosis disease in 79 children

Localization	n (%)	Symptoms	n (%)
Pulmonary	69 (87)	Fever	27 (34.2)
Lymph node	15 (19)	Chronic cough	27 (34.2)
Osteoarticular	4 (5)	Lymphadenopathy	18 (22.8)
CNS	2 (2.5)	Weight loss	8 (10.1)
Intestinal	2 (2.5)	Night sweat	5 (6.3)
		Haemoptysis	3 (3.8)

in the outcome parameters between symptomatic children and those diagnosed by radiology. Extrapulmonary manifestations were observed in 20 (25 %) patients. The disease localization and the symptoms reported are listed in Table 1. Biological specimens for culture were obtained in 43/79 (54 %) and *M. tuberculosis* was isolated in 53 % of them. Thirty children (38 %) had at least one foreign-born parent, although only 10 (12.7 %) children were born in a foreign country. Considering that foreign people account for only 2.8 % of resident population in Campania region (Istituto nazionale di statistica 2013), the observed TB disease incidence was about 21-fold higher in the foreign-origin than in the Italian-origin population. Forty-nine (62 %) children were Italian, while 30 (38 %) were of non-Italian origin. Among children of foreign origin, 16 (20.3 %) were from Eastern Europe (12 from Romania), 8 (10.1 %) from Africa, 4 (5.1 %) from Asia, and 2 (2.5 %) from South-America. Notably, 16 families were from Eastern Europe, 12 of which were from Romania, and 7 were of Roma ethnicity coming from Romania.

The socioeconomic features of the children's families according to their origin are shown in Table 2. A comparative evaluation showed that 49 Italian and 30 foreign-origin families did not differ in parental education, family income and employment rates. However, language skills were significantly different, with half of foreign parents having poor understanding of either Italian or English ($P < 0.0001$).

Local and foreign-origin children showed a different trend in the outcome parameters (Tables 3a and 3b). While all the Italian children had a favorable outcome, 6/30 (20 %) foreign-origin children had a complicated disease course ($P = 0.002$). Foreign-origin children also showed a higher rate of drug resistant TB (20 % vs 2 %; $P = 0.011$) and proportion of missed scheduled visits on follow-up (15.7 % ± 16.3 vs 8.6 % ± 9.6; $P = 0.042$). Likewise, hospital stay was longer in foreign-origin than in Italian origin children (12 ± 13.1 vs 5.1 ± 6.5 days; $P = 0.012$). No differences in the two cohorts were observed in the adherence to TB treatment. Seven children were infected with resistant strains, six of them of foreign origin: 5 (83.3 %) families were from Eastern Europe and 4 of them had MDR-TB. The resistance pattern is shown in Table 4. According to the univariate analysis Table 3b. the following were risk factors for running a complicated course: harboring drug resistant *M. tuberculosis*

Table 2 Characteristics of 79 children with tuberculosis and their families according to family origin

		Italian	Foreign origin	X^2/*t-value*	*P*
Age at diagnosis (months, m ± SD)		77.65 ± 53.2	63.07 ± 61.1	−1.107	.272
Household residents (n, m ± SD)		4.8 ± 1.5	4.04 ± 1	−2.046	.046
Gender n (%)	M	28 (57.1)	13 (43.3)	1.421	.255
	F	21 (42.9)	17 (56.7)		
Mother education n (%)*	Primary or less	16 (53.3)	9 (56.2)	0.036	.850
	Secondary or more	14 (46.6)	7 (43.7)		
Father education n (%)*	Primary or less	21 (72.4)	9 (56.2)	1.212	.331
	Secondary or more	8 (27.6)	7 (43.8)		
Income n (%)*	<20.000 €/year	22 (79.2)	21 (87.5)	1.162	.281
	≥ 20.000 €/year	7 (2.8)	3 (12.5)		
Language comprehension n (%)	Adequate (3 or more)	49 (100)	15 (50)	**30.242**	**<.0001**
		0 (0)	15 (50)		
	Inadequate (2 or less)				
Lag time from source identification (days, m ± SD)		33.2 ± 42	30.4 ± 16	−0.192	.849

*Data not available for all the enrolled patients

Table 3a Outcomes parameters of tuberculosis according to children origin

	Italian	Foreign origin	X^2/*t-value*	*p*
Complicated disease course* (n, %)	0 (0)	6 (20)	**10.605**	**0.002**
Resistant TB (n, %)	1 (2)	6 (20)	**7.432**	**0.011**
Hospital stay (days, m ± SD)	5.1 ± 6.5	12.04 ± 13.1	**2.598**	**0.012**
Missed scheduled visits (%, m ± SD)	8.6 ± 9.6	15.7 ± 16.3	**2.079**	**0.042**
Non adherence to TB treatment (n,%)	7 (25)	6 (14.3)	1.275	0.206

*Culture positivity or clinical and/or radiologic findings of active TB persisted after 3 months of therapy or relapsed within 6 months after the end of the treatment, or treatment omitted for at least 2 months

strains (OR: 46.7; 95 %CI: 5.9–363.7), Eastern European origin (OR: 10.16; 95 %CI: 1.7–61.9), at least one culture positivity for *M.tuberculosis* (OR: 8.0; 95 %CI: 1.2–52.79) and the presence in the household of people with chronic cough (OR: 7.3; 95 %CI: 1.07–50.4). However, multivariate analysis showed that harboring resistant strains rather than Eastern European origin, culture positivity, contacts with chronic cough is an independent risk factor for a complicated course (OR: 72.98 95 %CI: 1.54–3468.58; $P = 0.029$).

Nevertheless, children from Eastern European families showed a higher risk or resistant TB compared with children from other countries at either the univariate (OR:6.667; 95 %CI: 1.319–33.69; $P = 0.022$) or the multivariate analysis (OR: 10.6; 95 %CI: 1.63–68.9; $P = 0.013$), whereas Italian children were lower at risk of being infected with MDR-TB compared with foreign-origin children (OR: 0.083; 95 %CI: 0.009–0.732; $P = 0.025$). Interestingly, 6 children in our cohort were of Roma ethnicity: 4 of them harbored resistant strains of which, 3 had a complicated disease course. Roma children had a higher hospital stay (23.17 ± 20.86 days vs 6.72 ± 7.7 days; $P < 0.0001$) and a higher rate of missed scheduled visits (22.8 % ± 18.05 vs 10.75 % ± 12.59; $P = 0.037$) compared to the other children. Moreover, Roma ethnicity was independently correlated with hospital stay ($r^2 = 0.253$, $P < 0.0001$) and with the rate of missed visits ($r^2 = 0.073$, $P = 0.038$), suggesting that this subset of patients raise

Table 3b Univariate analysis of outcome determinants of tuberculosis

	Favourable outcome (n, %)	Complicated disease course (n, %)	*OR (95%CI)*	*p*
Extrapulmonary TB (n, %)	18/73 (24.7)	1/6 (16.7)	0.661 (0.07–5.58)	0.663
Mother education primary or less (n, %)	22/42 (52.4)	3/4 (75)	2.7 (0.26–28.4)	0.401
Presence of TB symptoms	46/72 (64.)	4/6 (66.7)	1.13 (0.19–6.6)	0.892
Presence of elderly at home	5/52 (9.6)	1/5 (20.0)	2.35 (0.22–25.32)	0.481
Cohabitants on cytostatic therapy	1/53 (1.9)	1/5 (20.0)	13 (0.68–249)	0.089
Cohabitants with chronic cough	9/53 (17)	3/5 (60)	**7.3 (1.07–50.4)**	**0.043**
Inadequate language comprehension (2 or less)	12/73 (16.4)	3/6 (50)	5.08 (0.91–28.3)	0.063
Successful case tracking	50/72 (69.4)	5/6 (83.3)	2.2 (0.24–19.9)	0.483
Culture positivity	18/71 (25.4)	5/6 (83.3)	8.0 (1.2–52.79)	**0.031**
Drug resistant TB*	3/70 (4.1)	4/6 (66.7)	**46.7 (5.9–363.7)**	**<0.001**
Non adherence to TB treatment	10/64 (15.6)	3/6 (50.0)	5.4 (0.95–30.6)	0.057
Eastern EU origin	12/73 (16.4)	4/6 (66.7)	**10.16 (1.7–61.9)**	**0.012**

*Drug resistant TB is the only variable proved to be an independent risk factor at the multivariate analysis (OR: 72.98; 95%CI: − 1.54–3468.58. $P = 0.029$)

Table 4 Features of the seven drug resistant TB cases

Gender/ age (years)	Country	TB site	Culture	rpbO PCR (RIF)	Resistance (patient)	Resistance (adult source)	Treatment	Outcome
F/5.6	Romania	CNS	−	+	−	INH, RIF, SPM, PZA	AMK, MFX, LIN, CS, EMB	Improved
M/12.2	Ivory Coast	Lung (cavitary)	+	−	INH	–	INH, RIF, PZA, EMB, PZA	Relapse, improved
F/1	Romania	Lung	+	+	INH, RIF	INH, RIF, SPM, PZA	INH, PZA, EMB, AMK, MFX	Relapse, improved
M/3.8	Romania	Lung	−	+	−	INH, RIF	INH, PZA, EMB, AMK, MFX	Improved
F/2	Peru	Lung	+	nd	INH, RIF, EMB	–	PZA, EMB, KAN, ETH, MFX, CS	Improved
M/1	Romania	Lung	−	+	−	INH, RIF, SPM, PZA	EMB, AMK, MFX, LIN	Relapse, improved
F/14	Italy	Lung	−	nd	–	RIF	INH, PZA, EMB, MFX	Improved

INH isoniazid, *RIF* rifampicin, *EMB* ethambutol, *SPM* streptomycin, *PZA* pyrazinamide, *AMK* amikacin, *MFX* moxifloxacin, *LIN* linezolid, *CS* cycloserine, *KAN* kanamycin

particular difficulties in management. Neither socio-economic factors nor other clinical variables were associated with a risk of complicated disease course. No correlation was found between family income, schooling or language skills, adherence to treatment, missed appointments, total hospital stay and any other outcome determinants.

4 Discussion

The epidemiology of childhood tuberculosis has a different pattern in high and low incidence countries, and in the latter immigration plays a pivotal role (Langlois-Klassen et al. 2011). Unlike the observed decrease in high incidence regions, pediatric notifications have increased between 2000 and 2009, by 6.1 % in children below 1 year of age and 7.4 % in those aged 1–4 years in low incidence countries, where about 30 % of all cases are of foreign origin (Sandgren et al. 2011). Our data showed an even more relevant increase of pediatric TB, with a 140 % increase in the incidence between 2009 and 2014 with about 40 % of children with active TB coming from families with at least one foreign parent. Prevention and control of TB is hindered in several European countries by the lack of specific measures to protect at risk populations such as refugees (Menzies et al. 2010; Guh et al. 2011; Mulder et al. 2012). We looked for possible risk factors related to geographical origin and socioeconomic features. Except for language skills, the socio-economic features of resident and immigrant population were similar, and confirmed a major role of deprivation as a general risk of TB. Although deprivation was similarly distributed in Italian and non-Italian families of affected children, a higher risk of mismanagement was detected in foreign-origin children. However, our data show that foreign origin children are more likely to harbor drug resistant strains and that drug resistance rather than socio-economic factors is closely related with a complicated course. Recently, a multicentre study has shown that the risk of drug resistance is about 4–20 times higher in immigrants than in Italian citizens (Fattorini et al. 2012), a pattern similar to other low incidence settings (Pasticci et al. 2012; Syridou et al. 2012). Our data support this pattern. Multidrug resistance was detected in 20 % of foreign origin cases and children harboring drug-resistant strains had a more severe course, as judged by their relapses and persisting symptoms and longer hospital stays. It is of note that in three out of seven drug resistant cases, culture was not obtained at time of first active TB episode and possible multidrug resistance was not recognized before a relapse occurred. We also showed that drug resistance, culture positivity and the origin from Eastern Europe were the only risk factors for a complicated disease course, even though only drug resistance proved to be an independent risk factor. According to the latest WHO report, a scale-up of rapid testing and detection of the multidrug resistant TB cases is urgently needed to stop the epidemic worldwide (WHO 2014). This can be achieved implementing drug susceptibility detection strategies in high-risk settings.

In addition to the higher incidence of drug resistance, foreign patients are prone to miss scheduled medical visits, suggesting a more difficult access to transports and social services. This probably contributes to the poorer outcome of the disease in foreign children, although there is no direct proof of such a link. However, reminders and recall policies may be routinely implemented in these vulnerable populations (Liu et al. 2014). Notably, in our sample a substantial proportion of families from Eastern European were of Roma ethnicity who often live in overcrowded and unhygienic conditions (Karim et al. 2012; Cluver et al. 2013; Tornee et al. 2003). Poor integration and access to healthcare could ultimately worsen the overall outcomes of TB.

However low adherence to treatment was a risk factor equally distributed in Italian and non-Italian children, and it can be speculated that the disease was perceived as mild by the parents of many affected children. In this field, education and counseling by nurses have proved more effective than counseling by physicians in

improving adherence (Casals et al. 2012; M'imunya et al. 2012). Finally, health care professionals should be aware of the frequent intrafamilial pattern of transmission, especially in immigrants. The high rate of infected children detected through case tracking in foreign origin families reflects the importance of looking for source cases in this setting, allowing optimal disease control and prevention, translation of drug susceptibility information from the source case to the affected child and the opportunity of immunization.

5 Conclusions

In foreign origin children the incidence of TB is markedly increased, mismanagement occurs more frequently and the disease course is more complicated compared to indigenous population. This complicated course is related to the presence of MDR strains rather than to socioeconomic features. On the other hand, in immigrant families there is a lower efficacy of medical care as judged by the longer hospital stay and more missed visits as a consequence of social and cultural background, so that cultural and epidemiologic factors act synergistically in this frail population. Effective medical and social protocols should be developed for at risk children to achieve a better control of TB. These should include a closer interaction between health care and social services, the presence of cultural-linguistic mediators, and the implementation of integrated strategies for effective management. This might include routine microbiological investigations with routine drug susceptibility tests, associated with clinical controls with close monitoring of adherence to therapy, check of recovery and case tracking.

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Genotyping and Antifungal Susceptibility of *Dipodascus capitatus* Isolated in a Neonatal Intensive Care Unit of a Sicilian Hospital

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Abstract

In August 2015, *Dipodascus capitatus* was isolated from two patients admitted to the neonatal intensive care unit. Nosocomial acquisition of the fungus was suspected and epidemiological studies were undertaken. The patients were simultaneously hospitalized, and the comparison of the two isolates by two independent molecular typing methods have confirmed clonal dissemination of a single strain of *D. capitatus*. Antimicrobial susceptibility testing was useful for identifying the appropriated antifungal therapy in micafungin. To our knowledge these are the first described cases of neonatal *D. capitatus* infection and also the first report of successful treatment by micafungin.

Keywords

Dipodascus capitatus • Nosocomial Acquisition • Genotyping • Antifungal Susceptibility

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1 Introduction

Dipodascus capitatus, the teleomorph form of *Geotrichum capitatum,* also known as *Blastoschizomyces capitatus* or *Magnusiomyces capitatus*, is ubiquitary in nature, and is occasionally part of the normal human microbiota (Guého et al. 1987; DeHoog and Vitale 2007). Phylogenetically this yeast is considered as an Ascomycota, and morphologically as Trichosporon spp. by its ability to produce anelloconidia and arthoconidia

It has been described as cause of invasive fatal infection in immunocompromised patients with hematological malignances and severe neutropenia (Girmenia et al. 2005, Lafayette et al. 2011). In these patients an invasive infection is rarely caused by *D. capitatus*, but if it occurs it is associated with unfavorable outcome and mortality rate exceeding 50% (Saghrouni et al. 2012).

The acute disseminated infection may be associated with skin lesions characterized initially by purpuric nodules progressing to centrally necrotic lesions, then early diagnosis and appropriate management will improve its prognosis (Trabelsi et al. 2015).

It may resemble invasive candidiasis but is associated with high bloodstream recovery rates, deep organ involvement, and an awful prognosis. In the disseminated infection the galattomannan antigen could be positive suggesting a false diagnosis of invasive aspergillosis (Özkaya-Parlakay et al. 2012). This type of geotrichosis is reported more frequently in Europe than in the USA (85% and 10% of cases, respectively). Furthermore, 87% of the European cases occurred in Italy (Central and Southern), Spain and France suggesting that climatic factors can influence the epidemiology of geotrichosis (Girmenia et al. 2005).

The most favorable therapy is not established because of limited data on antifungal susceptibility and on different therapeutic strategies. The efficacy of treatment for systemic therapy remains controversial: multidrug combination is considered a more efficient regime, even if there is no evidence that this is more effective than single drug regime (Özkaya-Parlakay et al. 2012). In vitro activities of amphotericin B, fluconazole, itraconazole and voriconazole have been investigated. High activity of amphotericin B was observed and poor susceptibility of some strains to fluconazole and itraconazole have been described (Girmenia et al. 2003, Venditti et al. 1991). In some cases, voriconazole or caspofungin are used as first-line therapy, or amphotericin B in combination with voriconazole (Giacchino et al. 2006).

In the present report, we emphasize the importance of achieving appropriate diagnosis through the investigation of rarely encountered fungi, such as *D. capitatus*, the difficulties of identification, the role of molecular typing and susceptibility testing.

2 Case Reports

2.1 Patient 1

A 28 weeks-gestation extremely low birth weight (600 g) male infant, with severe intrauterine growth restriction (IUGR) and mild respiratory distress, was referred to our Neonatal Intensive Care Unit (NICU) on day 9 of life for surgical management of suspected necrotizing enterocolitis, already in therapy with sulbactam/ampicillin, amikacin, metronidazole, ranitidine, and insulin. Blood culture obtained at admission was negative. Fluconazole prophylaxis (3 mg/kg/48 h) was started and dedicated total parenteral nutrition, via peripherally inserted central catheter (PICC) positioned in the birth unit, was maintained. He received platelets and blood transfusions for persistent thrombocytopenia and anemia, but never presented neutropenia.

On day 21 of life, because of persistent abdominal distension, a gastrografin enema was performed and demonstrated meconium ileum. After 5 days, when baby's weight reached 800 g, an exploratory laparotomy and a temporary ileostomy were performed. In the 48 h after surgery, the baby developed clinical symptoms

of infection with fever and pulmonary floccular infiltration at chest X-rays, treated with teicoplanin and meropenem; PICC was then removed and replaced on day 28 of life. PICC culture developed fungal growth which was 5 days later further identified as *D. capitatus*. Because of initial evidence of in vitro resistance to amphotericin B (by Kirby-Bauer method), and limited spectrum of tested antimycotic drugs suitable for neonates, fluconazole was continued at treatment dosage (6 mg/kg/die) until results of another antimicrobial susceptibility testing. On day 38 of life, intravenous micafungin (8 mg/kg/die) was started, according to the second antimicrobial susceptibility testing (colorimetric microdilution test). Targeted antifungal treatment appeared to be effective with regression of clinical symptoms and full recovery of X-ray pattern. *D. capitatus* was not further detected in the subsequent samples (PICC and blood culture). On day 60 of life, patient was back-transferred to birth NICU for laser treatment of retinopathy of prematurity and died at 5 months of life for complications of severe prematurity.

2.2 Patient 2

A male term neonate with prenatal diagnosis of left-sided congenital diaphragmatic hernia (CDH) was born by caesarean section in our unit in the same week (Assumption day holiday period) when patient 1 claimed the onset of clinical symptoms of infection. He presented a polymalformative condition with CDH, narrow thorax, pulmonary hypertension, severe IUGR, postaxial polydactyly, dysmorphic facial features, blue sclerae, congenital hypothyroidism, no hematological abnormalities. Further investigations showed normal karyotype, array comparative genomic hybridization and faciogenital dysplasia 1 gene analyses. Blood culture collected at birth was negative. An umbilical venous catheter (UVC), inserted at birth to guarantee parenteral nutrition, was replaced on second day by PICC. The baby was permanently depending on assisted ventilation and antibiotics (ampicillin/sulbactam and gentamicin) were administered since birth.

