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Gianfranco Donelli *Editor*

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Differential Immune Response of *Lactobacillus plantarum* 286 Against *Salmonella* Typhimurium Infection in Conventional and Germ-Free Mice

Tizá Teles Santos, Roberta Maria Dos Santos Ornellas, Leonardo Borges Acurcio, Sávio Henrique Cicco Sandes, Andréa Miura da Costa, Ana Paula Trovatti Uetanabaro, Jacques Robert Nicoli, and Gabriel Vinderola

Abstract

We aimed at evaluating *in vivo* the probiotic potential of *Lactobacillus plantarum* 286 against *Salmonella enterica* serov. Typhimurium. Colonization capacity and antagonistic activity were determined in feces of gnotobiotic mice. Survival to infection, translocation, histopathology, IgA and cytokine levels (IL-10, IL-6, IFN-γ, TNF-α, TGF-β) were determined both in conventional and germ-free mice followed *L. plantarum* 286 administration and *Salmonella* infection. *L. plantarum* 286 colonized the intestine of gnotobiotic mice, where it produced antagonistic substances against *S.* Typhimurium. In conventional animals, the administration of this strain increased intestinal IgA levels and reduced the inflammatory response and the tissue damage caused by *S.* Typhimurium. Reduction of tissue damage in the intestine and liver of germ-free animals was also observed, however the immune response elicited was different in either model. *L. plantarum* 286 showed *in vivo* probiotic properties in both murine models. Probiotic capacity results may depend on the animal model chosen.

Keywords

IgA · Interleukin · Lactic bacteria · Probiotic · Salmonellosis

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1 Introduction

The food industry has been following the growing market of functional foods searching for new ingredients that are attractive to consumers more

and more aware of the link between nutrition and health. Among functional foods, those containing probiotics lead the market. Lactic acid bacteria is the group of microorganisms most used in food products, because they are naturally found in traditional fermented foods and in the intestines of healthy people (Saad et al. 2013).

New strains with probiotics characteristics are of interest to the food industry, with special emphasis in autochthonous strains. The cocoa fermentation is a potential source of new probiotics, as great diversity of lactic acid bacteria is naturally found there (Saito et al. 2014). The assessment of new strains in animal models is mandatory before double-blind placebo controlled trials are carried out in humans.

Food pathogens are major cause of infections that affect millions of people worldwide. Salmonellosis is one of the most common food infections, which can cause from enterocolitis to sepsis, as well as enteric or typhoid fevers. In mice, *Salmonella enterica* serovar. Typhimurium promotes the invasion of intestinal cells and macrophages, setting an infection by intracellular parasitism (Anderson and Kendall 2017). The invasion triggers an inflammatory process with the release of pro-inflammatory cytokines (Hobbie et al. 1997). The salmonellosis model in conventional mice has been largely used for the characterization of probiotic strains (Zacarías et al. 2014; Silva et al. 2017). Germ-free mice have been also used for the characterization of probiotics (Sandes et al. 2017). The gnotobiotic mouse model is a complex *in vivo* approach, from the point of view that it offers a diverse population of immune cells in the intestinal mucosa, but at the same time is simplified as these animals do not possess intestinal microbiota. In addition, the germ-free mice model is important to evaluate the specific interaction of the strain under study with the host's immune system. It was recently reported that experimental toxoplasmosis established differently in conventional and germ-free mice (Nascimento et al. 2017). In this context, the aim of this work was to evaluate the *in vivo* probiotic capacity of *Lactobacillus plantarum* 286, a strain whose probiotic potential *in vitro* was previously studied (Santos et al. 2016), in conventional and germ-free mice, using the murine model of salmonellosis.

2 Materials and Methods

2.1 Strains

L. plantarum 286 was provided by Mars Cocoa (Mars Center for cocoa science – MCCS, Barro Preto, Bahia, Brazil). The strain was isolated and identified by Mars Cocoa and belongs to the company's collection. The bacteria was kept frozen at −70 °C in de Man, Rogosa and Sharpe broth (MRS, Acumedia, Neogen, Lansing, MI, USA) with 15% (v/v) of glycerol and it was reactivated in MRS broth, under aerobic conditions (37 °C, 18 h), reaching a final concentration of approximately 9 $\log_{10}$ of Colony Forming Units (CFU) per ml. *Salmonella* Typhimurium was a clinic isolate supplied by the Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. The strain was kept at −70 °C in Brain Heart Infusion (BHI, Acumedia) broth with the addition of 15% (v/v) of glycerol. The reactivation was carried out in BHI broth (37 ° C, 24 h), under aerobic conditions.

