ORIGINAL RESEARCH PAPER
Volume-7 | Issue-10 | October-2018 | PRINT ISSN No 2277 - 8179
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

BACTERIA-BACTERIA AND HOST-BACTERIA INTERACTION OF BACTERIA ISOLATED FROM A PATIENT WITH CYSTIC FIBROSIS.

Microbiology

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ABSTRACT

Bacterial populations and inflammation in the airways have been suggested as the main causes of cystic fibrosis (CF) pulmonary exacerbation. In this work we studied the interactions of pathogenic bacteria *Pseudomonas aeruginosa* (Pae) and of *Burkholderia cepacia* complex (BCC) with the oropharyngeal flora *Streptococcus milleri* group (SMG) isolated from the sputum of a CF patient with pulmonary exacerbation. We investigated the virulence of single and mixed bacteria by evaluating the *in vitro* production of pyocyanin by spectrophotometric analysis and of rhamnolipids by haemolysis and growth inhibition of *Bacillus subtilis*. Elastase production was analyzed in a mucus-like medium with the addition of polymorphonuclear leukocytes (PMN) or DNA. Also, necrosis and netosis induced by bacteria in PMN were measured by flow cytometry and spectrofluorometry, respectively. Bacterial infections in a murine experimental model were analyzed.

Combinations of Pae with BCC and SMG enhanced pyocyanin and rhamnolipids production ($p<0.01$). Pae and BCC induced the highest elastase release from PMN, but it was lower when SMG was added ($p<0.05$). Highest PMN necrosis levels were induced by the mixture of SMG, BCC, and Pae ($p<0.01$). Pae was the major inducer of PMN netosis, followed by BCC and SMG. When Pae was combined with BCC and/or SMG, netosis was reduced. Netosis correlated with elastase values.

In mouse models, infections combining SMG with Pae increase pulmonary inflammation.

Commensal strains can increase the virulence of pathogenic bacteria. The exact role of Netosis and elastase in polymicrobial infections remains to be determined.

KEYWORDS

Cystic Fibrosis, Polymicrobial Infection, *Streptococcus Milleri*, Neutrophils, Netosis.

INTRODUCTION

The biological relevance of microbial interactions remains largely unknown. A deeper understanding of the mechanisms of these interactions will provide a new perspective on the role of known virulence determinants and of the factors relevant to polymicrobial disease. Rather than being thought of simply as a host-pathogen relationship, these interactions should be envisioned as a whole spectrum of host-pathogenic microbe mechanisms, microbe-microbe interactions, and environmental factors [1].

Cystic fibrosis (CF) is a disease in which an understanding of these events is crucial.

It is an autosomal recessive disorder that leads to the abnormal composition of airway secretions and determines a predisposition for chronic polymicrobial bronchopulmonary infections [2] with a prolonged inflammatory response exhibiting various degrees of neutrophil influx, these neutrophils being major contributors to this disease [3,4].

Pathogens commonly reported in CF include *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* (Pae), which is the most relevant. Other opportunists include nosocomial pathogens such as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Burkholderia cepacia complex* (BCC). These bacteria occur in many environments as complex surface-attached communities termed biofilms [5].

Polymorphonuclears (PMN) home in the site of infection and kill pathogens by phagocytosis. Apart from the more traditional mechanisms of phagocytosis and degranulation, neutrophils can also generate NETs (neutrophil extracellular traps) to directly combat microbes during inflammation and infection. NETs are cast as decondensed chromatin fibers coated with antimicrobial proteins,

forming web-like structures that trap and kill a variety of microbes during inflammation and infection. They contain DNA, modified extracellular histones, proteases and cytotoxic enzymes such as elastase [6].

Biofilm bacteria respond to the presence of PMN by up regulating the synthesis of several virulence determinants like rhamnolipids and pyocyanin, mainly from Pae, which are capable of inducing PMN necrosis or apoptosis [7]**.** In addition, these microbes and several host factors can stimulate NETs formation. Contact with biofilm causes PMN in the site of infection to undergo a frustrated phagocytosis or release NETs, trapping and killing various microbes. Consequently, PMN release enzymes and reactive oxygen intermediates that damage surrounding tissues [6, 8].