On day 3 of life, surgical correction of CDH was performed and fluconazole prophylaxis (3 mg/kg/48 h) was started. Because of funguria on the next day, fluconazole dosage was increased to 6 mg/kg/die and intravesical transcatheter instillation of amphotericin B was performed.

After 5 days of incubation, fungal growth from UVC culture was observed, and the isolates were further identified as *D. capitatus*. On day 15 of life, targeted antifungal treatment with intravenous micafungin (8 mg/kg/die) was started, according to antimicrobial susceptibility testings (Kirby- Bauer and colorimetric microdilution test). The patient died two days later because of severe pulmonary hypertension as a complication of his multiple congenital malformations. On cultures performed at death, *D. capitatus* was not isolated from PICC and pleural fluid but only from endotracheal tube, likely as a sign of environmental contamination.

3 Methods

PICC from patient 1 and UVC from patient 2 were routinely cultured on Columbia blood agar and Sabouraud dextrose agar. The colonies grown on plates were further inoculated to CHROMagar-Candida (Beckton Dickinson, Heidelberg, Germany), and were identified by microbiological investigations and sequence analysis.

Sequencing analysis of the Internal Transcribed Spacer (ITS) of 18S rDNA in the rRNA gene was performed to ensure identification of these organisms according to (Subramanya Supram et al. 2016).

The sequence data were analyzed using the National Center for Biotechnology Information (Bethesda, Md., USA) BLAST system (available at http://www.ncbi.nlm.nih.gov/BLAST/).

Two techniques were used for molecular typing, the random amplification of polymorphic DNA (RAPD) method and PCR fingerprinting. The RAPD primer selected for genotyping was OPE-4 (5- GTGACATGCC-3). The PCR fingerprinting technique was performed with the

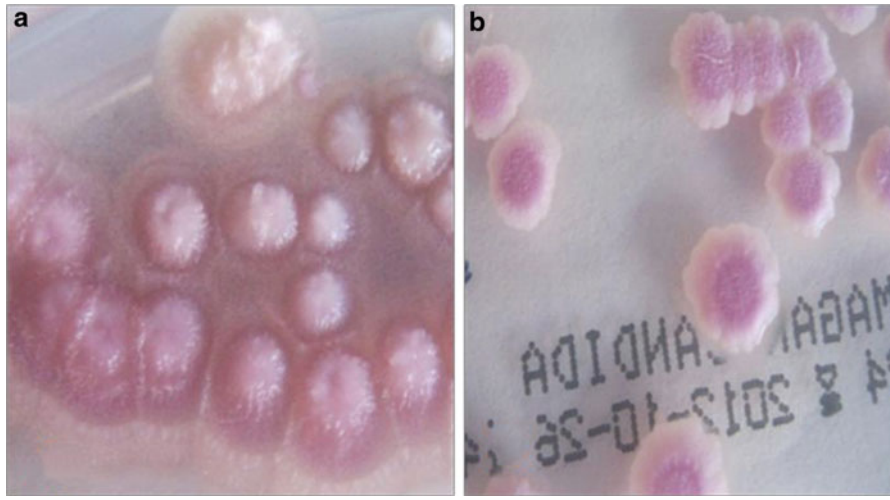

Fig. 1 *D. capitatus* (**a**) and *C. krusei* (**b**) on CHROMagar-candida

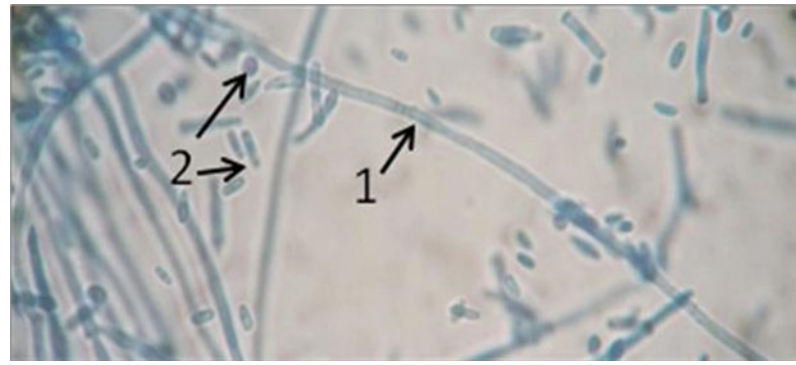

Fig. 2 Presence of septate hyphae (*1*) and cylindrical or clavate conidia (*2*) observed in lactophenol cotton blue preparation

core sequence of phage primer M13-GAGGGTGGCGGTTCT-' as a single primer according to Gadea and Erzoz. (Gadea et al. 2004; Ersoz et al. 2004).

Typing techniques were performed at least two times on separate days. Band patterns were electrophoresed through 1.2% agarose gels (Pronadisa, Madrid, Spain), stained with ethidium bromide (Sigma Aldrich Química), and photographed under UV light by Gel Doc BIORAD. Finally, antifungal susceptibility of the yeasts was determined both by Kirby-Bauer method that by colorimetric microdilution test (SENSITITRE® YEASTONE®), and the results were interpreted according to the breakpoints of CLSI guidelines M27-S4 (CLSI 2012).

4 Results

After 5 days at 30 °C the colonies on Sabouraud dextrose agar were whitish, glassy, and funiculose with a smooth expanding zone. The morphotype on CHROMagar-Candida showed white-pink and wrinkled and fimbriate colonies that were easily distinguishable in color and morphology from those of *C. krusei* (Fig. 1).

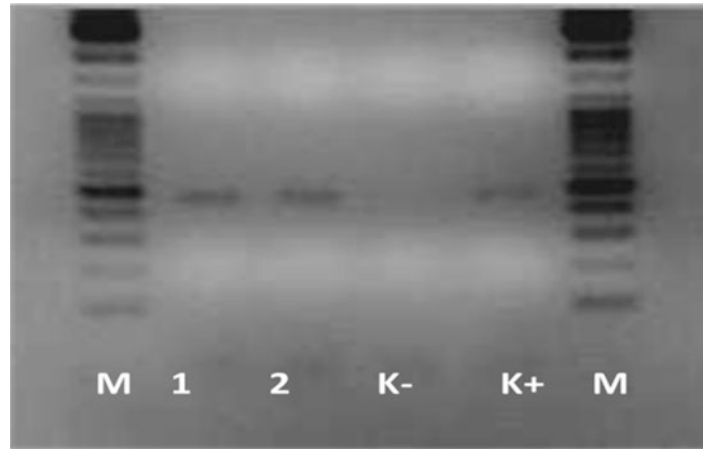

Fig. 3 PCR for 18S rDNA, M ladder 100 bp Fermentas, lane 1 for patient 1, and lane 2 for patient 2, K- and K+ control negative and positive

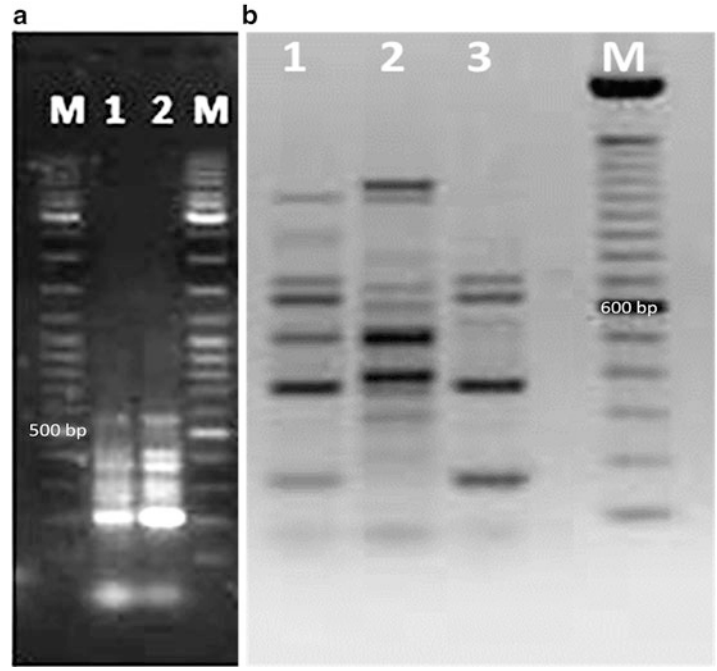

Fig. 4 (**a**) Pattern obtained with primer M13: lane M ladder 100 bp Fermentas, lane 1 for patient 1, and lane 2 for patient 2. (**b**) Pattern obtained with primer M13 (ladder 100 bp Invitrogen) of strains isolated from three adult patients in the same hospital in 2012

Microscopic examination, with lactophenol cotton blue preparation, revealed hyphae and conidiophores creeping or ascending, profusely branched at acute angles with conidiogenous cells which formed long, cicatrized stems of hyphal growth (rachides) on which the conidia were born. Conidia were cylindrical to clavate, with a rounded apex and flat base. Also, arthroconidia and endoconidia were often present (Yarrow 1998) (Fig. 2).

The PCR for gene ITS revealed a template with molecular weight equal to 480 bp as showed in Fig. 3

The DNA sequences of all the isolates were completely matched to that of *D. capitatus* (*G. capitatum*) from the Gen Bank DNA database (Accession number AY788305).

Table 1 Minimum inhibitory concentration (μg/ml) of antifungal drugs displayed against isolates obtained by colorimetric microdilution test and Kirby-Bauer

	1G	2C
MIC (μg/ml)		
AB ≤1 (S)	1	1
5FY ≤ 1 (S)	0,5	0,5
AN ≤ 2 (S)	2	2
CAS ≤ 2 (S)	3	3
MIC ≤ 2 (S)	2	2
FLC 16–32 (SD)	32	32
ITC ≤ 2 (S)	1	1
POS ≤ (S)	1	1
VRC ≤ 2 (SD)	1	2
Kirby-Bauer Alone size		
AB (20 μg)	8	8
KCA (10 μg)	22	22
ECN (10 μg)	24	24
NY (100 μg)	24	22
MCL (10 μg)	22	27
FLC (100 μg)	12	12

AB amphotericin B, *5FY* 5 fluorocytosine, *AN* anidulafungin, *CAS* caspofungin, *MIC* micafungin, *FLC* fluconazole, *ITC* itraconazole, *POS* posaconazole, *VRC* voriconazole *KCA* Ketoconazole, *ECN* econazole, *NY* nystatin, *MCL* miconazole, *S* susceptible, *SD* susceptible dose dependent

Therefore, all the isolates were identified as *D. capitatus*. The Patterns obtained with primer M13 are shown in Fig. 4. The identifying of the isolated strains was also confirmed by RAPD PCR with primer OPE-4. The discriminatory power was comparable for each of the two primers. The profiles were reproducible between different DNA preparations from the same strain as well as between runs when samples were run a second or a third time. This would indicate that the two cases of disseminated infection caused by *D. capitatus* could be related epidemiologically.

In addition, the genome profiles were compared with those of three other *D. capitatus* isolates, that acted as control organism, performing RAPD PCR and PCR fingerprinting. These controls were not temporally related and were isolated from sputum of adult patients that were hospitalized in the hematologic and surgical wards of the same hospital in 2012 (Fig. 4b).

The susceptibility results for *D. capitatus* strains performed by Kirby-Bauer (K-B) method showed the two strains resistant to amphotericin B but susceptible for micafungin, econazole, ketoconazole, and nystatin. Fluconazole showed intermediated sensitivity. The results of the colorimetric microdilution test, used as confirmation test, are displayed in Table 1. Both *D. capitatus* isolates showed homogeneous results in antifungal susceptibility testing. Differences in voriconazole MICs were not clinically significant. While the resistance to amphotericin B obtained by K-B method was not confirmed.

5 Discussion

D. capitatus infection is rare but can cause life-threatening invasive infections with high morbidity and mortality (Trabelsi et al. 2015). The septicemia is frequently not responsible for clinical signs and symptoms of yeast infection, and respect to candidemia it develops more frequently deep organ dissemination as secondary localization (60–80% vs 10–20% of patients) (Miglietta et al. 2015).

During septicemia, *D. capitatus* produces a soluble antigen cross-reactive with the enzyme immunosorbent assay for *Aspergillus* that offers high sensitive diagnostic tool in managing invasive infection, but determines false-positive results versus invasive aspergillosis (Trabelsi et al. 2015).

The diagnosis of *D. capitatus* invasive infection appears to be easier compared with other fungal infections because hemoculture is positive in more than 70% of invasive cases versus 50% of *Candida* spp. and 10% of *Aspergillus* spp. cases (Girmenia et al. 2005).

The molecular typing is relevant in producing information on genetic variability of isolates and useful in recognizing the source of infection.

We report the up to date first isolation of *D. capitatus* in neonatal age. Our patients were critically ill newborns in NICU with severe general conditions characterized by fetal malnutrition, poor immune defenses, need of empirical broad spectrum antibiotics, intensive use of

multiple invasive devices, and moreover had undergone high-risk neonatal surgery. These characteristics present some overlapping with those of other pediatric and adult patients reported in the literature (Girmenia et al. 2005) and may be triggering factors for the onset of *D. capitatus* infection.

Both our patients seem to confirm that the *D. capitatus* acts as an opportunistic agent responsible for invasive infection only if host defenses are insufficient. As well as reported in adults with haemato-oncological or other severe diseases, critical general conditions which determine acidosis, cardiopulmonary insufficiency, poor immune response and low physical and biological defenses may predispose to *D. capitatus* invasive infection (Viscoli et al. 1999).

In our patients, we identified the isolates performing both phenotypic and genotypic evaluation, and we demonstrated the same molecular profile of these two isolates at migration patterns.

History of both patients demonstrated a likely epidemiological relationship. Patient 1 (outborn) being the index case was transferred from a NICU of another hospital and then patient 2 (inborn) has acquired the same microorganism in our NICU soon after birth, which occurred 15 days after admission of patient 1.

We suppose that patient 1 has been exposed to a pre-transfer PICC contamination by *D. capitatus* in the birth NICU, without classical sign and symptom of infection until a trigger factor (such as main abdominal surgery in an 800 g newborn and its general consequences) allowed invasive infection with emergence of systemic signs (fever, acidosis, pulmonary floccular infiltrations) that lead to diagnosis and identification of *D. capitatus* as the responsible etiologic agent. It is possible that in the pre-clinical period *D. capitatus* has been cross-transmitted from patient 1 to patient 2, likely through instruments or hands of health care workers. Many other concurrent factors may have played a role in allowing such transmission: the first-line use of fluconazole in neonatal unit which demonstrated to be not protective versus *D. capitatus* infection that can therefore easily spread, and the concomitant period of overcrowding (with many newborns in the NICU) and understaffing (because of personnel shortage during the Assumption week holidays in August) that increases the risk of cross-infections. After evidence of isolation of *D. capitatus*, infected newborns were immediately isolated and contact precautions, hand hygiene and sterile procedures have been implemented in the NICU. No other cases of infection by *D. capitatus* were registered.

We cannot exclude that *D. capitatus* has been imported from the former hospital but we do not have any clear evidence because no microbiological investigations have been performed prior to transfer. By typing the two cases confirm the emergence of *D. capitatus* as an opportunistic agent in which the use of central vascular catheters can be identified as a major risk factor of infection. Other cases of geotrichosis by contamination of central venous catheter were described but in adult patients with acute leukemia or polytraumatism (D'Antonio et al. 1994; Trabelsi et al. 2015).

D. capitatus was not responsible for the outcome of our patients. Patient 1 fully recovered from acute abdomen and fungal infection after introduction of adequate antimicrobial treatment, therefore he was back-transferred to the former hospital and died at 5 months of age for complications related to severe prematurity and IUGR. Patient 2 died at 18 days of life because of multiple congenital anomalies and subsequent pulmonary hypertension, before adequate antimicrobial treatment could demonstrate its efficacy.

There is no general optimal treatment strategy for *D. capitatus* infections. In vitro antifungal susceptibility findings are reported contradictory to those observed in the clinical practice (Arendrup et al. 2014). However, the evidence of in vitro intermediate sensitivity to fluconazole suggests that prophylaxis with fluconazole (whose efficacy and safety in preventing Candida infection in high risk newborns has been largely demonstrated by Kaufman 2010) is not protective versus *D. capitatus* infection, that can therefore easily spread.

6 Conclusion

Therapeutic choice against *D. capitatus* in newborns is very limited because most antifungal drugs cannot be used at this stage of life. Amphotericin B and at least in our cases micafungin appear to be the most appropriate and safe drugs in newborns and should be used as first choice treatment.

Low number of clinical reports do not allow to establish appropriate therapeutic protocols, and further studies are necessary to acquire data and to identify efficient target therapy.

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The Correlation Between Biofilm Production and Catheter-Related Bloodstream Infections Sustained by *Candida*. A Case Control Study

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Abstract

Biofilm forming capacity of yeasts colonizing the intravenous devices is considered a key factor involved in the pathogenesis of *Candida* - catheter-related bloodstream infections (CCRBSI). The biofilm production of strains of *Candida* spp. isolated both from the CVC and from the blood of patients with CCRBSI was compared to that of strains isolated from patients not having CCRBSI. Results, expressed in terms of Biofilm Index (BI), revealed that biofilm-producing strains were isolated in the CCRBSI group with a frequency significantly higher than in the non-CCRBSI group ($\chi^2 = 4.25$, $p = 0.03$). The species more frequently cultured was *C. parapsilosis* complex (including *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*). When this species was isolated from the CVC tip cultures of the CCRBSI group it showed BIs significantly ($p = 0.05$) higher than those found in the non-CCRBSI group. All the strains of *C. tropicalis* isolated from the CCRBSI group produced biofilm. Instead most of the isolates of *C. glabrata* were non-producers. The cumulative BI of non-albicans *Candida* strains isolated from CCRBSI patients was significantly higher than that of non-albicans strains cultured from patients non-CCRBSI ($\chi^2 = 6.91$; $p = 0.008$). *C. albicans* was a biofilm producer both in the CCRBSI and in the non-CCRBSI group. When isolated from the blood it showed enhanced biofilm production in the CCRBSI group only, while when

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colonizing the CVC it displayed high BIs both in the CCRBSI group and in non-CCRBSI group. Our data seem to indicate that the biofilm production capacity should be considered in the clinical management of CCRBSI.

Keywords
Biofilm • *Candida* catheter-related bloodstream infections • Biofilm Index

1 Introduction

The bloodstream infections (BSI) are associated with high mortality rates, enhanced cost of care, and increased duration of hospitalization (Bassetti et al. 2014). Candidemia has been identified as one of the leading cause of BSI worldwide (Pfaller and Diekema 2007; Wisplinghoff et al. 2004; Tortorano et al. 2006; Bouza et al. 2015). The presence of intravascular devices such as central venous catheter (CVC) is one of the most common risk factors for the development of candidemia (Kojic and Darouiche 2004; Lynch and Robertson 2008) and the suggested therapeutic approach for the patient with candidemia may lead to catheter removal (Mermel et al. 2009). Furthermore, the sessile growth of *Candida* organisms embedded in the biofilm on medical devices has been frequently associated to candidemia (Kojic and Darouiche 2004; Douglas 2003).

The incidence of biofilm-producing *Candida* strains recovered from the blood was reported to be 83.3% (Bouza et al. 2015), with *C. parapsilosis* complex and *C. albicans* accounting for the species most frequently involved (Escribano et al. 2013), the latter scoring 3rd among the microorganisms causing catheter-related-BSI (Kojic and Darouiche 2004). The presence of an indwelling venous catheter is considered a key predictor for lethality and hospital length of stay in the patients infected with biofilm forming yeasts. Indeed, biofilm generating strains of *Candida spp*. have been associated with high mortality rates. (Estivill et al. 2011; Tumbarello et al. 2007; Trofa et al. 2008; Quindós 2010).