2.2 Mice

Male and female germ-free NIH/Swiss mice (21–23 days of life) (Taconic, Germantown, NY, USA) were used in this study. Animals were maintained in flexible plastic insulators (Standard Safety Equipments, McHenry, U.S.A.) and treated as described previously (Martins et al. 2013). Conventional male BALB/c mice (21–23 days of life) were obtained from the animal facility of the Federal University of Minas Gerais (UFMG). Animals were kept in micro-isolators (UNO Roestsvaal, Zevenar, Holland), receiving *ad libitum* autoclaved food (Nuvital, Nuvilab, Curitiba, Brazil) and sterile water (121 °C, 15 min). The micro-isolators were stored in ventilated chambers (Alesco, São Paulo, Brazil) with light period (12 h light/12 h darkness), humidity (60–80%) and temperature

(22 ± 1 °C). The experiments were made by the standards of the National Council of Animal Experiments Control – CONCEA (2016). The study was approved by the Ethics Committee in Animal Experiments of the Federal University of Minas Gerais (CETEA/UFMG, protocol n° 24/2015).

2.3 *L. plantarum* 286 Administration and Pathogenic Challenge in Germ-Free Mice

Fourteen germ-free animals were used in this part of the study. The experimental design is shown in Fig. 1a. Animals received by gavage a single dose (0.1 ml, 10^9 CFU per ml) of *L. plantarum* 286 in 0.85% (w/v) sterile saline solution (10^8 cells/mouse). For the infection, animals received by gavage a single infective dose (10^6 UFC) of *S.* Typhimurium contained in 0.1 ml of 0.85% (w/v) sterile saline solution, 5 days after mono-association with *L. plantarum* 286. Success of *L. plantarum* 286 colonization was confirmed by plating feces on MRS agar, 5 days after the administration of the strain.

2.4 Colonization of Germ-Free Mice with *L. plantarum* 286 and *Ex Vivo* Antagonism

The capacity of intestinal colonization of the strain under study was assessed as described by Martins et al. (2010). Counts of lactobacilli in the feces of germ-free mice on the fifth day after mono-association with *L. plantarum* 286 were performed (Fig. 1a, group 286G). Freshly feces of mono-associated mice were collected by anal stimulation and transferred aseptically into a sterile microtube, previously weighed. Samples were vigorously vortexed until homogenization, serial decimal dilutions (0.85% w/v saline solution) were made and 0.1 ml of each dilution was plated on MRS agar (Acumedia). Plates were incubated (37 °C, 48 h, aerobic conditions). Results were expressed in $\log_{10}$ CFU per gram of feces. Two repetitions of the experiment were made.

The *ex vivo* antagonism was performed according to Alvim et al. (2016). A feces sample (approximately 50 mg) was collected from mice on the fifth day after mono-association with *L. plantarum* 286 (Fig. 1a, group 286G). and placed on the center of a plate with MRS agar (Acumedia). In order to inactivate microorganisms in feces, plates were exposed to chloroform vapor for 30 min, followed by 30 min additional exposure to air in a laminar hood for complete evaporation of the solvent. Plates with the feces were incubated at 4 °C for 24 h. After this incubation, an inoculum containing 10^6 CFU per ml of *S.* Typhimurium in semi-solid (0.75% agar) BHI (Acumedia) was poured on the surface of the plate, following incubation (37 °C, 24 h, aerobiosis). The inhibition halos around feces were measured with a Mitutoyo digital pachymeter (São Paulo, Brazil). Two repetitions of the experiment were made.

2.5 Survival of *L. plantarum* 286-Fed Conventional Mice to *Salmonella* Infection

Twenty conventional animals were used for the mortality assay. The experimental design is shown in Fig. 1b. Ten mice received the same dose of *L. plantarum* 286 whereas ten animals were used as control (receiving only 0.1 ml of 0.85% (w/v) sterile saline solution), daily, for seven consecutive days prior to infection. The administration of *L. plantarum* 286 continued after infection until the end of the experimental period, according to Silva et al. (2004). Conventional animals were infected in the same way as germ-free animals.

The cumulative mortality was recorded for 28 days after infection, during which the oral administration of *L. plantarum* 286 continued. The evaluation of the development of the disease was carried out according to Gill et al. (2001). General aspects of the mice health were recorded at days 1, 3 and 6 after pathogenic challenge. A general health scoring scale (GHS) was used as follows: (3) mice with bright eyes and alert, smooth bright coat, responding to stimuli and