In bronchial secretions studied with conventional culture techniques, species of mouth-dwelling microbiota were considered as sputum contaminants. However, these bacteria considered as commensals could be involved in pulmonary exacerbation. The role of these commensals is interesting since they are probably able to enhance Pae pathogenicity. This interaction is the focus of extensive studies [9]. The confusion surrounding streptococcocal taxonomy together with the inability of the clinical microbiology laboratory to identify streptococci and their association with commensal microbiota has created an environment whereby the medical significance of these organisms is easily overlooked. The *Streptococcus anginosus* group, herein referred to as the *Streptococcus milleri* group (SMG), which includes the species *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*, has been implicated in pulmonary exacerbations in CF patients [10]**.**

Communication between bacteria (pathogens and commensals) in the CF lung occurs through molecules known as autoinducers, which coordinate the production of biofilm and virulence factors in a process called quorum sensing (QS). Pulmonary exacerbation due to co-colonization could result from pathogenic activity of all microbial species or from the re-expression of virulence factors of pathogens induced by the presence of commensals [11].

The above data suggest that pathogen-commensal interactions and pathogen-commensal–neutrophil interactions are highly relevant to CF airway pathophysiology.

We still lack a solid understanding of how multiespecies interactions govern the scope, progression and severity of CF, and even less is known about the way in which the host responds to polymicrobial infection compared to monomicrobial infection. In order to provide some information about this topic, we analysed the behaviour of three strains, Pae, BCC and SMG, isolated from the sputum of a CF patient with severe pulmonary exacerbation**.**

Bacterial activities of both single and mixed strains were compared. *In vitro* studies of pyocyanin and rhamnolipid production by bacteria were performed using a conventional medium; elastase was measured in bacterial cultures in a mucus-like medium supplemented with PMN or DNA and necrosis and netosis were determined in human PMN cultures challenged with bacteria. Besides, *in vivo* bacterial interactions were studied in a mouse pulmonary infection model.

Materials and methods

1-CF patient:

An 8-year-old female with a ΔF 508 mutation, admitted to the Hospital del Niño Jesus, Tucumán, Argentina, with an infection with Pae*,* BCC and SMG.

The first microbiological study was conducted by our team at the time of the patient's admission to the hospital during an acute pulmonary exacerbation with a forced expiratory volume in one second (FEV1) of 44%. Intravenous antibiotic treatment with tobramycin, ceftazidime and colistine was initiated on admission. Antibiotic therapy with inhaled tobramycin was continued for the following 10 days, as the patient had not returned to the baseline functional status. The patient remained hospitalized for 15 days and achieved only modest clinical remission.

Three months later, the patient was admitted with a second episode of exacerbation and was administered the same antibiotic treatment. During this period, FEV1 was decreased to 26% until her death 15 days later.

2-Sputum samples:

Sputum was processed using standard clinical microbiological methods [12] after approval of the Niño Jesus Hospital Ethics Committee and written parental consent. Pae*,* BCC and SMG were all isolated from sputum samples of the CF patient. Samples were collected in sterile containers during periods of remission and two exacerbations (before antimicrobial therapy).

Samples were inoculated onto plates of Columbia blood agar, chocolate agar and Columbia sheep blood agar 5% (Biomerieux©, Argentina), Levine and BCSA agar, Sodium Azide agar and Mannitol Salt agar (Britania, Argentina).

Plates were incubated for 48-72 h at 37 °C in air alone or 5% CO_2 (Levine and BCSA agar). Phenotypic identification was based on conventional biochemical properties, API NE20 and API Strep (Biomeriex©, Argentina).

3-Bacterial strains isolation:

Pae sensitive to ceftazidime, ciprofloxacin, gentamicin, amikacin, imipenem, meropenem, piperacillin/tazobactam and colistine and BCC were isolated during the first pulmonary exacerbation. During a second exacerbation after the remission period, Pae resistant to all the above antibiotics and sensitive to colistine was isolated besides the *Streptococcus anginosus* group called SMG. BCC was determined by a PCR assay.

Analysis of the nucleotide sequence of the sensitive strain of Pae and the multi-resistant Pae strains showed that they were not genetically related.

Multi-resistant Pae, BCC, and SMG strains were used in the subsequent experiments.