Aim of this study was to ascertain the capacity of strains responsible for *Candida*-catheter-related-BSIs (CCRBSI) to produce biofilm, and to compare it to the production by yeasts isolated from patients not affected by CCRBSI.

2 Materials and Methods

2.1 Data Collection and Study Design

This case-control study was conducted from January 2011 till June 2015 in patients hospitalized in the medical and surgical wards, as well as in the intensive care units (ICU) of a 1.300 beds-multiple building, tertiary care, University Hospital (Policlinico Umberto I of Rome, Italy) with an average admission of 38.000 patients/year. Strains of *Candida* spp. were collected from patients affected by *Candida*-catheter-related bloodstream infection (CCRBSI) defined according to microbiological criteria, when the cultures of both catheter tip and blood derived from a peripheral vein yielded the growth of the same species of *Candida* with identical antifungal profile. Only the first episode of CCRBSI was recorded in patients with recurrent or subsequent episodes of the infection, and only one strain from both blood and CVC was considered in the experiments. Strains isolated from patients with either blood or CVC tip culture positive for *Candida* spp., hospitalized during the same period of time, not matching the aforementioned microbiological criteria for CCRBSI served as a control (non-CCRBSI). The controls were selected by an internal

database according to the parameters of sex, age, and the ward of hospitalization (Intensive Care Units ICU, General Medicine and Surgery wards). Strains cultured from neutropenic patients i.e. those hospitalized in the onco-hematology unit were not included in the study. Written patients consent was not required because of the observational nature of this study. The blood samples and CVCs were drawn by the healthcare professionals at the hospitalization site of the patients and sent to the laboratory for analysis, and therefore the authors did not collect clinical samples. Patient characteristics were collected from an electronic database. These records and information were anonymized by laboratory staff members not involved in the study.

2.2 Microbiological Methods

Blood samples were drawn in Bactec Mycosis IC/F or Bactec Plus Aerobic Broth and incubated in an automated culture system Bactec 9420 (Becton-Dickinson, Inc., Sparks, MD). Positive samples were inoculated on Sabouraud dextrose agar supplemented with chloramphenicol (Oxoid spa, Milan, Italy) and cultured for at least 24 h at 37 °C. Catheter tips were microbiologically examined within 15 min after removal, according to the quantitative culture method of Cleri et al. (1980) with slight modifications. In brief, approximately 5 cm of the distal part of the CVC was placed in 1 ml of brain-heart-infusion broth and vigorously shaken on a vortex for 30 s. A total of 10 μl of the suspension was then streaked on agar plates. After incubation at 37 °C for 24 h, any growth of *Candida* was identified, both by API ID 32C System (Biomerieux Diagnostic System, Inc., St. Louis, MI), and by MALDI-TOF technique (Bruker Daltonik GmbH, Bremen, Germany) performed in duplicate, accepting score values $\geq$1.8 (Ghosh et al. 2015; Pinto et al. 2011). Susceptibility of the yeasts to the antifungal drugs was tested through the Sensititre Yeast One 10 (Trek diagnostic systems, East Grinstead, West Sussex, UK) according to the manufacturer's instructions.

2.3 Biofilm Production

The formation of metabolic active fungal biofilm of the isolated strains was measured in vitro by means of XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H–tetrazolium-5-carboxanilide, Sigma-Aldrich s.r.l., Milan, Italy] reduction assay (Ramage et al. 2001) with slight modifications. Briefly, each *Candida* strain in the log-phase of growth (200 μl of 10^6 cells/ml in RPMI 1640) was plated on flat-bottomed 96-well polystyrene microtiter plates and incubated at 37 °C for 48 h. After incubation, the plates were washed three times in sterile PBS to remove planktonic cells, and each well was added with 100 μL of XTT-menadione (0.67 g/L XTT, 1 μM menadione, Sigma-Aldrich). The resulting color change was spectrophotometrically quantified at 490 nm with a microtiter plate reader (Eti System Reader, Bio-Tek Instruments Inc., Winooski, VT, USA). In separate wells, 200 μl of RPMI 1640 medium was used as the negative control, whereas 100 μl of a 50 mg/mL solution in water of phenazine methosulfate (Sigma Aldrich) served as positive colorimetric control. Results were expressed in terms of Biofilm Index (BI), relative to a control biofilm producer strain of *Candida albicans* (*C. albicans* SA40, kindly provided by Flavia De Bernardis) (Sandini et al. 2011), according to the following formula: BI = (absorbance test strain)/[0.5 (absorbance *C. albicans* SA 40)] (Ceccarelli et al. 2016). All experiments were performed in triplicate. The species were defined producer if BI was $\geq$0.5.

2.4 Statistical Analysis

The incidence rate of the CCRBSI was calculated as the ratio between the number of new cases reported in the wards and the total number of patients hospitalized in the same ward each year, and expressed as the number of episodes/1000 admissions. The mean ($\pm$SD) biofilm production was calculated for each species. Statistical analysis was performed using Stata software

11. Proportion comparisons for the categorical variables were done using Chi-square tests of Pearson. Significance was set at $\alpha < 0.05$.

3 Results

3.1 CCRBSI Distribution

Ninety-eight patients (averaging 57.20 ± 21.5 years, 57.14% male) presented the microbiological criteria for CCRBSI during the study period, showing the growth of the same species of *Candida* with identical antifungal profiles both from the blood and from the CVC tip cultures. The overall incidence was of 0.73 episodes/1000 admissions, with an incidence in the ICU higher than that found in the surgical and in the medical wards (6.85; 0.29; and 0.1 episodes/1000 admission, respectively). The species more frequently isolated were *C. parapsilosis* complex (including *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*, 42.8%) followed by *C. albicans* (34.7%). The incidence of isolation of *C. parapsilosis* complex and *C.albicans* in the ICU (3.6 episodes/1000 admission and 2.23 episodes/1000 admission respectively) was higher than that observed in the medical and surgical wards, while *C. tropicalis* and *C. glabrata* were isolated in the ICU and in the surgical wards only [Table 1].

Table 1 Frequency of isolation in Candida catheter-related bloodstream infection in different wards

Frequency/1000 admission	Medicine	Surgery	ICU
C.parapsilosis complex	0.1	0.1	3.6
C. albicans	0.06	0.11	2.23
C. glabrata	0	0.04	0.51
C. tropicalis	0	0.06	0.17
Other species	0.02	0.007	0.34

Data are expressed as episodes/1000 admissions

3.2 Biofilm Production

The biofilm production of the isolated *Candida* strains by was measured by means of XTT reduction assay and the results were expressed in terms of Biofilm Index (BI). In the aggregate analysis of all the isolated yeasts, the proportion of biofilm producing strains was significantly higher than that of the non-biofilm producing fungal cells ($\chi^2 = 74.39$; $p < 0.001$). *C. albicans* and *C. tropicalis* had a significantly ($p < 0.05$) higher proportion of biofilm producing strains [Fig. 1]. Up to 36% of the isolates of *C. parapsilosis* complex did not produce biofilm, whereas all the strains but one of *C. glabrata* were non-producers.

To find out differences in the biofilm production capacity between the CCRBSI and non-CCRBSI isolates, experiments were performed on 86 *Candida* spp. strains isolated both from the CVC and from the blood of 43 out of the 98 patients with CCRBSI. Results were compared to the BIs of 86 *Candida* strains isolated either from the CVC (n°= 43) or from the blood (n°= 43) of patients non-CCRBSI. *Candida* isolates of the CCRBSI group showed identical BIs both for the strains isolated from the blood and for those isolated from the CVC tip culture, with a statistically significant correlation (95% of the cases, $\chi^2 = 243.62$; $p < 0.001$). The biofilm-producing strains were isolated more frequently, and with a significantly enhanced production in the CCRBSI group relative to the non-CCRBSI group ($\chi^2 = 4.25$, $p = 0.03$) [Fig. 2]. No significant differences were observed in the biofilm production by strains of *C. albicans* isolated from both the CCRBSI and from the non-CCRBSI group. However, when the strains of *C. albicans* were isolated from the CVC tip culture, BIs higher than those found for the other species were observed both in the CCRBSI group ($\chi^2 = 35.97$; $p < 0.001$) and in non-CCRBSI group ($\chi^2 = 5.18$; $p = 0.03$). Furthermore, when the strains of *C. albicans* were isolated from the blood cultures, an enhanced biofilm production was observed in the CCRBSI

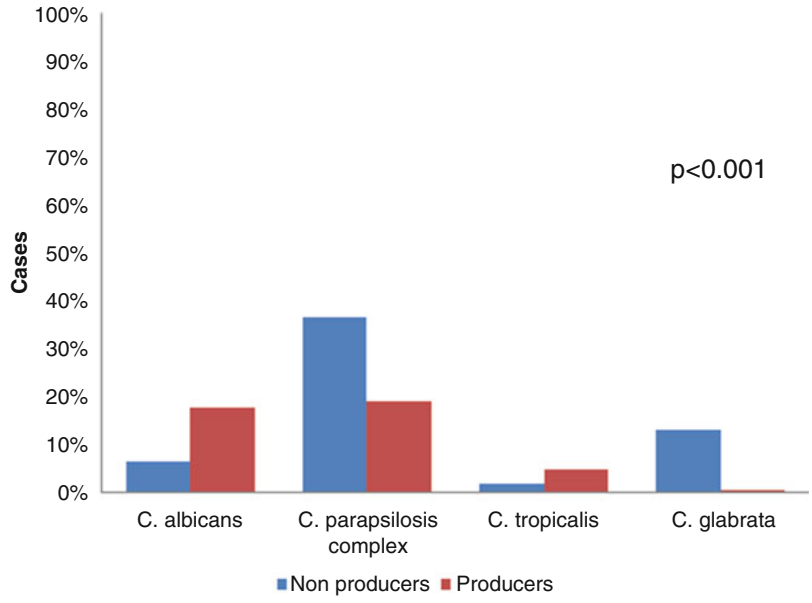

Fig. 1 Biofilm production by the different *Candida* species. In the aggregate analysis the percentage of biofilm producing strains was significantly higher than that of the non-biofilm producing fungal cells ($\chi^2 = 74.39$, $p < 0.001$)

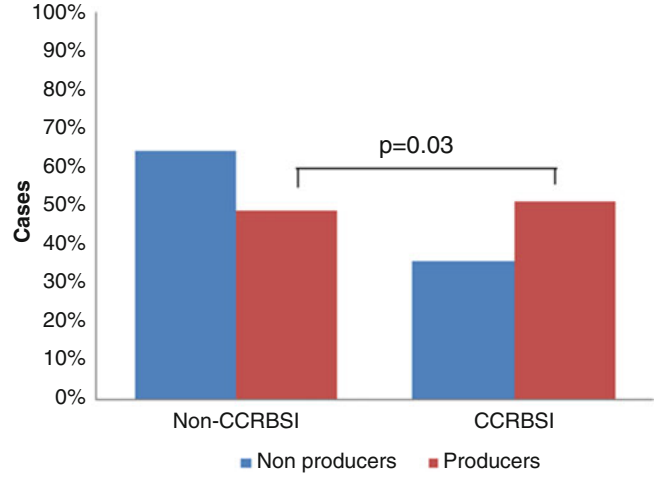

Fig. 2 Biofilm production by the strains isolated from the CCRBSI and non-CCRBSI study groups. The biofilm-producing strains were isolated more frequently, and with a significantly enhanced production in the CCRBSI group relative to the non-CCRBSI group ($\chi^2 = 4.25$, $p = 0.03$)

group only ($\chi^2 = 19.05$; $p = 0.08$ vs $\chi^2 = 10.52$; $p = 0.03$). Strains of *C. tropicalis* produced biofilm almost exclusively when isolated from the CCRBSI group ($\chi^2 = 4.95$; $p = 0.02$) [Fig. 3]. Moreover, strains of *C. parapsilosis* complex isolated from the CVC tip culture of the CCRBSI group showed BIs significantly higher than those found in the non-CCRBSI group ($\chi^2 = 3.6$; $p = 0.05$) [Fig. 4]. Altogether, the cumulative BI of non albicans *Candida* strains isolated from CCRBSI patients was significantly higher than that of non albicans strains isolated from patients non-CCRBSI ($\chi^2 = 6.91$; $p = 0.008$).

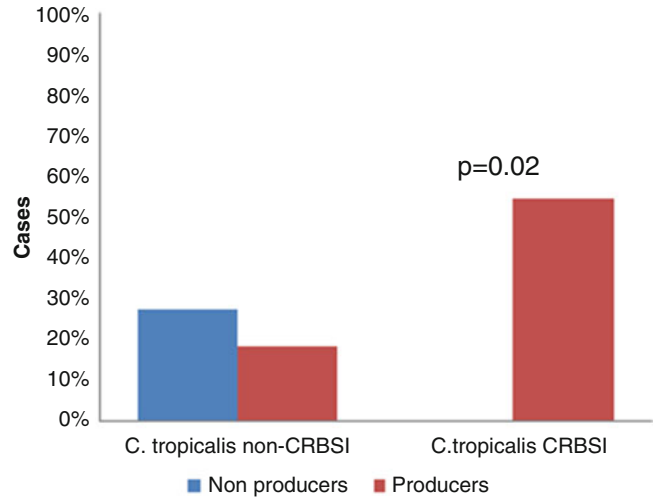

Fig. 3 Biofilm production by *C. tropicalis* strains isolated from the CCRBSI and non-CCRBSI study groups. Strains of *C. tropicalis* produced biofilm almost exclusively when isolated from the CCRBSI group ($\chi^2 = 4.95$; $p = 0.02$)

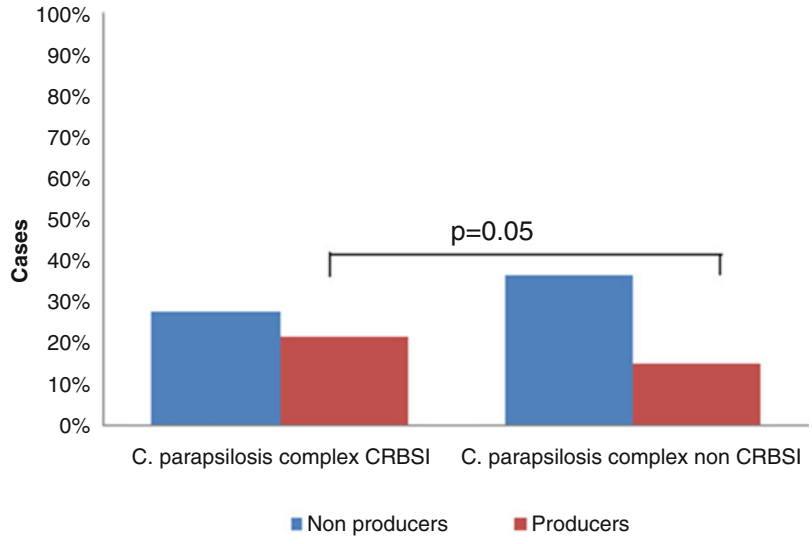

Fig. 4 Biofilm production by *C. parapsilosis* complex cultured from the CVC tips. Strains isolated in the CCRBSI group showed BIs significantly higher than those found in the non-CCRBSI group ($\chi^2 = 3.6$; $p = 0.05$)

4 Discussion

The biofilm production by the colonizing yeasts has been shown to play a key role in the pathogenesis of *Candida*-catheter-related-BSIs (CCRBSI) (Tumbarello et al. 2007). In this study, the biofilm production capacity by the yeasts isolated from CCRBSI-patients was compared to the biofilm production by *Candida* strains cultured from non-CCRBSI patients. Several assays have been considered for measuring the biofilm production by *Candida* (Seneviratne et al. 2009), but a gold standard method has not yet been defined. In our study the XTT assay was used. This method was generally considered as a highly reliable test for the indirect quantification of biofilm mass, which correlates with cell viability (Tumbarello et al. 2007; Chandra et al. 2012; Adam et al. 2002; Ramage et al. 2001). However, even though reliable, the XTT method is not provided with

definitive standard cut-offs to establish whether a *Candida* strain can be classified as a biofilm producer or not. Therefore, in the attempt to standardize the interpretation of biofilm production we introduced an arbitrary index, the Biofilm Index (BI), which allowed us to compare the production by different species relative to an unrelated biofilm producer strain of *C. albicans* (Sandini et al. 2011; Ceccarelli et al. 2016). Considering a BI $\geq$ 0.5, our results showed that the percentage of biofilm producing strains was significantly higher than that of the non-biofilm producing fungal cells and that *C. albicans* and *C. tropicalis* had a significantly higher proportion of biofilm producing strains. However, our results showed that the species most frequently involved in causing CCRBSI was *C. parapsilosis* complex, mainly in males averaging 58 years hospitalized in the ICU, paralleling observations reported in other studies (Levin et al. 1998; Wisplinghoff et al. 2004; Esteve et al. 2011; Safdar et al. 2013; Hu et al. 2014). Although the aggregate analysis of all the yeasts isolated in our study showed that up to 36% of the strains of *C. parapsilosis* complex did not produce biofilm, *C. parapsilosis* complex isolated from the CVC cultures of the CCRBSI group had BIs higher than the strains collected from the non-CCRBSI group. Accordingly, Shin et al. demonstrated that *C. parapsilosis* complex was more likely to form biofilm if the isolates were derived from patients whose candidemia was central venous catheter related (Shin et al. 2002). Due to the relatively lower virulence and to the high diffusion in our hospital setting, it could be hypothesized that *C. parapsilosis* complex might have spread from the patient's skin at the insertion site of the catheter to the surfaces of the indwelling device to form biofilm. Since *C. parapsilosis* complex was reported as the species most frequently isolated from health care workers (HCW) (Clark et al. 2004, Bonassoli et al. 2005), the predominant source of CVC contamination could be found in the colonizing yeast flora of the hands of HCW. Indeed, Wong Song et al. confirmed that 74% of *C. parapsilosis* complex strains isolated from hand of HCW had the ability to produce biofilm (Song et al. 2005). While biofilm formation by *C. parapsilosis* complex was reported as less extensive in different isolates, *C. albicans* produced the most extensive biofilm structure (Kumamoto 2002). In our study, BI production by *C. albicans* isolates confirmed that this species was a strong producer both in CCRBSI and in the non-CCRBSI group. Furthermore, a significantly higher BI was observed in the strains of *C. albicans* isolated from the blood in the CCRBSI group, thus confirming that the capacity of *C. albicans* to produce biofilm is a major virulence factor, to be considered in the dissemination of the infection. *C. tropicalis* was mainly reported as a causative agent of nosocomial urinary tract infections (Rho et al. 2004) and as a common agent of candidemia, especially in oncology patients (Nucci and Colombo 2007) and it was recognized as a strong biofilm producer by many reports (Silva et al. 2011; Negri et al. 2016; Bizerra et al. 2008). In our study we demonstrated that all the strains of *C. tropicalis* isolated from the CCRBSI group produced biofilm. Therefore, biofilm production could be related to an intrinsic capacity of *C. tropicalis* to colonize the intravenous device and to spread to the bloodstream. This observation may parallel those of Silva-Dias et al. who reported that *C.tropicalis* was the species that displayed the higher values of hydrophobicity, thus allowing a more efficient adherence to the catheter surfaces. (Silva-Dias et al. 2015) In contrast, we evidenced that all but one of the *C. glabrata* isolates did not produce biofilm, even though a relatively small, yet significant, rate of CCRBSI was sustained by this species. Therefore, mechanisms other than biofilm production may be involved in the CCRBSI caused by *C.glabrata*, such as a metabolic activity higher than that of other species (Marcos-Zambrano et al. 2014). In fact, *C. glabrata* was correlated for this ability to a poor outcome of the infected patients (Klevay et al. 2008; Lee et al. 2010; Garnacho-Montero et al. 2010; Carrillo-Muñoz et al. 2010; Ruan et al. 2008; Segireddy et al. 2011). Moreover, it has been reported that even though the adherence properties of infecting

organisms are important, they are not the only pathogenic determinants of a catheter-related *Candida* infection (Tumbarello et al. 2012).

To our opinion, our study suffers of at least three limitations. Firstly, it was carried out as a single-center study and the results might not be transferable to the general population. Secondly, no molecular typing was performed to prove the identity of the isolated strains. However, Hasan et al. (2009) demonstrated that sequential isolates of Candida strains with stable biofilm exhibited identical genotype. Moreover, Escribano et al. (2013) showed that 91% of the yeasts obtained from both blood and catheter samples isolated in the same patients displayed identical genotypes. In our study in the absence of genotyping, we observed in the CCRBSI group 95% concordance between the BI of a *Candida* strain isolated from the blood and the BI of the strain isolated from the CVC. Finally, the last limitation resides in the quantification of biofilm production performed only with a single, non-standardized assay i.e. XTT. However, the arbitrary introduction of the Biofilm Index might be taken as an attempt to standardize the biofilm production capacity of a single *Candida* strain and perhaps it might be considered as a novel parameter in the clinical management of a suspected CCRBSI, especially in the absence of the molecular analysis of the strains.

In conclusion our study highlighted that the greater capacity to produce biofilm in the CCRBSI group compared to the non-CCRBSI group, along with the higher proportion of biofilm producers among non-albicans isolates in CCRBSI group, may be important factors to be considered in the pathogenesis of CRBSI. In such patients, catheter removal is nowadays a longstanding problem.

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Inhibition of Urease Enzyme Production and some Other Virulence Factors Expression in *Proteus mirabilis* by *N*-Acetyl Cysteine and Dipropyl Disulphide

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Abstract

Proteus mirabilis is one of the important pathogens that colonize the urinary tract and catheters resulting in various complications, such as blockage of the catheters and the formation of infective stones. **Purpose:** In this study we evaluated the effect of *N*-acetyl cysteine (NAC) and dipropyl disulphide on some virulence factors expressed by a *Proteus mirabilis* strain isolated from a catheterized patient. **Methods:** Antibacterial activity of both compounds was determined by broth microdilution method. Their effect on different types of motility was determined by LB medium with variable agar content and sub-MIC of each drug. Their effect on adherence and mature biofilms was tested by tissue culture plate assay. Inhibitory effect on urease production was determined and supported by molecular docking studies. **Results:** The minimum inhibitory concentration (MIC) of NAC and dipropyl disulphide was 25 mM and 100 mM, respectively. Both compounds decreased the swarming ability and biofilm formation of the tested isolate in a dose-dependent manner. NAC had higher urease inhibitory activity (IC50 249 ±0.05 mM) than that shown by dipropyl disulphide (IC_{50} 10±0.2 mM). Results were supported by molecular docking studies which showed that NAC and dipropyl disulphide interacted with urease enzyme with binding free energy of −4.8 and −8.528 kcal/mol,

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respectively. Docking studies showed that both compounds interacted with Ni ion and several amino acids (His-138, Gly-279, Cysteine-321, Met-366 and His-322) which are essential for the enzyme activity. **Conclusion:** NAC and dipropyl disulphide could be used in the control of *P. mirabilis* urinary tract infections.

Keywords
P. mirabilis • Urease • Biofilm • NAC • Dipropyl Disulphide • Molecular Docking • Motility

1 Introduction

Infective stones are responsible for approximately 15% of urinary stone diseases. The main cause for the formation of infective stones is the presence of urease positive organisms associated with the urinary tract infection mainly from *Proteus* species. *P. mirabilis* is second to *E. coli* as a common cause of urinary tract infections, especially in the elderly catheterized population (Peerbooms et al. 1985). UTIs associated with *P. mirabilis* usually lead to urinary tract obstruction, stone formation, blockage of urinary catheters, and bacteriuria. This may be due to the production of several virulence factors including urease, hemolysin, surface adherence and biofilm formation (Rózalski et al. 1997; Coker et al. 2000).

Urease enzyme is one of the most important virulence factors identified until today (Rózalski et al. 1997). Urease is a nickel containing enzyme, it generates ammonia from urea increasing the alkalinity of urine which favors formation of struvite and carbonate apatite crystals. The aggregation of these crystals is considered one of the primary causes of urinary stone formation. Once a stone begins to form, bacteria stick to the stone and live within its layers protecting them from antibiotics. In addition, urease is involved in the chemotactic reactions of the bacterium and reduces opsonization by human complement (Nakamura et al. 1998; Rokita et al. 1998; Hu and Mobley 1990). *Proteus mirabilis* urease enzyme differs from other bacterial ureases in which it is not only found in the cytoplasm of the cell but also on the bacterial surface (Mobley et al. 1995; Hawtin et al. 1990).

Swarming ability of *P. mirabilis* is another factor that can play a role in pathogenesis. Swarmer cells have been shown to move rapidly over catheter surfaces (Stickler and Hughes 1999). Therefore initiation of infection could possibly be mediated by the migration of the organism. *P. mirabilis* showed three types of movement: swimming, swarming and twitching. *Proteus* bacteria use swimming motility (SM) in liquid media. Swarming motility (SW) is used for movement on solid surfaces and twitching motility (TM), to twitch between two solid surfaces (Rózalski et al. 1997; Rashid and Kornberg 2000).

Biofilm formation facilitates survival and adaptation to the unfavorable conditions of the external environment and protects organisms from the host immune system and antimicrobials (Costerton 1999; Jacobsen and Shirtliff 2011). Biofilms, in particular crystalline biofilms, lead to encrustation and blockage of indwelling catheters in patients with UTIs (Mobley and Warren 1987).

Furthermore, the development of multiple drug resistant strains made treating *P. mirabilis* infections more difficult (Rózalski et al. 1997; Mokracka et al. 2012). So, finding antibiotics with new modes of action is urgently needed. Also, Neutralizing or suppressing the expression of the pathogen's virulence factors is needed to attenuate the pathogenicity of the bacterium and make it easier for the host innate immune system to clear the infection (Fernebro 2011).

Enzyme inhibitors, such as urease enzyme inhibitors, have attracted great attention of biomedical scientists the last couple of decades. In addition, inhibition of other virulence factors such as adherence, biofilm production and swarming ability of *P. mirabilis* will facilitate the organism's treatment and decrease its virulence.

Based on the previous data and the known urease inhibition properties of thiol and disulphide compounds (Mobley et al. 1995; Todd and Hausinger 1989), our study was done using NAC and dipropyl disulphide due to the presence of thiol group in NAC and disulphide moiety in dipropyl disulphide which may affect the urease activity. Also, NAC plays an important role in destroying biofilm due to its mucolytic properties(Millea 2009; Olofsson et al. 2003) by cleaving disulfide bonds which crosslink glycoproteins (Dodd et al. 2008). We aimed to test the urease inhibitory activity of *N*-acetyl cysteine (NAC) and dipropyl disulphide (a major component of onion oil) against *Proteus mirabilis* urease enzyme. Their effect on *P. mirabilis* adherence, biofilm formation and ability to inhibit different types of movements was also investigated. Finally, molecular docking and simulation studies were carried out to improve the reliability, accuracy of biological tests, and show possible interactions between both compounds and urease enzyme.

2 Materials and Methods

2.1 Bacterial Strain, Chemicals and Media

Two *Proteus mirabilis* isolates were collected from a urine sample and the stent of a catheterized patient undergoing a ureteral stent removal. Isolation and identification were performed according to standard procedures (Benson 2002). Dienes typing for the discrimination between strains of *Proteus mirabilis* was performed as previously described (Sabbuba et al. 2003). Isolates of *P. mirabilis* were plated for single colonies on CLED agar and incubated overnight at 37 °C. Single colonies from both isolates were then inoculated as macro-colonies onto tryptone soy agar plates. After overnight incubation at 37 °C, no zone of inhibition (dienes demarcation line) developed between the last waves of swarming of both isolates. Thus both isolates were regarded as the same strain. Biofilm formation assay was conducted by tissue culture plate method (TCP) as described by Christensen et al., stationary (18-h) trypticase soy broth(TSB) cultures of *P. mirabilis* were diluted 1:100 with fresh TSB .Individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates were filled with 0.2 ml aliquots of the diluted culture. The tissue culture plates were incubated for 18 h at 37 °C. The contents of each well were gently aspirated by tipping the plate and placing the aspirator tip in the lowest corner of the well. With an automatic hand pipette, the wells were washed four times with 0.2 ml of phosphate-buffered saline (pH 7.2). Then 25 µl of 1% solution of crystal violet was added to each well (this dye stains the cells but not the polystyrene) plates. The plates were incubated at room temperature for 15 min, rinsed thoroughly and repeatedly with water. After drying, the ODs of stained adherent bacterial films were read with a micro ELISA auto reader (model 680, Bio rad) wavelength of 570 nm, and the wavelength of values was considered as an index of bacteria adhering to surface and forming biofilms. Biofilm production is considered high (OD $>$ 0.240), moderate (OD = 0.120–0.240), or weak (OD $<$ 0.120) (Christensen et al. 1985).The isolate was cultured on trypticase soy agar (TSA, Difco) slants for daily use and stored in a trypticase soy broth medium (TSB, Difco) along with 15% glycerol, at −80 °C for subsequent uses. *N*-acetylcysteine was obtained from Sedico, Egypt. Dipropyl disulphide $\geq$ 97% was purchased from Sigma-Aldarich. About 163.19 mg of NAC was dissolved in 10 ml distilled water to prepare a final concentration

of 100 mM stock solution while 300.638 mg of dipropyl disulphide was dissolved in 10 ml of dimethyl sulphoxide (DMSO) to prepare a stock solution of 200 mM concentration.

2.2 Scanning Electron Microscopy (SEM)

The removed ureteral stent was examined by scanning electron microscope (SEM) for crystalline biofilm. The stent was fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco phosphate buffered saline PBS (pH 7.2) for 1.5 h, rinsed with PBS, and then dehydrated through an ethanol series. Samples were dried using critical-point drying method (Bray 2000) and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan) (Soboh et al. 1995).

2.3 Evaluation of the Antibacterial Effect of NAC and Dipropyl Disulphide and Determination of their MIC Using Broth Microdilution Method

Briefly, twofold serial dilutions of both agents were prepared in sterile Mueller Hinton Broth (MHB, Oxoid) for a testing concentration range of 1.5–200 mM. Then 100 µl from each dilution was transferred into the well of a microtiter plate and inoculated with 5 µl of standardized cell suspension (1.5×10^7CFU/ml). Plates were incubated at 37 °C overnight, and the lowest concentration of NAC and dipropyl disulphide that prevented visible growth was recorded as the MIC (CLSI 2011). Minimum bactericidal concentration (MBC) was determined by sub-culturing of 10 µl of broth from wells with no visible growth on TSA plates. The lowest concentration of the tested agents that killed 99.9% of the original inoculum was considered as MBC.

2.4 Effect of NAC and Dipropyl Disulphide on Different Virulence Factors of the Tested Strain

(a) *Effect on swarming (SW), Swimming (SM) and Twitching (TM) motility of tested isolate:* Luria-bertani (LB) medium was used with agar content appropriate for the type of motility being tested. Swarming medium contained 0.7% agar and the tested isolate was cultured by picking on the agar surface. Swimming medium contained 0.3% agar and the isolate was applied by puncture into the medium. Twitching medium contained 1.0% agar and the isolate was inoculated with a micropipette under the agar layer (10 µL of bacterial suspension). Sub-MIC concentrations of the tested agents were added to LB media to test their ability to inhibit different forms of movement. After cultivation for 24 h at 37 °C, the diameters of the formed zones were measured (SM and SW) (Jones et al. 2005). Also swarming of *P.mirabilis* over the tested catheter was performed using LB swarming medium. A strip of the medium was aseptically cut away from the agar plate. The prepared gap was bridged with a piece of the tested catheter. The tested strain was applied on the surface using a pick on one half of the agar. After 24 h cultivation at 37 °C, the ability to swarm over the tested catheter was determined (Hola et al. 2012).

(b) *Effect on adherence of the tested isolate:* 100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). TSB broth was used as a negative control. The plates were incubated at 37 °C for 90 min (adhesion period). Supernatants including planktonic cells were discarded and wells were gently washed with phosphate buffer saline (PBS) twice to remove any non-adherent cells. 100 µl of fresh TSB

broth containing one of the following: 1/4MIC, 1/2MIC, MIC, 2MIC and 4MIC concentrations of NAC and dipropyl disulphide (each alone) were added to each well. The plates were covered to prevent evaporation and incubated at 37 °C for 24 h. Liquid media containing the non-adherent cells were discarded and plates washed twice with PBS buffer. Adherent cells to the plastic surfaces were quantified using crystal violet assay (Wei et al. 2006). Experiment was performed in triplicate.

(c) *Effect on mature biofilms:* 100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). The plates were incubated at 37 °C for 48 h. After the incubation period, the supernatants from each well were aspirated and the wells washed twice with PBS without disturbing the biofilms at the bottom of the wells, then 100 µl of 1/4MIC, 1/2MIC, MIC, 2MIC and 4MIC concentrations of NAC and dipropyl disulphide (each alone) were added to the wells. Normal saline without any agents was added to the control wells. The plates were incubated at 37 °C for 24 h. Supernatants were discarded and plates washed twice with PBS buffer. Cells adherent to the plastic surfaces were quantified using crystal violet assay (Wei et al. 2006). Experiment was performed in triplicate.

(d) *Effect on urease production:* We determined the change of pH value of TSB containing 500 mM of urea (pH adjusted to 7.3) in presence of different concentrations (1/32, 1/16, 1/8, 1/4, 1/2 X MIC) of NAC and dipropyl disulphide (Bibby and Hukins 1992). Media containing urea were inoculated with standardized cell suspension (5×10^5 CFU/ml). *Proteus mirabilis* was considered as positive control while uninoculated media was used as negative control. The pH values of TSB containing urea (with the tested agents and control) were screened at 2 h interval for 18 h along the experiment using digital pH-meter (Elmetron CP-215). The increase in pH indicates the activity of urease enzyme.

Urease Activity Assay A total of 50 µl of overnight culture of the isolated *P. mirabilis* strain in MHB were transferred into 10 ml sterile MHB and additionally incubated 18 h at 37 °C with constant shaking. The cells were pelleted by centrifugation at 1258 *g* for 15 min (4 °C). The pellet was washed three times with 10 mM K_2HPO_4 solution and resuspended in 2 ml of the same solution. Thereafter, to release the urease, bacteria were sonicated for 90 s with 0.5 cycles at 100% amplitude using an ultrasonicator (UP200H, Hielscher Ultrasonics, Teltow, Germany) in an ice container. The resulting bacterial lysate was used for determination of urease activity in a microtiter plate using the phenol red colorimetric method. The assay mixture contained 10 mM K_2HPO_4 solution (pH 6.2), 0.002% phenol red and 500 mM urea (assay reagent). The increase in absorbance at 570 nm was recorded using a microplate reader (BioTek, USA) (Goldie et al. 1989; Tanaka et al. 2003). To determine the concentration of urease enzyme in the lysate, the lysate was centrifuged for 3 min. Then the absorbance (A) of the upper solution was determined in $\lambda = 278$ nm. By using the following eq. $A = \lambda bc$, where c is the concentration of solution (mol/L), b the length of the UV cell (Golbabaei et al. 2013; Akhtar et al. 2014). *P. mirabilis* ATCC 12453 reference strain served as a control during the procedure.

Determination of IC_{50} of the Tested Compounds In vitro inhibitory studies on urease were determined using indophenols method, which measures the liberation of ammonia from the reaction (Weatherburn 1967). The urease enzyme was dissolved in 20 mM sodium phosphate buffer and the pH was adjusted to 7 using 1 N NaOH. The assay mixture contained 50 µl

(2 mg/ml) of enzyme and 100 µl of different concentrations of the tested agents(NAC 0.39–50 mM and dipropyl disulphide 1.56–100 mM).The mixture was then added to 850 µl of 25 mM urea and pre-incubated for 0.5 h in water bath at 37 °C. The urease reaction was stopped after 30 min incubation by procedure. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn 1967. After pre-incubation, 500 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 500 µl of alkali reagent (1% w/v NaOH and 0.075% active chloride NaOCl) were added to 100 µl of incubation mixture and kept at 37 °C for 30 min. The absorbance was measured at 625 nm. All experiments were performed in triplicate in a final volume of 1 ml, and acetohydroxamic acid was used as a standard urease inhibitor.

Percentage inhibition was calculated using the formula (100 – (OD sample/OD control) × 100). The concentration that provokes an inhibition halfway between the minimum and maximum response of each compound (relative IC_{50}) was determined by monitoring the inhibition effect of various concentrations of compounds in the assay.

2.5 Protocol of the Docking Study

The automated docking simulation study is performed using Molecular Operating Environment (MOE®) version.09, 2014, at Assuit University Faculty of Pharmacy, Chemical Computing Group Inc., Montreal, Canada. The X-ray crystallographic structure of the target urease (1E9Y) was obtained from Protein Data Bank (PDB). The target compounds were constructed into a 3D model using the builder interface of the MOE program. After checking their structures and the formal charges on atoms by 2D depiction, the following steps were carried out: the target compounds were subjected to a conformational search, all conformers were subjected to energy minimization, all the minimizations were performed with MOE until a RMSD gradient of 0.01 Kcal/mole and RMS distance of 0.1 Å with MMFF 94 X force-field and the partial charges were automatically calculated. The enzyme was prepared for docking studies as follows: hydrogen atoms were added to the system with their standard geometry, the atoms connection and type were checked for any errors with automatic correction and selection of the receptor and its atoms potential were fixed. MOE alpha site finder was used for the active site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha spheres.

2.6 Statistical Analysis

One-Way ANOVA was employed to evaluate any significant difference between the values of optical densities (OD) obtained from biofilm formed by control (untreated) and the values obtained in the presence of different concentrations of NAC and dipropyl disulphide. Differences were done using SPSS, 17 statistical software (SPSS Inc., Chicago, IL).

3 Results & Discussion

E. coli, *Enterococcus faecalis*, *P. mirabilis* are more commonly associated with urinary tract infections (UTIs) in those individuals with structural or functional abnormalities, especially ascending infections in patients undergoing urinary catheterization (O'May et al. 2008; Garsin and Willems 2010; Goller and Seed 2010). *P. mirabilis* was found to have the greatest ability out of all gram-negative organisms to attach to many catheter materials (Hawthorn and Reid 1990; Roberts et al. 1990). Its high pathogenicity is due to the expression of many virulence factors. So, it is necessary to understand these virulence factors and their role in infection in order to find agents that can interact with these factors to prevent the establishment of infection and facilitate treatment.