5-Bacterial supernatants: Pae and BCC were selected from Levine and BCSA media. The SMG colonies were obtained from Columbia blood agar (Biomerieux©, Argentina). The monocultures were inoculated with a final inoculum corresponding to the McFarland 0.5 standard in 5 ml of BHI broth medium (Britannia, Argentina) for Gram-negative bacteria, and in Todd Hewitt broth supplemented with 0.5% yeast extract (Difco, Sparks, MD, USA) for SMG. Co-cultures of the strains with all possible combinations were performed. Bacteria were grown under aerobic conditions for 24 h**.** Supernatants of monocultures and mixed cultures were obtained by centrifugation at 30,000 g for 30 min followed by filtration with Millipore 0.22 μm filters. Broth was used as a negative control.

7-Pyocyanin assays: Pae and Pae mixed with BCC and SMG (V/V) cultures were grown for 24 h. Bacteria were removed by high speed centrifugation and pyocyanin concentrations in the culture supernatants were determined by subsequent chloroform/0.2 M HCl extraction steps. Absorbance was measured at 520 nm.

9-Rhamnolipids assays:

Two independent tests were conducted for rhamnolipids detection. They included haemolyses of erythrocytes by rhamnolipids (13) and growth inhibition of *B. subtilis* exerted by rhamnolipids (14). Supernatants were concentrated as follows: the pH of Pae supernatant culture and its respective mixtures with BCC and SMG supernatant cultures was adjusted to 6.5 and ZnCl2 was added to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) and extracted twice with an equal volume of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved in 100 ml of methanol. Concentrated culture supernatants were spotted onto paper filter discs (6.0-mm Whatman AA discs, Eurolab, Buenos Aires, Argentina), which were then put onto a layer of LB soft agar containing freshly grown *B. subtilis* cells (approximately 10^9/ ml) or onto agar plates containing 5% sheep blood (Becton Dickinson, Buenos Aires, Argentina). Typically, 10 ml of a culture supernatant was concentrated down to 100 ml, and 10 ml of aliquots was applied to the paper discs. After incubation of the *B. subtilis* plates at 37 °C overnight and the blood agar plates at room temperature for 2 days, the corresponding inhibition halos and haemolyses were measured.

10-XTT reduction assay: a balanced biofilm microbial community was reached following interactions among the numerous bacteria found in chronic infections. In order to determine the competency of the strains (Pae, BCC and SMG) inside the biofilm, we studied the

viability of bacteria in the biofilm when culture supernatants of planktonic bacteria were added. The turbidity of the bacterial suspensions (single or mixed) was adjusted to that of the McFarland 0.5 standard to achieve 10^8 CFU/ml. Then, 100 l of the suspensions were dispensed into 96-well Microtiter plates and incubated at 37 ˚C for 6 h. After incubation, when biofilms were obtained, 50 l of culture supernatants of single bacteria or bacteria mixed in different combinations were added. The plates were then incubated at 37 ˚C for 12 h. Then, semiquantitative measurements of metabolic activity of bacteria inside biofilms were performed with the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5[(phenylamino) carbonyl]-2*H*tetrazolium-hydroxide (XTT) reduction assay. Biofilms formed on the wells were washed twice with PBS to remove planktonic cells. Then, 50 µl of XTT salt solution (1mg/ml in PBS) (Promega, Madison, WI, USA) were added to each well. The plates were incubated at 37 ºC in the dark for 90 min. Bacterial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in a colorimetric change that can be measured spectrophotometrically at 492 nm, which was correlated with cell viability.

11-PMN isolation: PMN from human donors were isolated from heparinized venous blood samples by density gradient centrifugation using Ficoll-Hypaque and dextran T-500 (Sigma-Aldrich, St. Louis, MO, USA). PMN viability, determined by microscopy with Trypan Blue staining, was >96%.

12-Elastase assay: BM2 medium (plus DNA or PMN) was used for this assay. BM2 is a mucus-like medium developed by Yeung et al., which was formulated with: 62 mM potassium phosphate buffer (pH 7), 0.1% (wt/vol) Casamino Acids (CAA), 2mM MgSO4, 10 µM FeSO4, 0.4% (wt/vol), and 1% porcine gastric mucin. Mucin is a major component of respiratory mucus [15]. Since severe neutrophilic inflammation and extensive die-off of neutrophils is a distinctive feature of CF lung disease and vast amounts of free DNA build up in the airways in CF lungs, contributing to increased mucus viscosity [16], PMN (10^4/ml) or DNA (Nutrition Biochemical Corp., OH, USA) (1.4 mg/ml) were added to BM2 medium.