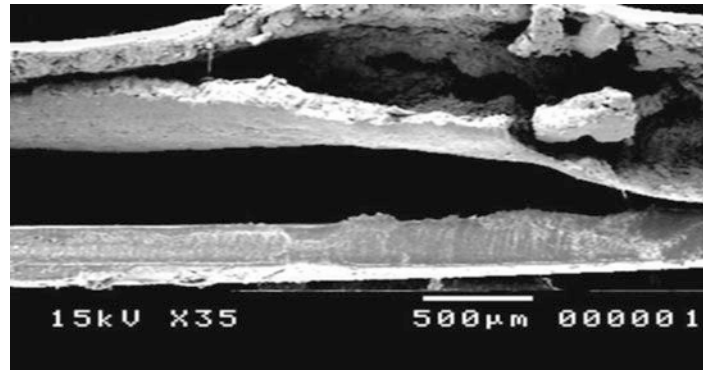

Fig. 1 Scanning electron micrograph showed the lumen of a ureteral stent obtained from a catheterized patient (×35). It showed a dense mass of biofilm and a high level of encrustation

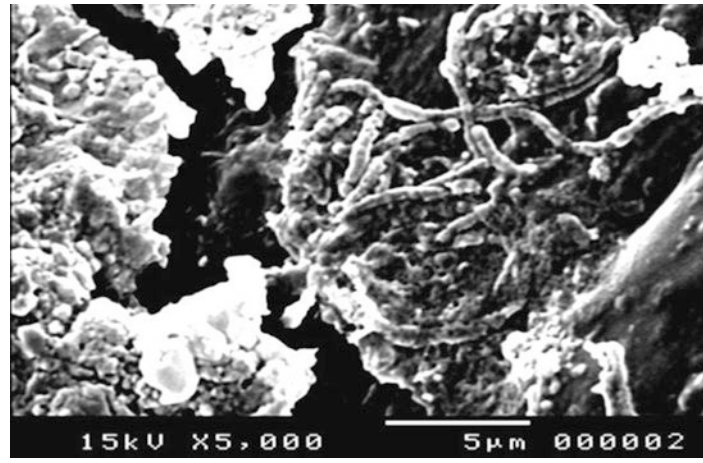

Fig. 2 Scanning electron micrograph showed the lumen of the ureteral stent covered with a dense mass of biofilm containing bacteria (*P. mirabilis*) and crystalline patches (white patches) (×5000)

In this work, the same *P. mirabilis* strain was isolated from two clinical samples taken from the same patient, which was proven by negative dienes reaction between both isolates. The *P. mirabilis* strain was negative for hemolysis, urease positive and strong biofilm producer according to tissue culture plate method (OD = 0.6). Stent removed from the patient was examined by scanning electron microscopy. SEM micrograph showed a dense mass of biofilm blocking the lumen of the stent (Fig. 1). Also, a high level of encrustation (crystalline patches) and a dense mass of biofilm containing *P. mirabilis* rods were observed using 2 different magnification powers (Figs. 2 and 3). Many studies reported the ability of *P. mirabilis* to encrust and block the lumen of catheters (Jones et al. 2005; Morris et al. 1997; Stickler and Sabbuba 2007; Vlamakis 2011).

The antibacterial activity, MIC and MBC of the tested agents were evaluated. The MIC of NAC was 25 mM while MBC was 50 mM. For dipropyl disulphide, MIC was 100 mM while MBC was > 200 mM. The antimicrobial and the anti-biofilm activity were previously reported by many researchers (Marchese et al. 2003; El-Feky et al. 2009; Abd El-Baky et al. 2014; Mohsen et al. 2015). They all showed that NAC has powerful effect on the disruption of mature biofilms formed by several types of microorganisms and can inhibit adherence. Also, it can increase the therapeutic activity of several antimicrobials helping in the inhibition and eradication of biofilms. Dipropyl disulphide

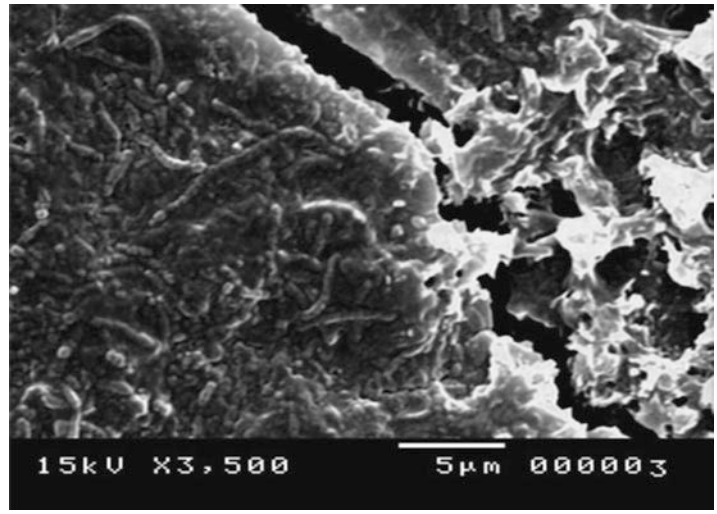

Fig. 3 Scanning electron micrograph showed the surface of the ureteral stent (×3500). It showed a dense mass of biofilm containing microorganisms and a high level of encrustation

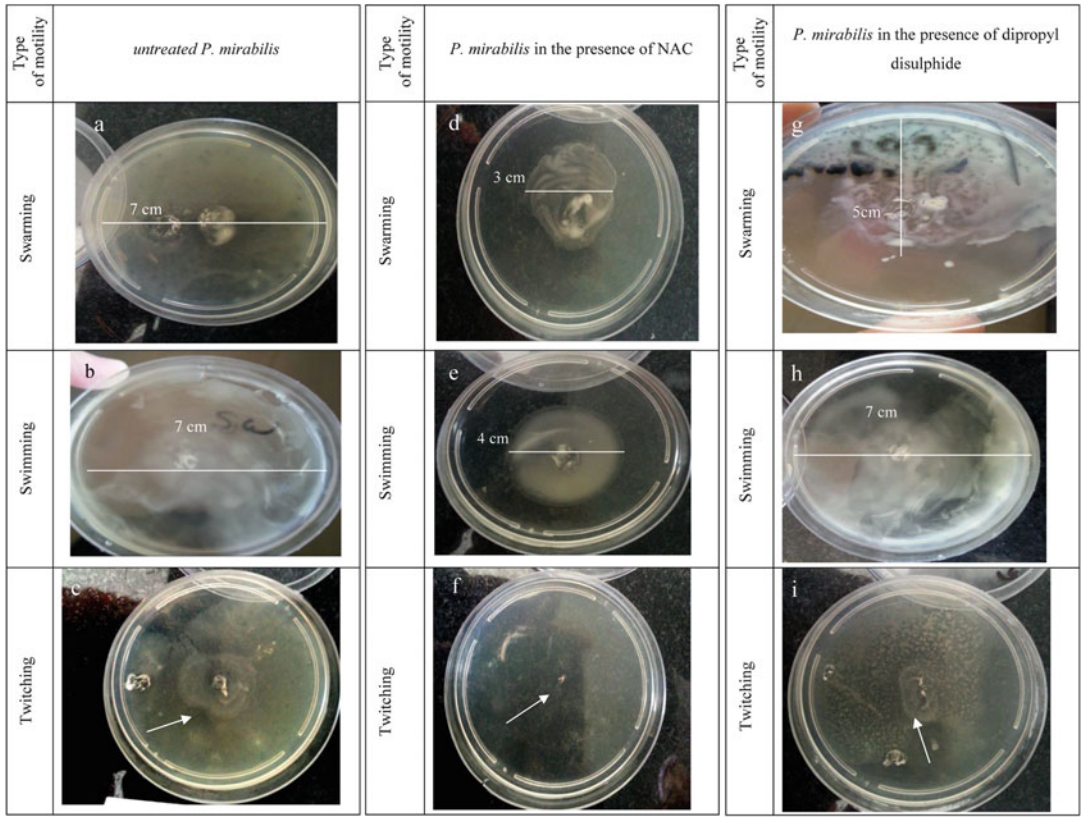

Fig. 4 (**a**–**c**) The ability of the tested strain to show different types of motility (swarming (*SW*), Swimming (*SM*) and Twitching (*TW*). (**d**–**f**) The effect of NAC on the ability of *P. mirabilis* to show the different types of motility. (**g**–**i**) The effect of dipropyl disulphide on the ability of *P. mirabilis* to show the different types of motility

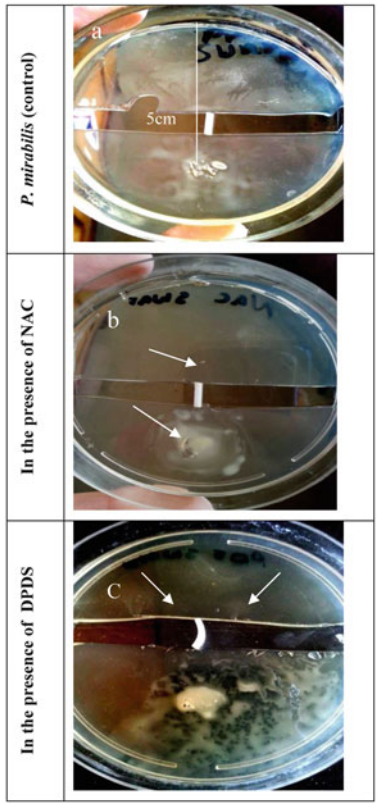

Fig. 5 (**a**) Swarming of the tested strain over the stent segment , (**b**) NAC inhibited the swarming motility of *P. mirabilis* , (**c**) dipropyl disulphide did not inhibit the swarming over the stent segment

is one of the main components of onion. Several studies showed that its antibacterial activity was scarce which agrees with our results (Kim et al. 2004; Mnayer et al. 2014).

The tested strain showed the ability to swarm over the whole plate. In addition, it was positive for swimming on 0.3% agar plate and twitching motility using 1% agar plates (Fig. 4a–c). Its ability to swarm over the stent was shown in Fig. 5a which indicates the ability of *P. mirabilis* to switch from swimmer cells in liquid media (urine) to swarmer cells on solid surfaces which helps in the dissemination of infection to other locations in the urinary tract. Naturally, the mucous layer covering the epithelial surfaces is highly viscous; it can trap many motile bacteria and prevent them from ascending the tract. But in case of *P. mirabilis*, entrapment of the organism in the mucus layer inhibits the flagellar rotation of trapped swimmer cells which induces swarmer cells' differentiation. Swarming motility may then facilitate the migration of this organism through the mucous layer and expand the population to new locations (Williams and Schwarzhoff 1978; Mobley and Belas 1995; Rather 2005).

By testing the changes in the ability of the organism to move in the presence of NAC and dipropyl disulphide, results revealed that NAC decreased the swarming ability of the tested isolate. As *P. mirabilis* showed a swarming zone of 3 cm while dipropyl disulphide decreased swarming zone to 5 cm in comparison to the control (untreated) strain that produced swarming zone of 7 cm (Fig. 4a, d, g). For swimming motility, the untreated strain showed swimming motility over the whole plate (Fig. 4b). Dipropyl disulphide didn't affect the swimming motility (Fig. 4h) while NAC decreased its ability to swim producing swimming zone of 4 cm (Fig. 4e). Figure 5f showed that twitching motility was blocked by NAC but no effect was observed by dipropyl disulphide (Fig. 4i). In the presence of NAC, *P. mirabilis* lost its ability to swarm over the stent surface while dipropyl disulphide didn't show any effect on its swarming activity (Fig. 5). Surface-associated swarming motility is implicated in enhanced bacterial spreading and virulence. Many studies tested different compounds that can block the swarming ability of motile microorganisms. As O'May et al. 2012 reported that tannic acid (TA) and epigallocathecin gallate (EGCG) and undefined cranberry powder (CP) can block swarming motility of *Pseudomonas aeruginosa*. Dusane et al. 2014 reported that Piperin and reserpine alkaloids decreased the swarming and the swimming ability of *E. coli*. Also, Ulrey et al. 2014 found that Cranberry proanthocyanidins reduced swarming and can disrupt biofilm of *P. aeruginosa*.

Stones are characteristic features of the biofilms developed during *P. mirabilis*-associated UTIs (Jones et al. 1990). The majority of patients with recurrent *P. mirabilis* catheter encrustation (62%) developed bladder stones, which led to the colonization of replacement

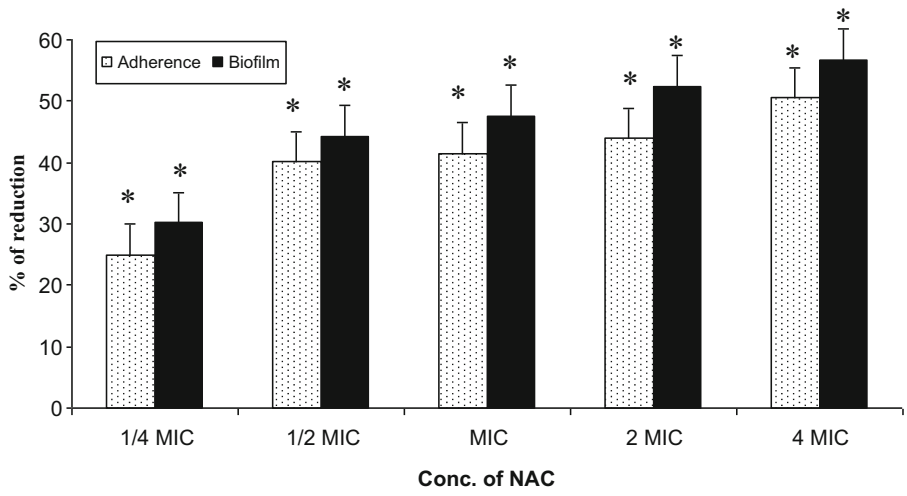

Fig. 6 Effect of different conc. of NAC on the adherence and mature biofilms of *P. mirabilis*. Results were obtained from three independent replicates. % of reduction was calculated in comparison to control and expressed as mean ± SD
*statistically significant ($P < 0.05$) compared with control

catheters with these organisms (Sabbuba et al. 2004). Both physical and chemical factors play a role in the initiation and development of the crystalline biofilms observed during *P. mirabilis* colonization. The pH of urine can be essential for bacterial attachment to polymer surfaces as macroscopic aggregates of cells and for crystals of calcium and magnesium phosphate to settle on the polymer surface and initiate crystalline biofilm development that blocks the flow of urine through the catheter (Jones et al. 1990). Therefore, the control of urine pH and subsequent crystallization could be critical in preventing biofilm formation on indwelling devices inserted in the urinary tracts of patients infected with *P. mirabilis*. Figures 6 and 7 showed the effect of *N*-acetyl cysteine and dipropyl disulphide on the adherence and mature biofilms of *P. mirabilis* isolate using tissue culture plate (TCP) assay. It was found that the inhibitory effect of both NAC and dipropyl disulphide was concentration dependent. The disruptive effect of NAC against mature biofilms (% of reduction ranged from 30.2 to 56.8 in comparison to control) was more than its ability to decrease the adherence of the tested isolate (% of reduction ranged from 25 to 50.5 in comparison to control). Dipropyl disulphide showed weak inhibitory effect on both the adherence and the mature biofilm of the tested isolate (% of reduction ranged from 12.2 to 28.5 and 11.3 to 26.1, respectively in comparison to control). All values were statistically significant ($P < 0.05$) compared with the control.

Urease inhibition by potent and specific compounds could provide an invaluable addition for the treatment of infections caused by urease-producing bacteria. Although several potent inhibitors of this enzyme have been reported, more effective, safe and potent inhibitors are considered necessary for the control of urease-related infections (Amtul et al. 2002, 2004). It was found that NAC and dipropyl disulphide had the ability to inhibit urease production at sub–MIC concentrations of the tested compounds. Change in pH value due to the production of ammonia from urea was determined at 2 h interval for 18 h. *P. mirabilis* incubated with urea showed an increase in the pH value ranged from 7.3 to 9. In the presence of increasing concentration of NAC (1/32 MIC-1/2 MIC), a decrease in the pH value was observed (7.1–6.2). Dipropyl disulphide caused a slight increase in the pH value from 7 (at 1/32 MIC) to 8 (at 1/2 MIC).

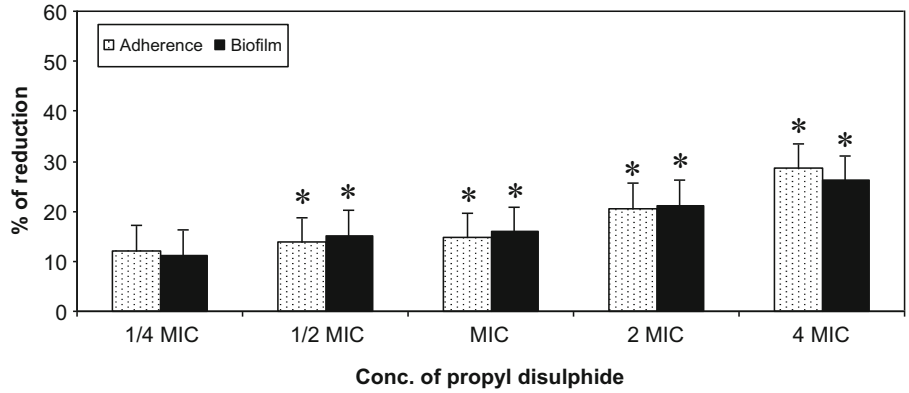

Fig. 7 Effect of different conc. of dipropyl disulphide on the adherence and mature biofilms of *P. mirabilis*. Results were obtained from three independent replicates. % of reduction was calculated in comparison to control and expressed as mean ± SD
*statistically significant ($P < 0.05$) compared with control

The urease inhibitory activity and IC_{50} of the tested compounds at different concentrations were evaluated. The results obtained were compared to that obtained by acetohydroxamic acid (standard urease inhibitor). It was found that the inhibitory activity ranged from 61.4 to 96.9% for NAC, 26 to 72.4% for dipropyl disulphide while acetohydroxamic acid (AHA) showed inhibitory activity ranging from 90 to 98% in comparison to control (without inhibitor). It was found that NAC had higher inhibitory activity (IC_{50} 249 ± 0.05 mM) than that shown by dipropyl disulphide (IC_{50} 10 ± 0.2 mM) when it is compared to the standard inhibitor acetohydroxamic acid (IC_{50} 120 ± 0.06 μM).

The role of urease enzyme in the degree of virulence was reported by many studies. These findings are further supported by the fact that treating the animals with acetohydroxamic acid, a potent urease inhibitor, reduces the severity of *P. mirabilis* infection (Musher et al. 1975). Despite the difference in the number of subunits forming urease enzyme among Jack bean (one), helicobacter (three) and all other bacterial species (two), the amino acid sequences are well conserved. The active site of the enzyme is found in the UreB subunit and comprises amino acid residues which are His-136, His-138, Lys-219, His-248, His-274, and Asp-362. These amino acids come in direct contact with the two nickel ions, urea, or a water molecule within the active site of the urease enzyme of *H. pylori* (Labigne et al. 1991). In addition, His-322 is near the active site and acts as a general base in the catalysis (Dixon et al. 1980). Urea binds in *O*-coordination to one nickel ion aided by His-221. His-322 (as an active base) activates a water molecule bound to the other nickel ion. An attack by the metal-coordinated hydroxide on the substrate carbon atom results in a tetrahedral intermediate that bridges the two nickel sites, a proton transfer to the intermediate with accompanying ammonia release, and a displacement to the carbamate by water to complete the cycle (Mobley 2001).