Bacteria (single or mixed) were grown in Eppendorf tubes containing BM2 medium to which DNA or PMN was added. Following incubation for 12 h at 37°C, the tubes were centrifuged, the supernatants collected, and the elastolytic activity was investigated using the Elastin-Congo red assay. Supernatants absorbance was measured at 495 nm [17].

13-Bacterial growth: bacteria (single or mixed) were grown on BM2 medium supplemented with DNA or PMN; after incubation for 24 h at 37°C, viable counts were determined by plating serial dilutions on selective media agar plates (blood, chocolate, Levin, Cled and BCSA).

14-PMN necrosis: briefly, PMN (10^6 cells/ml) were incubated for 1 h at 37 °C with Pae, BCC, and SMG cultures and their combinations. Cells were then washed with PBS and 1X binding buffer (0.1 M HEPES in NaOH, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$). Then, PMN were suspended in 100 µl of 1X binding buffer containing 5 µl of Propidium Iodide (PI) solution (50 µg PI/ml PBS, pH 7.4) and incubated in the dark at room temperature for 15 min. Within one hour after the addition of 400 µl of 1X binding buffer, the samples were analyzed in a Partec Pas II flow cytometer (FL2). The data for each population are given as percentage of PMN necrosis [18].

15-PMN Netosis: neutrophils suspended in HBSS containing Ca2+, Mg2+ and HEPES (20 mM) were aliquoted (5×10^4 cells/180 µl/well) into 96 well plates and left to settle for 30 min at 37°C. After that time, 20 µl of HBSS containing $5x10^5$ bacteria (single or in different mixtures) was added. In order to control NETs 100%, PMN were supplemented with $5x10^6$ Pae /180 µl /well. PMN supplemented with HBSS was used as a background control. The final volume in each well was 200 µl. Plates were incubated for 4 h and then SYTOX green (Molecular Probes, OR, USA) (6 µM final concentration), a cell-impermeable nucleic acid stain, was added and NET formation was observed by measuring mean fluorescence in a spectrofluorometer FLx800 (BIOTEK, VT, USA). Cells were also visualized by fluorescent microscopy carried out with a Zeiss Axiolab fluorescent microscope (Carl Zeiss, Germany) using SYTOX green to stain extracellular DNA and CMAC fluorescent cell-tracker (Molecular Probes, OR, USA) to stain bacteria [19].

16-*In vivo* model of lung infection and co-infection: the infection mouse-model of Hirche T. O. et al. [20] was used. All mice are housed with food and water ad libitum, and a 12 h light/dark cycle. All procedures were approved by the Animal Studies Committee of Washington University School of Medicine and endorsed by the Bioethics Committee of the Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucuman. Briefly, adult BALB/c mice from the random-bred colony kept in our department at the Institute of Microbiology were randomized in experimental groups of six mice each. Animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and medotomidine hydrochloride (1 mg/kg), and infected with single bacteria or co-infected with a bacterial mixture.

Single bacterial infection: each group was instilled intranasal with 25 µl of Pae, BCC or SMG suspensions (10^6CFU/ml) on each nostril for two consecutive days.

Bacterial co-infection: in order to follow the dynamics of infection observed in our patient, we developed the following scheme: mice were intranasally instilled with 25 µl of mixture of equal amounts of Pae or BCC suspensions (10^6CFU/ml) for two consecutive days, and 5 days later they were instilled with 25 µl (10^6CFU/ml) of SMG for three consecutive days. Animals were sacrificed by intraperitoneal injection with Pentobarbital (70 mg/kg) 5 days after infection and 3 days after co-infection for quantitative lung bacteriology, bronchoalveolar lavage and histopathology studies.

Negative control animals received an equal volume of sterile saline solution by intranasal instillation.

Quantitative lung bacteriology: in order to determine CFU/g of tissue, the whole lung of three mice per group (infected and co-infected) was aseptically excised, weighed and homogenized in 5 ml of sterile 0.9% saline, and 100 µl of serially diluted lung homogenate samples were plated on Levin, BCSA agar and Sodium Azide agar (Biomeriex©, Argentina) and incubated for 48 h at 37ºC.