The tested compounds were studied by docking them into the crystal structure of *Helicobacter pylori* urease enzyme (1E9Y) obtained from Protein Data Bank (PDB) to observe the common behavior of interaction of these compounds with the enzyme. In order to validate the docking reliability, the ligand acetohydroxamic acid was removed from the active site, and the Grid Box was set large enough to do blind docking. Figure 8a showed the docking studies of acetohydroxamic acid (standard urease inhibitor) with the active site. NAC interacts with urease with binding free energy of −4.8 kcal/mol. Docking studies of *N*-acetyl cysteine with the active site of the enzyme

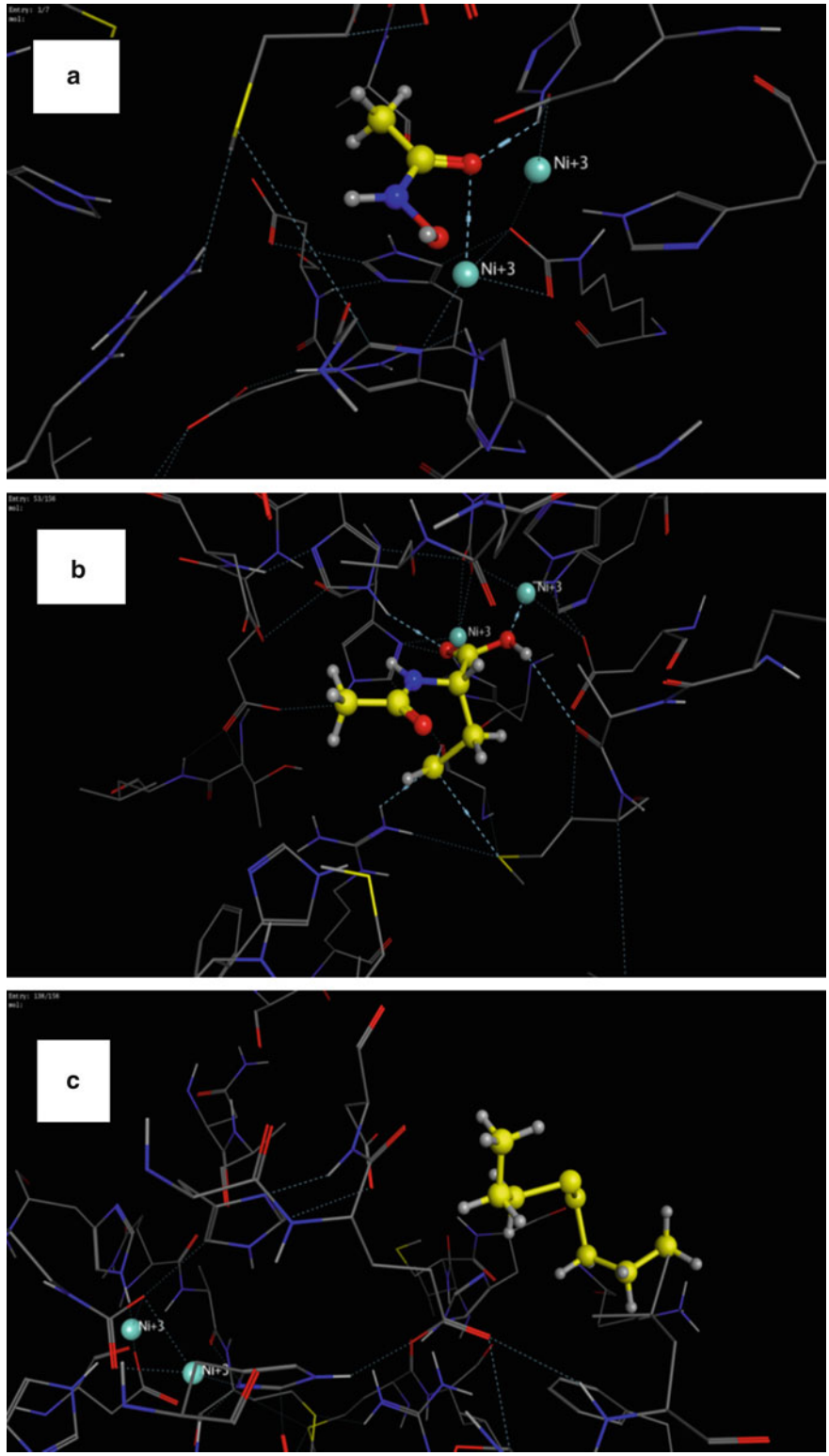

Fig. 8 Molecular docking of Acetohydroxamic acid AHA (**a**) NAC (**b**) and dipropyl disulphide (**c**) and their interactions with different residues of urease enzyme active site involved in interaction

(Fig. 8b) showed an *O*-coordinated bond with nickel 3002 and a hydrogen bond with His-138 which is in direct contact with Ni ion. The oxygen atom in the OH group makes a hydrogen bond with Gly 279. The thiol group forms two disulfide bonds with SH group of Cysteine 321 (Cys 321) and S-CH3 of methionine (Met366). So, these compounds lock the enzyme into a single conformation by covalently modifying the Cys residue in the flap. Kühler et al. reported that covalent modification of Cys321 will lead to an inactive enzyme, which indicated that Cys321 may be one of the key residues for the catalytic activity of urease (Kühler et al. 1995). Xiao et al. showed that quercetin inhibits urease enzyme due to the formation of hydrogen bonding with Cys 321, Met366 and Gly367 (Xiao et al. 2012). On the other hand, dipropyl disulphide interacts with urease with binding free energy of −8.528 kcal/mol. Diporpyl disulfide forms a hydrogen bond with His-322 located near to the active site (Fig. 8c); it plays an important role in the catalysis process and the release of ammonia. Many studies use several natural and chemical compounds to inhibit urease enzyme. Ranjbar-Omid et al. found that allicin extracted from garlic was able to inhibit urease, hemolysin and biofilm formation of *P. mirabilis* (Ranjbar-Omid et al. 2015). Prywer and Torzewska reported that curcumin may inhibit the activity of urease, not influencing the bacterium's vitality (Prywer and Torzewska 2012). Also, they noticed that in the presence of curcumin, pH of the urine increases much more slowly compared with the absence of curcumin which is similar to results obtained by dipropyl disulphide in our study.

4 Conclusion

NAC and dipropyl disulphide exhibited antimicrobial properties and can be used for the inhibition of crystalline biofilm formation by *P. mirabilis*. Both compounds decreased the swarming ability of the tested isolate in comparison to the untreated strain. NAC and dipropyl disulphide showed variable inhibitory activity against urease enzyme produced which was also supported by docking studies. Using NAC with antibiotic therapy may have an additive therapeutic effect in eradication of *P. mirabilis* and the protection of patients suffering from urinary tract infections or catheterized patients from developing infective stones.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Immunization with Streptococcal Heme Binding Protein (Shp) Protects Mice Against Group A *Streptococcus* Infection

Xiaolan Zhang, Yingli Song, Yuanmeng Li, Minghui Cai, Yuan Meng, and Hui Zhu

Abstract

Streptococcal heme binding protein (Shp) is a surface protein of the heme acquisition system that is an essential iron nutrient in Group A *Streptococcus* (GAS). Here, we tested whether Shp immunization protects mice from subcutaneous infection. Mice were immunized subcutaneously with recombinant Shp and then challenged with GAS. The protective effects against GAS challenge were evaluated two weeks after the last immunization. Immunization with Shp elicited a robust IgG response, resulting in high anti-Shp IgG titers in the serum. Immunized mice had a higher survival rate and smaller skin lesions than adjuvant control mice. Furthermore, immunized mice had lower GAS numbers at the skin lesions and in the liver, spleen and lung. Histological analysis with Gram staining showed that GAS invaded the surrounding area of the inoculation sites in the skin in control mice, but not in immunized mice. Thus, Shp immunization enhances GAS clearance and reduces GAS skin invasion and systemic dissemination. These findings indicate that Shp is a protective antigen.

Keywords

Immunization • Group A *Streptococcus* • *Streptococcus* Pyogenes • Shp • Heme Acquisition • Vaccine

1 Introduction

Group A *Streptococcus* (GAS), also known as *Streptococcus pyogenes*, causes diverse human infections ranging from mild pharyngitis to severe invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). Invasive infections account for over

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half a million deaths per year globally. In addition, GAS infection causes post-infection sequelae, including glomerulonephritis, acuterheumaticfever (ARF), and rheumatic heart disease (Walker et al. 2014). While GAS remains sensitive to all penicillins and cephalosporins, it can develop resistance to antibiotics such as macrolides, clindamycin, erythromycin and lincosamide (Hasenbein et al. 2004; Chen et al. 2012; Magnussen et al. 2016; Silva-Costa et al. 2015; Seppälä et al. 1992; Sayyahfar et al. 2015). Despite continued efforts of the academic and industrial communities for several decades, no licensed vaccine is available for this major human pathogen. Many vaccine candidates that mostly include membrane-associated proteins have been developed for over a hundred years (Tonooka et al. 1978; Beachey et al. 1981; Dale et al. 1996; McNeil et al. 2005; Sabharwal et al. 2006; Dale et al. 2015; Rivera-Hernandez et al. 2016; Huang et al. 2011). M protein is the most widely studied vaccine candidate. The first attempt to immunize human volunteers with crude whole M protein reduced colonization but latter studies raised the possibility that preparations of whole M protein induces autoimmune increases such as ARF (D'Alessandri et al. 1978; Fox et al. 1973; Massell et al. 1969). Subsequently, multivalent vaccines containing the N-terminal hypervariable region (this region may not be involved in autoimmune diseases) exhibited bactericidal activity against serotypes in developed countries but do not cover prevalent serotypes in developing countries (Dale et al. 2011; Wozniak et al. 2014). More recently, the highly conserved C-repeat regions of the M protein (J8, J14 and StreptInCor) have proved to elicit protective antibodies in animal-based trials but have not yet been tested in human clinical trials (Caro-Aguilar et al. 2013; Bauer et al. 2012; Moyle et al. 2013; De Amicis et al. 2014). In addition, secreted antigens such as streptococcal pyrogenic exotoxin A (SpeA) and secreted esterase (SsE) also represent promising vaccine candidates (Song and Zhang 2013). However, the conservation and serotype coverage of antigens, the geographical distribution of serotypes, the use of antigens devoid of autoimmune epitopes, and the selection of human-approved adjuvants restrict the approval and usage of a commercial human vaccine. Proteins or synthetic peptides that are highly conserved in GAS genomes and elicit protective immunity represent new targets for vaccine research.

Streptococcal heme binding protein (Shp) is located in the cell wall and is an essential component of the heme acquisition machinery in GAS. The GAS heme acquisition system also contains streptococcal hemoprotein receptor (Shr) and the ATP-binding cassette transporter (HtsABC). Shr extracts heme from hemoglobin, and Shp relays heme from Shr to HtsA, the lipoprotein component of HtsABC (Liu and Lei 2005; Nygaard et al. 2006; Ran et al. 2010). Shp is highly conserved in GAS, sharing 99% identity in its amino acid sequence with sequenced GAS strains of different serotypes. Shp-specific antibodies have been detected in the convalescent sera of patients with both invasive and pharyngeal infections (Lei et al. 2002). The objective of this study was to determine whether Shp is a protective antigen. We found that immunization with Shp elicited high anti-Shp IgG titers and protected mice against GAS infection.

2 Materials and Methods

2.1 Bacterial Strains, Vectors and Media

The pET21d vector (Novagen) was transformed into *E. coli* BL21 (DE3) cells (Novagen) to express recombinant Shp. All *E. coli* cells with plasmids were grown in LB medium or on LB agar plates at 37 °C supplemented with ampicillin (100 mg/l). The GAS strain MGAS5005 (serotype M1) was grown in Todd-Hewitt broth (Becton, Dickinson and Company) supplemented with 0.2% yeast extract (THY) at 37 °C with 5% CO_2.

2.2 Cloning, Expression and Purification of Recombinant Shp

Briefly, the genomic DNA of MGAS5005 was extracted and the *shp* gene was amplified using polymerase chain reaction with the sense 5′-ACC ATGGATAAAGGTCAAATTTATGGA TG-3′ and antisense 5′-CGAATTCTTAGTCTTTTTT AGACCGAAACTTATC-3′ primers. The protein obtained from this cloned fragment lacked the presumed secretion signal sequence (amino acids 1 to 29) and the transmembrane domain and charged tail (amino acids 259 to 291) located at the carboxy terminus, therefore, the size of the PCR product was approximately 690 bp. The underlined bases were added to introduce an *Nco*I restriction site-initiation codon and an *Eco*RI site-stop codon, respectively. The PCR product was digested and cloned into the *Nco*I and *Eco*RI sites of the pET-21d vector. The cloned fragment was sequenced to rule out spurious mutations. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells, with 1 mM isopropylthio-β galactoside (IPTG) induction at $OD_{600} = 0.6$ for 8 h. The bacteria were harvested by centrifugation, suspended in 25 ml 20 mM Tris-HCl (pH = 8.0) and sonicated on ice for 15 min. Cell debris was removed by centrifugation at 14,000 rpm for 10 min, and the resulting supernatant was loaded onto a Ni-Sepharose (GE Healthcare) column. Protein was eluted with imidazole (from low to high concentration) on a biological low pressure liquid chromatography system (Bio-Rad). Shp was identified by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining, and peak fractions were pooled. The protein was further purified using DEAE Sepharose (GE Healthcare) and Phenyl Sepharose (GE Healthcare) in turn. The purified recombinant protein was free of contaminating proteins, as determined by 10% SDS-PAGE with Coomassie Brilliant Blue staining. Protein concentration was measured with the BCA standard curve method.

2.3 Immunization

Female CD1 Swiss mice (4 to 5 weeks old) were purchased from Heilongjiang University of Chinese Medicine. Mice were immunized subcutaneously in the back with 200 μl of antigen-adjuvant suspension containing 50 μg recombinant Shp in 20 mM Tris-HCl (pH = 8.0) and 200 μl ALUM adjuvant at days 0, 14 and 28, or treated similarly with ALUM adjuvant control (n = 22 respectively). Blood samples were collected at days 13, 27 and 41 via tail bleeding, and then serum samples were prepared and stored at-80 °C for titer determination. Immunization experiments were performed at least twice. The protocols for the immunization procedures and the infection below were approved by the Institutional Ethics Committee of Harbin Medical University.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA kit (Neo Bioscience) was used to measure antigen-specific serum IgG titers. Briefly, 96-well ELISA plates were coated with 100 μl of 10 μg/ml rShp in 50 mM carbonate coating buffer (pH = 9.6) overnight at 4 °C and then blocked with 3% BSA in PBS for 1.5 h at 37 °C. The coated Shp was incubated with anti-sera (100- to 100,000- dilutions) at 37 °C for 2 h. Plates were washed with 0.2% Tween-20 in PBS three times and then incubated with horse radish peroxidase-conjugated goat anti-mouse IgG for 1 h. Plates were washed with 0.2% Tween-20 in PBS three times and then incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) for 40 min. The colorimetric reaction was stopped by the addition of 50 μl of 2 M H_2SO_4. To calculate IgG titers, A_{450} was measured using a microplate reader.

2.5 Mouse Infection

Immunized and control mice were subcutaneously inoculated with 1.0×10^9 CFU GAS in

0.2 ml PBS (MGAS5005) in the back 14 days after the final booster (n = 22 respectively). Fifteen mice in each group were observed twice a day for 15 days to determine survival rates. Blood, spleen, liver, lung and skin lesions were collected from 7 mice in each group 24 h after GAS inoculation to determine the number of bacteria. GAS numbers were determined by plating blood and homogenized tissues at the appropriate dilution onto THY agar plates. In addition, we measured skin lesion sizes of 20 mice 24 h after GAS inoculation. All protocols were approved by the Institutional Ethics Committee of Harbin Medical University.

2.6 Histological Analysis

Skin samples containing infection sites were excised from immunized and control mice 24 h after inoculation and fixed in 4% neutral-buffered formalin for 24 h. The skin samples were dehydrated with ethanol, cleared with xylene, and embedded in paraffin. Paraffin blocks were processed to obtain 5 μm sections, which were stained with 1% crystal violet and neutral fuchsin following standard Gram staining procedures. Stained samples were examined using an OLYMPUS BX51TF microscope.

2.7 Statistical Analysis

Antibody titer and bacterial CFU data were analyzed by Student's t-test using GraphPad Prism 5 software. The log-rank Kaplan-Meier test was used to analyze survival data.

3 Results

3.1 Cloning, Expression and Purification of Shp

The streptococcal heme binding protein gene, designated *shp*, was amplified by PCR. Shp lacked the presumed secretion signal sequence (amino acids 1 to 29) and the transmembrane domain and charged tail (amino acids 259 to 291), located at the carboxy terminus, to avoid potential toxicity associated with the secretion signal and charged tail. The fragment was then subcloned into pET21d to generate the recombinant plasmid under the control of an IPTG-inducible promoter, and then introduced into *E. coli* BL21 (DE3) cells. To maximize the yield of the recombinant protein in its soluble form, several induction conditions were tested; we found that induction with 1 mM IPTG at 37 °C for 8 h produced the more soluble Shp (data not shown). The protein was purified with affinity column chromatography. The final concentration was 3 μg/μl, with a single band determined by SDS-PAGE (Fig. 1).

3.2 Subcutaneous Immunization with Shp Elicited a Robust IgG Response

To examine the antigenic potential of Shp, recombinant Shp was administered subcutaneously into mice three times at days 0, 14 and 28. The serum anti-Shp IgG titers were evaluated and compared with their respective adjuvant control samples (Tris-HCl plus adjuvant) at days 13, 27 and 41. All mice immunized with Shp and adjuvant developed a significantly high anti-Shp IgG response that became almost completely saturated after the second booster (Fig. 2a–c). The IgG titer ratio at day 41 in Shp-immunized serum and mock-

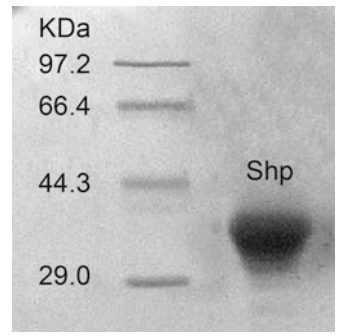

Fig. 1 Purified recombinant streptococcal heme binding protein (Shp). Protein purification with affinity column chromatography and identification by 10% SDS-PAGE

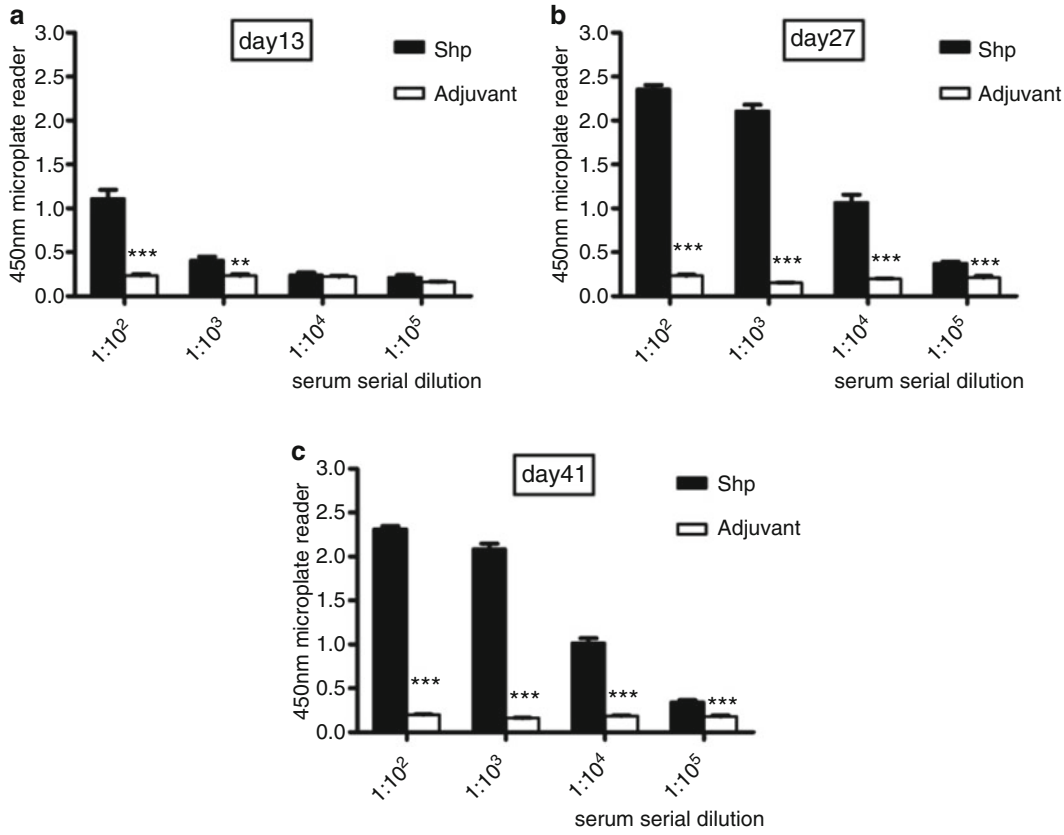

Fig. 2 Anti-Shp IgG response in serum after subcutaneous immunization. Mice were administered recombinant Shp and the resulting serum IgG titers were determined by ELISA. Day13: 2 weeks after the first immunization; Day27: 2 weeks after the second boost; Day41: 2 weeks after the third boost. Data represent the arithmetic mean ± SEM. **, $p < 0.01$ and ***, $p < 0.001$

immunized serum (4.47/0.37, at $1{:}10^2$ serum dilution) was 3.3-fold higher than the same titer ratio at day 13 (1.57/0.43, at $1{:}10^2$ serum dilution). However, the serum IgG titers of mock-immunized mice changed little and were only slightly triggered by adjuvant (Fig. 2a–c). These results suggest that subcutaneous immunization with Shp elicits a robust IgG response and that Shp is highly antigenic.

3.3 Shp Immunization Protected Mice against GAS Infection

The studies described above showed that Shp immunization administered via the subcutaneous route resulted in a significant serum anti-Shp IgG response. To examine whether the increased anti-Shp IgG can protect immunized mice, both the Shp-immunized group and the mock-immunized group were challenged with M1 GAS. Over the first two days, the percent survival of the two groups was the same (86%). However, no more mice died from the Shp-immunized group on the third day. The percent survival at the end point for mice immunized with Shp was 86%, whereas a 60% survival rate was observed in mock-immunized mice after GAS challenge ($p = 0.1536$, Fig. 3a). Although the difference in survival rate between immunized mice and control mice was not significant, immunized mice had higher survival rates. This protection

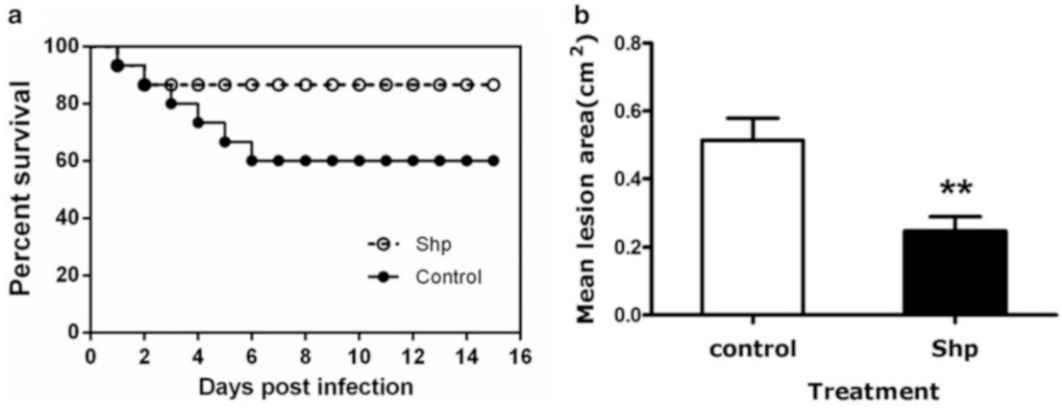

Fig. 3 Protection of mice against group A *Streptococcus* challenge after immunization with Shp. (**a**) Kaplan-Meier survival curves of mice challenged with 1×10^9 CFU bacteria in the back 2 weeks after the third boost with Shp+adjuvant or with adjuvant only are shown (n = 15). Statistical significance was determined by the log-rank test, $p = 0.1536$. (**b**) The skin lesion area was measured 24 h after GAS inoculation (n = 20). **, $p = 0.0014$

is further supported by the effect of Shp immunization on skin lesion size. The mean skin lesion size in immunized mice (0.25 ± 0.04 cm^2) was 49% of that in control mice (0.51 ± 0.06 cm^2), and the difference was significant ($p = 0.0014$, Fig. 3b).

3.4 Shp Immunization Enhanced GAS Clearance and Reduced GAS Skin Invasion

Twenty-four hours after inoculation, the average CFU at the skin infection site in immunized mice was lower (7.614 ± 0.1381) compared with that of control mice (8.212 ± 0.0859) (logCFU/100 mg, $p = 0.0032$, Fig. 4a). These results suggest that GAS is cleared more efficiently in immunized mice. To further examine the basis for the difference in CFU, the skin infection sites were analyzed by Gram staining. Gram staining analysis indicated that infection sites were surrounded by more inflammatory cells in immunized mice (Fig. 4c) than in control mice (Fig. 4b). More importantly, inflammatory cells (pink) engaged in bacteria at the edge of the infection site in immunized mice but not in control mice. Furthermore, inflammatory cells in immunized mice blocked the spreading of GAS in the skin, whereas GAS invaded the surrounding area of the inoculation site. These data suggest that Shp immunization enhances neutrophil infiltration and bacteria clearance and reduces skin invasion.

3.5 Shp Immunization Reduced Systemic GAS Dissemination

To further determine the effect of immunization with Shp on GAS virulence, we enumerated viable bacteria in the blood and from different organs 24 h after inoculation. There was a significant reduction of bacteria in the liver, spleen and lung of Shp-immunized mice compared to mock-immunized mice, except in the blood (Fig. 5a–d). In the blood, the mean colony number of mock-immunized mice was 2.858, in contrast with 2.295 of Shp-immunized mice (log CFU/ml, $p = 0.066$). In the liver homogenate, the colony number (log CFU/100 mg) of mock-immunized mice (4.668 ± 0.1446) was higher than that of Shp-immunized mice (3.404 ± 0.2790, $p = 0.0017$), suggesting that immunization with Shp restricted bacteria dissemination to the liver. Moreover, there was more bacteria in immune organs, including the spleen (6.140 ± 0.1874) in mock-immunized mice compared to Shp

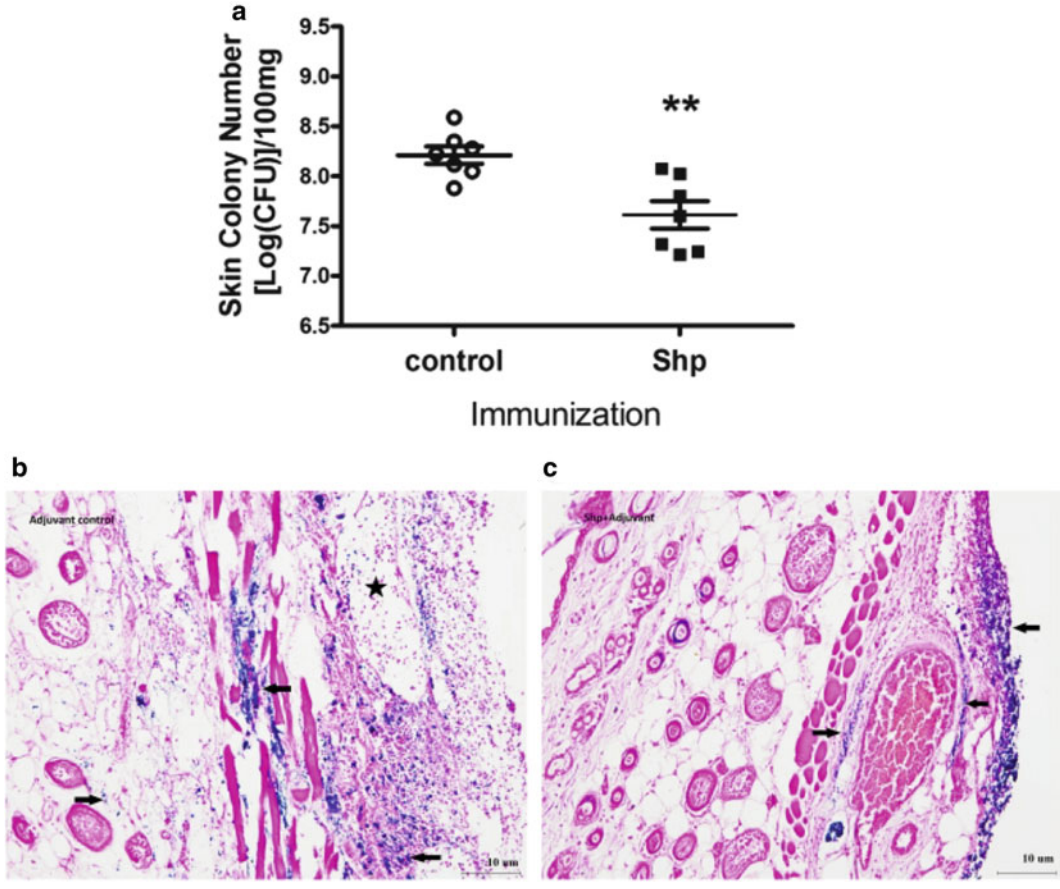

Fig. 4 Growth and spread of group A *Streptococcus* at the skin lesion site. (**a**) GAS growth at the skin lesion site after challenge for 24 h, as measured by colony counting. **, $p = 0.0032$. (**b**) and (**c**): Microscopic pictures of Gram-stained MGAS5005 at the inoculation site at a magnification of×200. (**b**): Skin of mock-immunized mice; (**c**): Skin of Shp-immunized mice. *Black arrow*: bacteria (*blue*); *Black star*: abscess

immunized mice (4.674 ± 0.1985) (log CFU/100 mg, $p = 0.0002$). Thus, Shp immunization significantly reduces systemic GAS dissemination.

4 Discussion

The cell surface heme-uptake proteins of GAS may represent a new target for molecular therapies. There are extremely few free iron molecules that can be acquired by GAS when they enter the human body due to the low solubility of ferric iron in the blood and tissue fluid at physiological pH. Thus, GAS must depend on cell surface heme-uptake proteins to bind to iron-containing proteins or iron carriers such as heme. Shp was the first cell surface heme binding protein identified in gram-positive pathogens (Lei et al. 2002). Shp is highly conserved among the different GAS serotypes and would provide a broader serotype coverage. Thus, we determined whether Shp is a protective antigen.

In this study, we purified recombinant Shp and determined the serum immune response

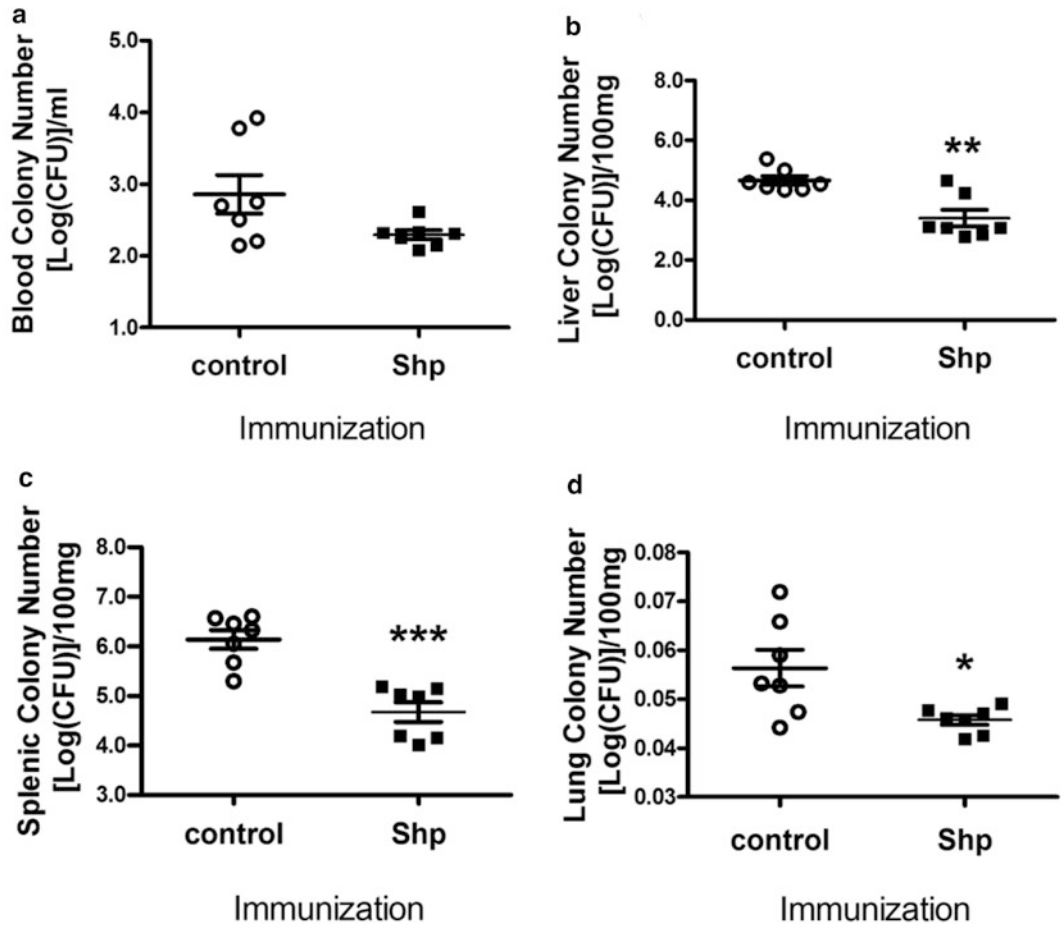

Fig. 5 Group A *Streptococcus* survival in mouse blood and organs, as measured by colony counting at 24 h post challenge. Comparison of systemic GAS dissemination in Shp-immunized mice and mock-immunized mice. CD1 mice were subcutaneously inoculated with 1×10^9 CFU MGAS5005 at 14 days after the final booster. (**a**) GAS survival in the blood, $p = 0.066$. (**b**) GAS survival in the liver, $^{**}p < 0.01$. (**c**) GAS survival in the spleen, $^{***}p < 0.001$. (**d**) GAS survival in the lung, $^{*}p < 0.05$. Data are shown as the mean ± SEM, n = 7

triggered by Shp immunization using alum as an adjuvant. Immunization with Shp elicited a high antibody response. The serum IgG titers were almost saturated after the second booster. This suggests that Shp is immunogenic and only requires one more booster to increase the anti-Shp IgG titer. In addition, clinical data showed that patients suffering from GAS diseases (caused by distinct serotypes) have significantly higher levels of anti-Shp antibodies in convalescent-phase sera, also suggesting that Shp is immunogenic in humans and has broad serotype coverage (Lei et al. 2002).

Although the difference in survival rate was not statistically significant, immunization with Shp caused less mice to die after GAS infection. Moreover, the protective effect could be illustrated by the skin lesions at the GAS inoculation sites. The average skin lesion size in Shp-immunized mice was 49% of that in control mice, and the average CFU in skin lesions of Shp-immunized mice (4.11×10^7) was 25.2% of that in control mice (1.63×10^8), suggesting that Shp immunization significantly reduces skin lesions and may influence the initial step of GAS invasion, which is crucial for bacterial pathogenesis.

There were significant reductions of bacteria CFU in the organs and at the inoculation sites of Shp-immunized mice, indicating that Shp immunization enhances GAS clearance and reduces GAS skin invasion. These results support that Shp represents a promising antigen that can induce protective effect after immunization. It was interesting that most bacteria CFU was in spleen compared to those in the liver and lung, suggesting that GAS is able to disseminate to the spleen from the blood. However, the CFU in the spleen of Shp-immunized mice was significantly less than that in control mice. The most likely reason for this phenomenon is the protective immune response after Shp immunization. As GAS disseminated to the immune organ (spleen), the majority of bacteria were killed. Thus, we assumed that the clearance ability would be enhanced after Shp immunization. The enhanced clearance abilities were also present in the liver and lung rather than the blood. Further, histological examination showed that Shp-immunized mice formed small and mild lesions at the inoculation site, which differed from the large abscesses and the spread of bacteria even to the smooth muscle layer in mock-immunized mice. These results demonstrate that immunization with Shp reverses the severe inflammatory response on the skin and hinders the spread of bacteria at the infection site. In summary, the surface protein Shp is immunogenic and Shp immunization can protect mice against GAS infection. Another cell surface heme binding protein, Shr, could also provide defense from systemic GAS infections (Huang et al. 2011). It shows that Shr serum antibodies are likely to interfere with Shr iron acquisition and thus may act directly to limit GAS growth and spread. Together with our result, targeting the heme acquisition system may be a promising direction for GAS vaccines. Additional studies would be worthwhile to determine the coverage of distinct GAS serotypes, which may contribute to the development of GAS vaccines.

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The Public Health Impact of the So-Called "Fluad Effect" on the 2014/2015 Influenza Vaccination Campaign in Italy: Ethical Implications for Health-Care Workers and Health Communication Practitioners

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Abstract

Seasonal influenza, causing complications, hospitalizations and deaths, generates a serious socio-economic burden, especially among elderly and high-risk subjects, as well as among adult individuals. Despite the availability and active free-of charge offer of influenza vaccines, vaccine coverage rates remain low and far from the target established by the Ministry of Health. Notwithstanding their effectiveness, vaccines are victims of prejudices and false myths, that contribute to the increasing phenomenon of vaccine hesitancy and loss of confidence. Media and, in particular, new media and information and communication technologies (ICTs) play a major role in disseminating health-related information. They are extremely promising devices for delivering health education and promoting disease prevention, including immunization. However, they can also have a negative impact on population's health attitudes

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and behaviors when channeling wrong, misleading information. During the 2014/2015 influenza vaccination campaign, the report of four deaths allegedly caused by administration of an adjuvanted influenza vaccine, Fluad – the so-called "Fluad case" – received an important media coverage, which contributed to the failure of the vaccination campaign, dramatically reducing the influenza vaccine uptake. In the extant literature, there is a dearth of information concerning the effect of the "Fluad case". The current study aims at quantifying the impact of the "Fluad effect" at the level of the Local Health Unit 3 (LHU3) ASL3 Genovese, Genoa, Italy. Ethical implications for health-care workers and health communication practitioners are also envisaged.

Keywords
Ethics of vaccination • Fluad influenza vaccine • Influenza vaccination campaign • Media coverage

1 Introduction

Influenza is a highly infectious airborne disease known since ancient times, caused by a negative-sense, single-stranded RNA virus which belongs to the *Orthomyxoviridae* family (Barberis et al. 2016). In particular, seasonal influenza, causing complications, hospitalizations and deaths (250,000–500,000 yearly deaths worldwide, according to an estimate of the World Health Organization, WHO) (Gasparini et al. 2012), generates a serious socio-economic burden, especially among elderly and high-risk subjects (Gasparini et al. 2012). It imposes a high economic impact among adult individuals as well, with an estimated cost of €940.39 per case (Gasparini et al. 2012).

Despite the availability and active free-of charge offer of influenza vaccines (Gasparini et al. 2014a; Gasparini et al. 2014b), vaccine coverage rates remain low and far from the target established by the Ministry of Health and agreed upon by the scientific community, set at 75% as minimum (Bonanni et al. 2015; Gasparini et al. 2013b). Notwithstanding their effectiveness, vaccines are victims of histories, prejudices, false allegations, controversies, "urban myths" and, even, shams and frauds (Gasparini et al. 2015, 2016; Signorelli 2015; Terracciano et al. 2016), that contribute to increasing concerns towards vaccination (Rosselli et al. 2016). This phenomenon is known as vaccine hesitancy, and according to the Strategic Advisory Group of Experts (SAGE), can be defined as "delay in acceptance or refusal of vaccines despite availability of vaccinations services. Vaccine hesitancy is complex and context specific, varying across time, place, and vaccines. It is influenced by factors such as complacency, convenience, and confidence" (SAGE 2014; Salmon et al. 2015).

Media and, in particular, new media and information and communication technologies (ICTs) play a major role in disseminating health-related information. They are extremely promising devices for delivering health education and promoting disease prevention, including immunization (Amicizia et al. 2013; Odone et al. 2015b, c). However, they can also have a negative impact on population's health attitudes and behaviors when channeling wrong, misleading information.

During the 2014/2015 influenza vaccination campaign, the report of four deaths allegedly caused by the administration of a MF59-adjuvanted influenza vaccine, Fluad (Gasparini et al. 2013a, b; Tsai 2013) – the so-called "Fluad case" (Odone et al. 2015a; Signorelli et al. 2015) – received an important media coverage, which contributed, at least partially, to the failure of the vaccination campaign, dramatically reducing the influenza vaccine uptake. Fluad is an inactivated

subunit vaccine adjuvanted with an oil-in-water emulsion (Vogel et al. 2009), approved and licensed in 1997. Several clinical trials have shown that it is capable to foster higher levels of hemagglutination inhibition antibodies and, as such, to confer greater protection with respect to non-adjuvanted vaccines (De Donato et al. 1999; Frey et al. 2003; Gasparini et al. 2013a; Minutello et al. 1999; Nolan et al. 2014; O'Hagan et al. 2013; Podda 2001).