Bronchoalveolar lavage: (BAL) was collected by lavaging both lungs. Briefly, 0.5 ml saline containing 10 U/ml of heparin was injected and aspirated through the tracheostomy tube three times. Total volume of BAL fluid was pooled. Then, BAL cells were counted in a haemocytometer and smears were made and stained with Giemsa to count differentials. Haemoglobin was determined by spectrophotometry [21].

17-Histopathology: after sacrifice, the lungs of three mice per group were instilled with 1 ml of 10% formaldehyde using a tracheal catheter. The whole lung was removed and processed by histological routine haematoxylin-eosin staining. The degree of inflammation was scored on a scale from 0 to 3+, where (0) means absence, (+) means mild focal inflammation, (++) means moderate to severe focal inflammation and (+++) means severe inflammation to necrosis, or severe inflammation throughout the lung. Histopathology evaluation was performed in a blind fashion.

18-Statistical analysis: data were analyzed by paired *t* tests or ANOVA with Turkey's test. Results were considered statistically significant where $p<0.05$.

RESULTS

Detection and quantification of rhamnolipids and pyocyanin: Table 1 shows rhamnolipid production determined with two techniques (*Bacillus subtilis* growth and haemolysis halos) and pyocyanin measured by absorbance.

Highest rhamnolipids producer for a single strain was Pae ($p<0.005$). These production levels increased markedly in the mixture of Pae+BCC+SMG ($p<0.001$). There is a correlation between rhamnolipids and pyocyanin production; values were higher for Pae and mixture of three bacteria.

TABLE 1

XTT: as shown in Table 2, substantial inhibition of viability inside biofilm was found when Pae and BCC biofilm was incubated with mixtures of culture supernatants from 2 or 3 bacteria. In contrast, these mixtures had no effect on SMG biofilm viability. The SMG culture supernatant inhibited the viability of BCC and Pae. This suggests that Pae, BCC and SMG (combinations of 2 or 3 bacterial supernatants)

antagonize by soluble factors that decrease the viability of Pae and BCC bacteria in biofilm but do not affect SMG survival. This competition partly agrees with the dynamics of the infection of the patient under study, in which BCC was negative and could only be detected by PCR, while Pae and SMG could be isolated by plate culture.

TABLE 2.

Elastase assay: As shown in Table 3, Pae and BCC monocultures in BM2 medium + PMN showed the highest elastase level compared to the other assays ($p<0.01$). This elastase probably comes from PMN as suggested by the elastase lower values obtained when Pae and BCC bacteria were cultured in medium without PMN (BM2+DNA and BM2 medium alone). If PMN are not challenged with bacteria (control: BM2+PMN), they do not release elastase. Elastase activity was decreased in mixtures of 2 and 3 bacteria by SMG.

TABLE 3.

Netosis and Necrosis of PMN

Netosis and necrosis induction by bacteria on PMN are presented in Table 4. Pae was the highest netosis inducer followed by BCC and SMG ($p<0.01$). Combination of Pae with BCC or SMG induced lower netosis values than Pae alone ($p<0.05$), but higher than BCC or SMG alone and BCC+SMG ($p<0.05$).

As also shown in Table 3, PMN necrosis induced by single bacteria were significantly lower compared to necrosis induction by a mix of 2 or 3 bacteria ($p<0.01$). No difference in necrosis induction was found between Pae, BCC and SMG, or between mixtures of 2 or 3 bacteria.

TABLE 4.

***In vivo* model of lung infection and co-infection:** bacterial virulence *in vivo* (single and mixed) was tested using a mouse lung infection model. Bronchoalveolar lavage (BAL) cell counts (Table 5) and histologic lung changes (Fig. 1) are expressed. Mice were inoculated with Pae, BCC and SMG alone and their mixture. Negative control (animals instilled with sterile 0.9% saline) did not reveal histological lung modifications (Fig 1.A) and only a small number of resident alveolar macrophages were found in BAL fluids. Mice infected with Pae (Fig. 1B) or BCC showed moderate lung inflammation with milder accumulation of neutrophils, particularly in peribronchiolar and perivascular areas. Leucocytes in BAL (80% of PMN) were higher than SMG ($p<0.05$), in which we observed a lung with mild inflammation (Fig. 1C). Moderate to severe inflammation was observed in lung from mice infected with Pae+BCC, Pae+SMG (Fig. 1D), BCC+SMG and Pae+BCC+SMG (Fig 1.E) with an increase in interstitial and perivascular neutrophilic infiltration, milder lung haemorrhage and significant lung oedema. In addition, we found a higher number of BAL cells compared with the lung of mice infected with Pae or BCC alone.

The highest numbers of retro-cultured bacteria from lungs were found in mice co-infected with Pae+SMG (Pae: $1.6x10^6$ CFU/g, SMG: $2x10^6$ CFU/g) and Pae+SMG+BCC (Pae: $1.2x10^6$ CFU/g, SMG: $2.5x10^6$ CFU/g, BCC: $2x10^5$ CFU/g) compared with Pae alone ($2.5x10^5$ CFU/g) ($p<0.01$).

TABLE 5.

FIG.1.

DISCUSSION

Our results provide an experimental demonstration of how bacteria isolated from a CF patient, Pae, BCC and SMG can, through interactions with each other and with the host, modify behaviours involved in bacterial virulence and inflammatory response.

Initially, the airways of CF children display a rich and diverse microbiome. With the progression of the disease over time, the bacterial community decreases in terms of diversity, often displaying a predominant pathogen (e.g. Pae and/or BCC) [22]. In addition, members of the SMG have emerged as clinically relevant in chronic airway infections in CF patients and have been implicated as etiologic agents of pulmonary exacerbation [10, 11, 23]. At the time of her admission to the hospital, our patient, a CF child, was undergoing an acute pulmonary exacerbation because of chronic infection with Pae and BCC. After 5 months of regular follow-up, she experienced a period of clinical remission that was interrupted by a second pulmonary exacerbation. At this time, multi-resistant Pae strains and *Streptococcus anginosus* (SMG) were isolated. The patient's clinical condition rapidly deteriorated despite treatment with broad-spectrum antibiotics.

These results are consistent with several reports in which co-infections involving different bacterial species are common and probably the norm. Co-infecting species interact both synergistic and antagonistically within the lung in response to selection pressures exerted by the within-host environment and by other microorganisms of the community. Synergism can result in increased disease severity in mixed species infections [24]. In the present work, *in vitro* interactions of BCC and Pae increased pyocyanin and rhamnolipid production. This production was enhanced even more when Pae and BCC were co-cultured with SMG. Consequently, the associations of 2 or 3 species of bacteria enhanced PMN necrosis when compared with necrosis induction of each bacterium.

The analysis of bacterial spent culture medium on the viability of the each bacterium in the biofilm showed that mixtures of 2 or 3 bacterial supernatants reduced viability of BCC, Pae and SMG in the biofilm. SMG reduced the viability of Pae and BCC while none of the supernatants significantly inhibited SMG. During chronic infection, pathogens will experience changing selection pressures as they encounter new habitats and different co-infecting species and as they respond to medical intervention [25]**.** This interaction between bacteria partly reflects the dynamics of infection in the patient studied here; at the end stage Pae and SMG were detectable by plate culture because they were metabolically active while BCC, which may be confined to biofilm, was detected only by PCR.

Chronic airway infection with Pae and/or BCC is clearly associated with increased airway inflammation, faster lung function decline and poorer prognosis [26]. SMG within complex and dynamic communities can establish chronic pulmonary infections when SMG is the numerically dominant pathogen [10].

Inflammation is mediated mostly by PMN. Neutrophil primary granule components, myeloperoxidase and elastase, are inflammatory markers in CF airways, and their increased levels are associated with poor lung function. In addition, when neutrophils necrose, they release DNA and actin into the airways, increasing the viscoelasticity of airway secretions [27].

Elastase release induced by Pae or BCC on PMN is significantly higher than those induced by SMG or a mixture of 2 or 3 bacteria**.** This is not correlated with PMN necrosis when challenged with bacteria; necrosis induced by Pae or BCC is significantly lower than necrosis induced by mixed bacteria. In addition, the elastase released is only partially correlated with the production of bacterial virulence factors because more rhamnolipids and pyocyanin were detected when three bacteria were mixed (Pae+BCC+SMG).