During the 2014/2015 two women and two men aged between 67 and 87 died, after being vaccinated with Fluad. These deaths, as reported by the Italian Network of Pharmacovigilance, occurred in the southern regions of Sicily and Molise. During the safety assessment, further presumed post-vaccination deaths occurred and the number of fatal adverse events grew to 19. The link between the Fluad uptake and the deaths is purely due to chance and not to causality, as rigorously assessed by the Italian and European drug regulators (European Medicines Agency (EMA). Press release. 3 December 2014; Italian Medicines Agency (AIFA). Press release. 23 December 2014). However, this uncertainty has impacted on vaccination coverage.

In the extant literature, there is a dearth of information concerning the effect of the "Fluad case" and its public health impact. The current study aims at quantifying the impact of the "Fluad effect" at the level of the Local Health Unit 3 (LHU3) ASL3 Genovese, Genoa, Italy.

2 Material and Methods

2.1 Data Extraction

Data concerning the number of vaccinations delivered at ambulatories of LHU3 Genoa, Genoa (Italy) were extracted from 27th November 2014 to 27th December 2014. Data were also broken down according to the age of the subjects (subjects $\geq$65 years, and subjects $\leq$64 years) and were, finally, compared with the data concerning the previous vaccination campaign (2013/2014).

More in details, since the two time series of absolute number of vaccinations delivered per year could have reflected differences in logistics and organization – during the 2014/2015 campaign, the number of *ad hoc* ambulatories (that is to say, specifically dedicated to influenza vaccination) increased – data were normalized in order to minimize the impact of this variable. Specifically, absolute figures of vaccination per year were rescaled in the range 0–1 by applying the formula $z_i = \frac{x_i - \min(x)}{\max(x) - \min(z)}$, where $x = (x_1, \ldots, x_n)$, and z_i is the ith normalized value, and the relative number of vaccinations delivered before and after 27th November were compared for both years (Hinkle et al. 2003). To compute the eventual impact of the so-called "Fluad case", we summed up the number of vaccinations delivered before and after 27th November and we calculated the decrease in percentage.

2.2 Big Data Analysis

Google News (freely available at https://news.google.it/) and Twitter (accessible at https://twitter.com/) have been mined in the same period, searching for Fluad-related content written in Italian language, using "Fluad" as key-word and exploiting an *in-house* script, as previously described in details in (Bragazzi et al. 2016a) and in (Bragazzi et al. 2016b).

3 Results

The main events associated with the study period are detailed in Table 1. The two time series of number of vaccinations delivered per day during the influenza vaccination campaign 2013/2014 and 2014/2015 are shown in Fig. 1. The decision of the Italian Medicines Agency (AIFA) to withdraw two Fluad batches (namely, the batches 142701 and 143301) after the report of four deaths wrongly related to the Fluad vaccine uptake (Italian Medicines Agency (AIFA) 2014) was associated with a decrease in accesses to vaccine ambulatories, whilst the report of the

Table 1 The key media events during the Fluad case and the main events of the 2014/2015 influenza vaccination campaign at LHU ASL3 Genoa, Italy

Day	Event
27th October 2014	Launch of the influenza vaccination campaign at LHU3 ASL3 Genovese, Genoa (Italy)
27th November 2014	AIFA decided to suspend two Fluad batches because of three deaths allegedly associated with Fluad vaccine administration
1st December 2014	ISS released the first safety results
3rd December 2014	EMA released the PRAC conclusion
23rd December 2014	ISS released the final safety results
27th December 2014	End of the influenza vaccination campaign at LHU3 ASL3 Genovese, Genoa (Italy)

Abbreviations: *AIFA* Italian Medicines Agency, *EMA* European Medicines Agency, *ISS* Italian Institute of Health, *LHU* Local Health Unit, *PRAC* Pharmacovigilance Risk Assessment Committee

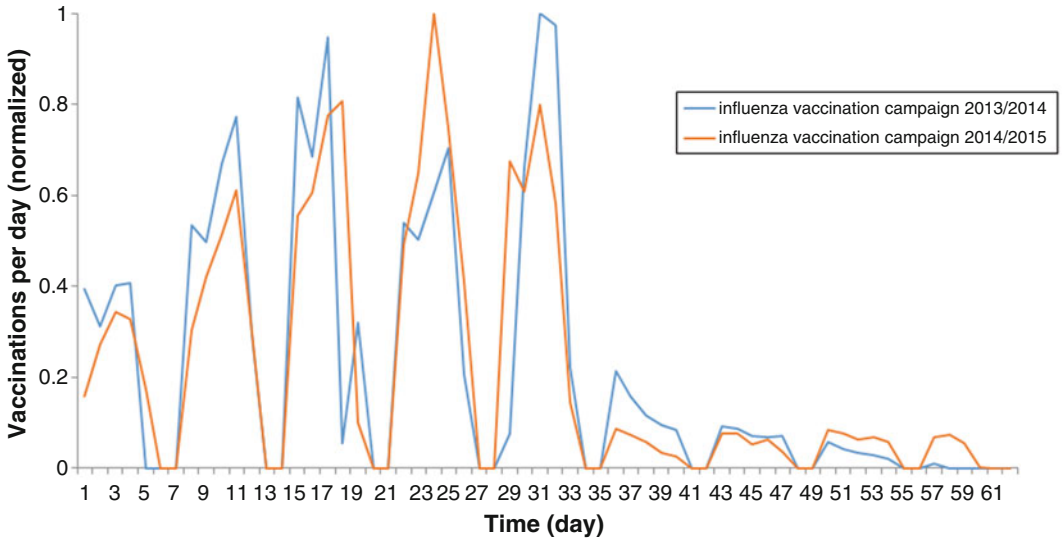

Fig. 1 The normalized number of vaccinations per day delivered during the influenza vaccination campaigns 2013/2014 and 2014/2015 at the Local Health Unit ASL3 Genoa, Italy. Figures have been rescaled in the range 0–1

PRAC's safety results and the European Medicines Agency (EMA)'s conclusions (European Medicines Agency (EMA) 2014) did not contribute to increasing the reduced influenza vaccine uptake (Fig. 2).

In terms of raw figures, the number of vaccinations delivered at ambulatories of LHU3 decreased by 84.7% (in particular, by 85.0% for subjects ≥65 years and by 80.2% for subjects ≤64 years). Then, we compared this trend with that occurred in the previous year: we found that the number of vaccinations had decreased by 78.5%. As such, the decrease that could be attributed to the "Fluad effect" is 6.2% (1.2% for subjects ≥65 years and 14.6% for subjects ≤64 years).

Our analysis of Fluad-related *corpus* of 1613 tweets retrieved and processed (Fig. 3a) has shown a remarked activity at the news of the putatively Fluad-related deaths, which has gradually decreased throughout time. More in details, the tweet peak corresponding to the announcement of the recall of Fluad batches (27th November 2014) is 2-times higher than the

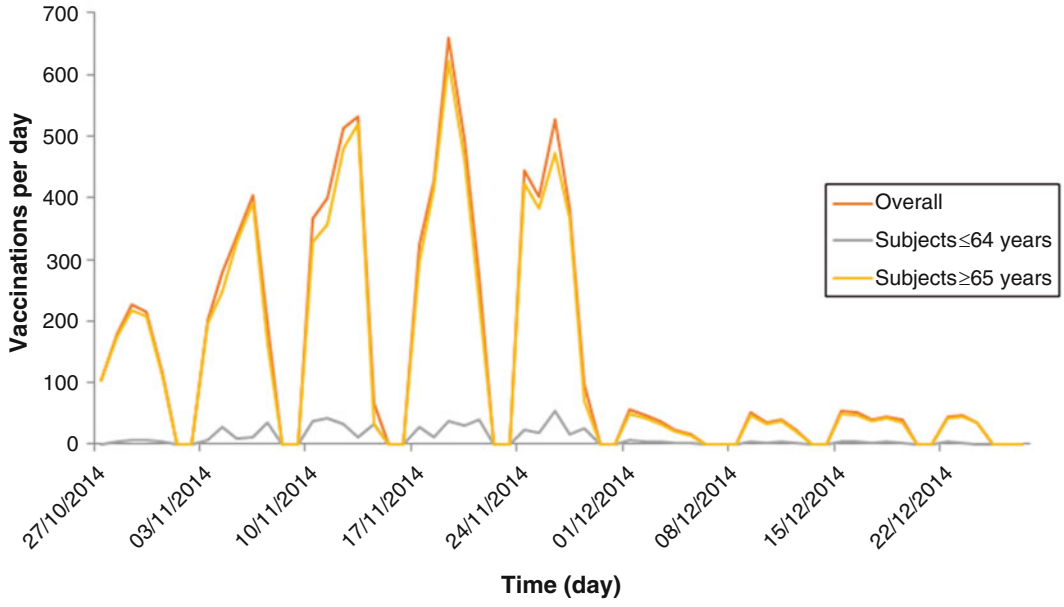

Fig. 2 The number of vaccinations per day delivered during the influenza vaccination campaign 2014/2015 at the Local Health Unit ASL3 Genoa, Italy

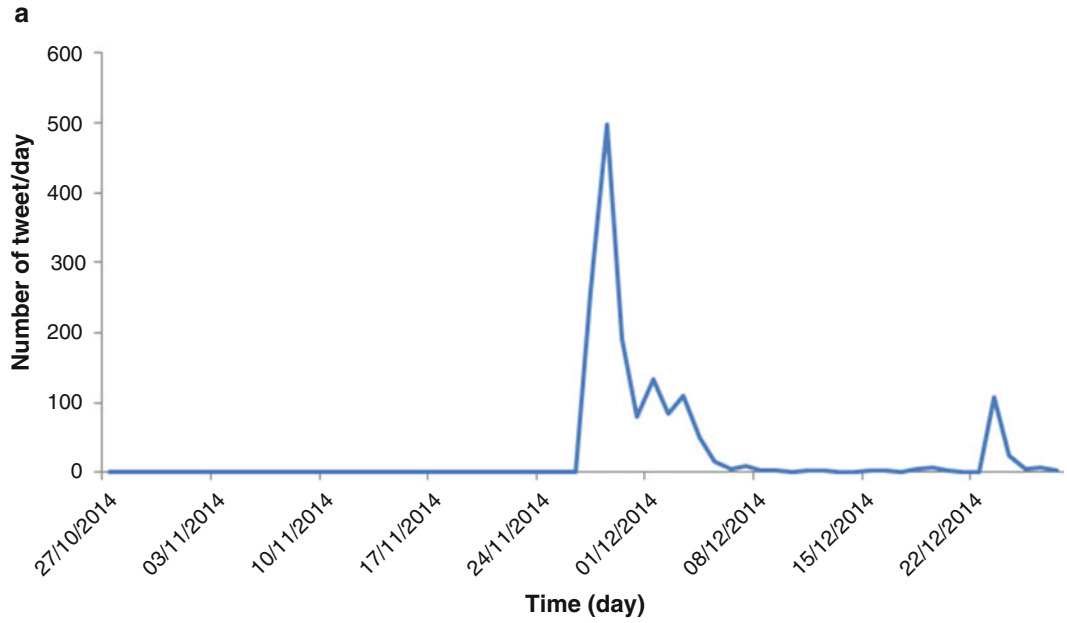

Fig. 3a Time series of Fluad-related tweets during the study period

peak corresponding with the first ISS' announcement of safety of Fluad vaccine (1st December 2014) and 2.4-fold higher than the peak in coincidence with the EMA's and the ISS' final clarification of no relation between deaths and Fluad administration (3rd and 23rd December 2014). When considering the highest peak occurred 1 day after the announcement of

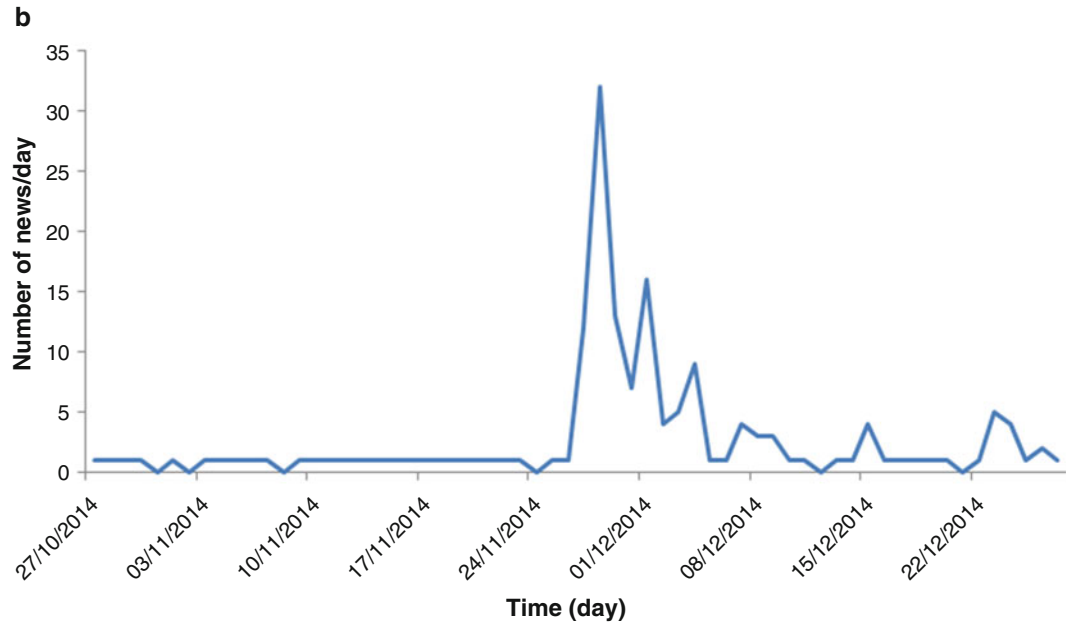

Fig. 3b Time-series of Fluad-related news during the study period, as captured by Google News

Novartis of the precautionary recall (28th November 2014), these figures are 3.7 and 4.6, respectively. Also the analysis of Fluad-related news has yielded similar results (Fig. 3b).

4 Discussion

To the best of our knowledge, our study is one of the few studies to quantitatively assess the impact of health communication on vaccine uptake.

Health communication can be defined as "a multifaceted and multidisciplinary field of research, theory, and practice concerned with reaching different populations and groups to exchange health-related information, ideas, and methods in order to influence, engage, empower, and support individuals, communities, health-care professionals, patients, policymakers, organizations, special groups, and the public so that they will champion, introduce, adopt, or sustain a health or social behavior, practice, or policy that will ultimately improve individual, community, and public health outcomes" (Schiavo et al. 2014).

Specifically focusing on the "Fluad case", Odone and coworkers performed a quantitative analysis of the media coverage during the 2014/2015 influenza vaccination campaign and found that whilst no articles were published at the launch of the immunization campaign, most articles were released during the "uncertainty period", that is to say the time window between the report of the three deaths putatively related to the administration of two Fluad batches and the PRAC's safety results and the EMA's conclusions. Furthermore, during this period, articles were rather longer and located in the main page or main sections of Italian newspapers (Odone et al. 2015a).

This finding has been replicated by our analysis of Fluad-related tweets and news written in Italian language and posted/released during the study period 27th October–27th December 2014.

Capanna and collaborators investigated LHU coordinators' perceptions concerning vaccination adherence assessment during the 2014/2015 influenza vaccination campaign in Lazio, using *ad hoc* questionnaires sent *via* email. The authors found that LHU coordinators perceived that the "Fluad case" was a major media event

with a negative impact on anti-flu vaccination program adherence. The LHU coordinators underlined a failure in communication and joint management of Public Health Institutions in Italy about efficacy and safety information of flu vaccine (Capanna et al. 2015).

Generally speaking, health-protective behavioral intentions are positively influenced by media coverage, when health communication is well-thought, planned, proactive and highly integrated. Media represent, indeed, an important information source for the general population (Bragazzi 2013; Gargano et al. 2015). For example, in Russia in the-1990s, communication helped to increase diphtheria vaccination coverage after a number of outbreaks due to a drop in diphtheria, tetanus and pertussis (DTP) coverage (Porter et al. 2000; Waisbord and Larson 2005). In particular, Kononova and collaborators have shown that variables such as multitasking, polychronicity (preference for multitasking), and strength of health-related arguments play a crucial role in shaping attitudes to vaccinating (Kononova et al. 2016). On the contrary, a poor, distorted, or biased health communication can contribute to vaccine hesitancy and loss of confidence (Goldstein et al. 2015; Odone et al. 2015b, c). For example, the need of reducing thimerosal in vaccines was poorly communicated in 1999 and contributed to undermine confidence in vaccination practices (Goldstein et al. 2015).

A proper communication is of crucial importance especially when there is the need to communicate risk and uncertainty in a rigorous, credible, evidence-based and transparent but accessible way (Terracciano et al. 2016). For example, during the 2009 influenza A (H1N1) pandemic, throughout Europe, collaboration between experts and the media was quite poor and professionals felt misunderstood (Cloes et al. 2015). This issue is not so new: already during the swine flu, as reported by Rubin and Hendy, the media coverage was superficial even though not inaccurate or sensational. However, television newsmen and wire reporters were generally unprepared for such a complex story. The authors concluded that a better and closer cooperation between the medical/scientific community and the media would have resulted in an improved immunization coverage (Rubin and Hendy 1977).

Our data show a 6% decrease in vaccine uptake after the precautionary suspension of Fluad decided by AIFA. This figure is in line with the decrease of the overall influenza vaccination coverage in people aged $\geq$65 years from 55.4% to 48.6% at a national level (Levi et al. 2016). Even though this decrease is less than what one could potentially expect (Capanna et al. 2015), some aspects should be considered. On one hand, the announcement of the Italy's drug regulator occurred during the second part of the vaccination campaign, that is to say in the period when most people had already undergone immunization practice. On the other hand, it is not the first time that the Fluad vaccine has been banned: it had already been suspended in October 2012 and since then influenza coverage rates have been particularly low (Capanna et al. 2015). Thus, it is noteworthy that the ban in 2014 has reduced the vaccine uptake of 6%.

In conclusion, as in the swine flu case and in the H1N1 case, also the "Fluad case" demonstrates the need of a proper communication and the onus of a collaboration between health-care workers in the field of public health and health communication practitioners.

5 Conclusion

Seasonal influenza vaccines can effectively prevent influenza cases and its complications in terms of hospitalizations and deaths, thus mitigating the influenza-related direct and indirect costs and societal burden. However, influenza immunization coverage in Italy remains unacceptably low. During the 2014/2015 influenza season, the Fluad case negatively influenced influenza vaccine uptake and confidence. Our study shows that the report of the four deaths originally wrongly associated with the uptake of the Fluad influenza vaccine had, at least partially, a detrimental effect on population health and prevention behaviors at the level of LHU3 ASL3 Genovese, Genoa (Italy). The report of

the PRAC's safety results and final EMA's conclusions had no effect on vaccine uptake, suggesting a gap in communication. Public institutions, health authorities and agencies as well as the scientific community should make serious efforts in developing *ad hoc* innovative, multidisciplinary strategies for better conveying and disseminating health education messages and actively engaging with the media and ICTs for creating connectivity, relationship and trust towards vaccination practice among population (Jit et al. 2015; Orr et al. 2016; Philip et al. 2016; Stahl et al. 2016). In this way, it will be possible to design tailored interventions meeting with the users' needs and reaching out different populations (Philip et al. 2016). Decision-makers and stakeholders should properly found communication campaigns to raise awareness of vaccination practices (Waisbord and Larson 2005) and the different actors involved should properly coordinate and collaborate with each other to attain the ambitious goal of adequate vaccination coverage rates, as a shared ethical responsibility (Biasio et al. 2016; Odone et al. 2015b, c; Odone and Signorelli 2015).

Conflicts of Interest None.

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