Moreover, we observed that human neutrophils release large amounts of neutrophil extracellular traps (NETs) in the presence of Pae. Elastase release from PMN is related to NETs formation as demonstrated elsewhere [28]. NETs formation induced by Pae was higher than BCC and SMG and combination of two or three bacteria have an intermediate value of NETosis induces by each alone bacteria. Therefore, the bacteria do not interfere with the induction of NETosis.

Clinical data clearly indicate that neutrophils are unable to remove *Pseudomonas aeruginosa* effectively from CF lung since both NET-mediated and classical intracellular killing mechanisms fail. Neutrophils release their dangerous granule contents into the airway lumen. The balance between the benefit of the bactericidal activity that promotes NET formation and the cost of the concomitant tissue destruction in NETosis should be considered as the balance between the decrease in CF mucus viscoelasticity using DNase and the release of enzymes that damage the surrounding tissue [29].

In our *in vivo* assays, the mice pulmonary infection model showed that Pae virulence was significantly enhanced by the presence of SMG and BCC compared with the pathogenic activity of each bacterium by itself. The pathogenic strains associated with SMG induced a remarkable lung infiltration of PMN, oedema and vascular congestion as shown by histological studies.

Whether this pulmonary exacerbation in which PMN necrosis is enhanced as observed in the in vitro assays is due to the up-regulation

of virulence factors of Pae induced by SMG, or is caused by the virulence of SMG has not been determined yet.

Our results suggest that other bacteria considered oral flora, such as SMG, may play a significant role as pathogens in the CF population when they are the prevalent bacteria. It was demonstrated that antimicrobial therapy directed at the SMG proved more effective than anti-Pseudomonal therapy in these cases, and the clinical resolution of these exacerbations was correlated with a decrease in SMG [10]. This treatment was not performed on our patient because it is not a common practice in our hospital as SMG is not considered a relevant pathogen, which prevented the resolution of the second exacerbation period.

It is possible that during the second exacerbation Pae virulence was increased by SMG and BCC as we observed in both *in vitro* and *in vivo* assays.

In summary, our study demonstrates that interactions between pathogens, commensals and host could affect the manifestation of the disease.

Netosis as an antimicrobial process must be evaluated in the context of the release of substances that promote not only elimination of infection but also provide DNA which increases mucoviscosity and mediators responsible for tissue destruction.

Homeostasis between NETs formation and clearance is essential in sustaining a healthy immune defence against pathogens in lung.

FIGURE LEGENDS.

FIG.1. Micrograph of bacteria-infected lung tissue. Histological study (H-E) of lung from control mice intranasally instilled with PBS (A), mice infected with Pae (B), SMG (C), Pae+SMG (D), and Pae+BCC+SMG (E). The insets in panel E is magnification of the boxed area showing neutrophil accumulation. Analysis of neutrophil influx, oedema and vascular congestion is expressed in Table 5 and described in the text.

Bacteria	Rhamnolipids		Pyocyanin
	Halo of haemolysis[a]	Halo of *B. subtilis* Inhibition[a]	OD 520nm
Pae	9±2*	10±2*	0.320±0.060**
BCC	3±1	4±1	0.090±0.033
SMG	2±1	3±1	0.080±0.056
Pae+BCC	8±2	15±3	0.290±0.032
Pae+SMG	7±2	11±3	0.260±0.053
BCC+SMG	6±1	10±2	0.090±0.027
Pae+BCC+SMG	17±2**	21±3**	0.430±0.080**
Control (-)	0	2±1	0.007±0.003

TABLE 1: Quantification of rhamnolipids and pyocianin produced by single Pae and Pae mixed with other bacteria. a diameter (mm)

Values represent the mean of three independent experiments ± SD after basal values were subtracted from individual values. Statistical significance: (*) $p<0.005$; (**) $p<0.001$

	Bacterial viability (OD 492nm)		
Bacterial Culture Supernatants	**Pae**	**BCC**	**SMG**
Pae	**286±66**	295±83	162±54
BCC	201±67	**359±74**	148±30
SMG	164±56*	114±46*	**150±50**
Pae+BCC	93±48*	106±32*	73±30
Pae+SMG	145±40*	185±38*	98±42
BCC+SMG	36±14**	67±25**	82±46
Pae+BCC+SMG	107±19**	130±46*	66±32

TABLE 2. Absorbance values of XTT reduction obtained from biofilms of bacteria grown in the presence of bacterial conditioned medium. Data represent the mean±SD of three independent experiments, after basal values were subtracted from individual values. Statistical significance between bacteria grown in their own spent culture medium (bold) and supernatants of other cultures: (*) $p<0.05$ and (**) $p<0.01$.

Bacteria	BM2+PMN	BM2+DNA	BM2
Pae	0.489±0.017*	0.183±0.022	0.194±0.006
BCC	0.466±0.040*	0.137±0.006	0.145±0.007
SMG	0.224±0.020	0.117±0.005	0.113±0.005
Pae+SMG	0.216±0.015	0.152+0.003	0.115±0.008
Pae+BCC	0.372±0.007	0.175±0.006	0.187±0.007
BCC+SMG	0.304±0.012	0.142±0.003	0.156±0.005
Pae+BCC+SMG	0.315±0.010	0.173±0.004	0.185±0.008
Control BM2 medium	0.039±0.010	0.021±0.011	0.013±0.009

TABLE 3: Elastolytic activity of single or mixed bacteria cultured in BM2 medium and BM2 medium supplemented with PMN or DNA. Values (OD495nm) represent the mean of three independent experiments ± S.D. Statistical significance: (*) $p< 0.01$).

Bacteria	Netosis (4 h)		Necrosis
	RFU[a]	%	%
Pae	$11.014 \pm 0.115\times10^3$	64.5±1,8**	18.7±1,5
BCC	$6.523 \pm 1.750\times10^3$	38.3±3,0	19.3±3,2
SMG	$5.399 \pm 0.456\times10^3$	31.9±5,0	17.0±3,6
Pae + SMG	$9.325 \pm 0.506\times10^3$	54.9±5,4*	28.0±4,6**
Pae + BCC	$8.483 \pm 0.218\times10^3$	49.9±2,1*	29.3±3,5**
BCC + SMG	$6.010 \pm 0.206\times10^3$	31.7±3,3	27.3±2,5**
Pae + BCC.+ SMG	$8.369 \pm 0.425\times10^3$	49.1±2,6*	
Control 100% (Pae 5×10^6 bacteria)	$17.049 \pm 1.148\times10^3$	100%	

TABLE 4: Netosis and necrosis quantification of neutrophils stimulated with single and mixed bacteria [MOI 10:1]. Data represent the mean±SD of three independent replicate experiments after basal values were subtracted from individual values. Statistical significance: (*) $p<0.05$ and (**) $p<0.01$. a RFU: Relative Fluorescence Units.

Bacteria	BAL cells/ul	Lung Histopathology			
		Edema	Alveolar leukocyte infiltration	Interstitial leukocyte infiltration	Vascular Congestion
Pae	4740±336	+	0	+	++
BCC	4040±644	+	0	0	++
SMG	1100±100*	0	0	0	0
Pae+BCC	16000±490**	++	+	+	+++
Pae+SMG	12880±550**	++	+	+	++
BCC+SMG	7870±320	++	+	+	+++
Pae+BCC+ SMG	8600±608	++	+	++	++

TABLE 5. BAL cells and histology of lung from mice infected with single and mixed bacteria.

Statistical significance: *($p<0.05$) SMG vs Pae or BCC. **($p<0.01$) Pae+BCC and Pae+SMG vs Pae or BCC. The degree of lung inflammation hallmarks was scored on a scale from 0 to 3+, where (0) indicates absence, (+) mild (++) moderate to severe and (+++) severe.

ACKNOWLEDGEMENTS

We thank all the patients and hospital del Niño Jesús staff who generously helped with this study. This work was supported by grant from Consejo de Investigación de la Universidad Nacional de Tucumán, Programa CIUNT N° 26/D453, Argentina.

Author contributions

G.D., J.C.V., N.G., S.O., P.S., C.S, M.O.M., L.H. M.R., and A.T. performed the experiments. J.C.V., G.D., N.G., and S.O. designed the study. J.C.V, G.D. and N.G. wrote the paper.

Transparency Declaration:

Funding sources played no role in either the design or the analysis of this study. All authors declare that they have no commercial or other associations that might pose a conflict of interests.

Abbreviations used:

CF: cystic fibrosis
Pae: *Pseudomonas aeruginosa*
BCC: *Burkholderia cepacia* complex
SMG: *Streptococcus milleri* group
PMN: polymorphonuclear
NETs: neutrophil extracellular traps

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