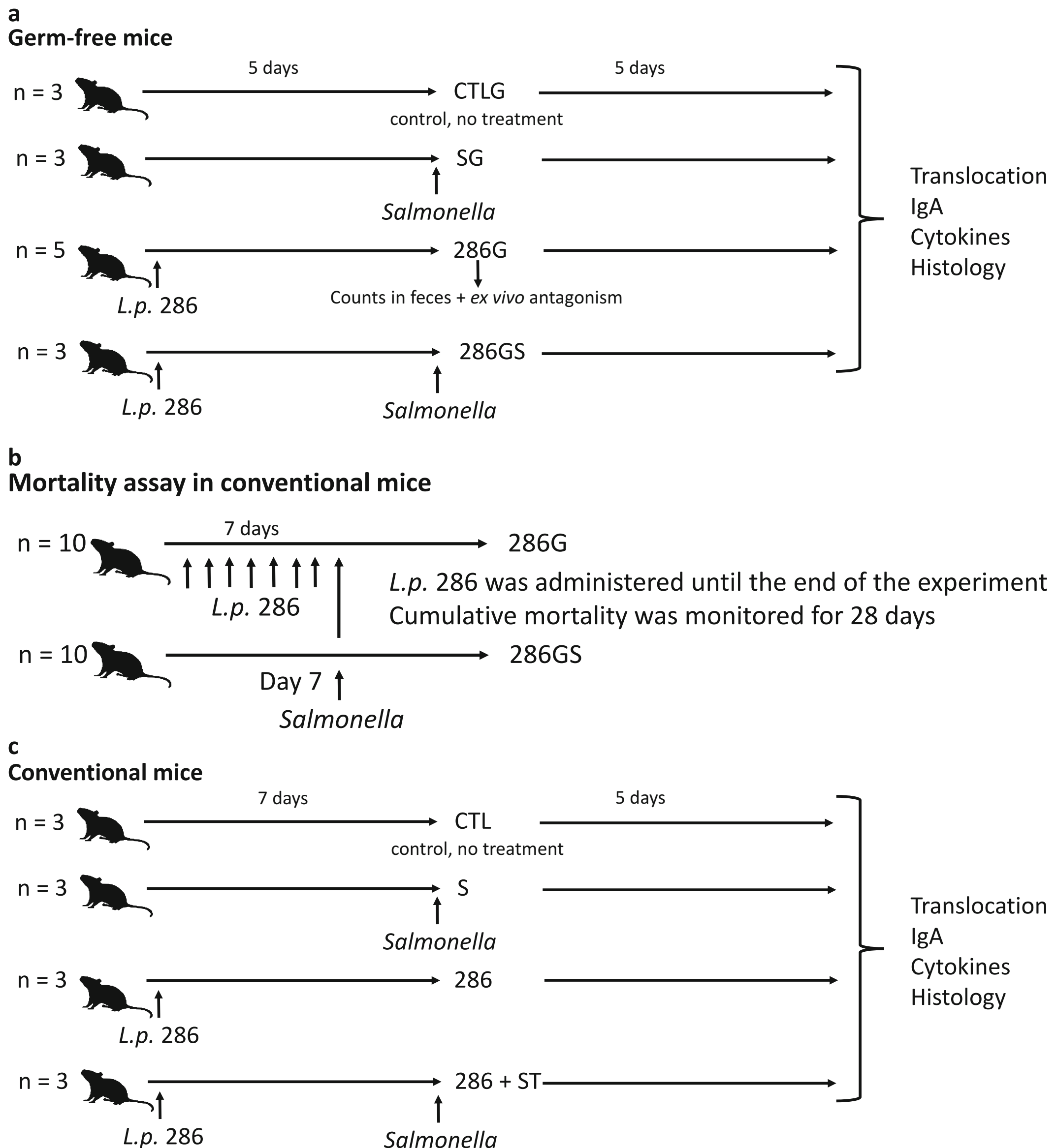

Fig. 1 Experimental designs used in this study

showing interest in its environment; (2) mice with coat noticeably jumpy and forming tuft of hair, not as alert or active, less interested in the environment out of the cage, and with signs of hyperventilation when handled; (1) mice without reaction to stimuli, very spiky hair, showing overturned posture, preferring to sleep than reacting to the environment, low body temperature and feet cold. The determination of body weight completed this assessment.

2.6 Traslocation Assay in Conventional and Germ-Free Mice

2.6.1 *L. plantarum* 286 Administration and *Salmonella* Challenge

For conventional mice, four groups (three animals/group) were set (Fig. 1c) as follow: a control group that received only sterile saline solution (named CTL); a group that received

only *L. plantarum* 286 (named 286); a group that received *L. plantarum* 286 for 7 days and then it was challenged with *S.* Typhimurium (named 286 + ST) and a group that was treated with sterile saline solution for 7 days and then it was challenged with *S.* Typhimurium (named S). Five days after the challenge, animals were euthanized by cervical dislocation for the analyses described below.

For germ-free mice, four groups (three animals/group) were set (Fig. 1a) as follow: a control group (CTLG), a group that was mono-associated for 5 days with *L. plantarum* 286 (286G); a group similar to the 286 group, which was challenged with *S.* Typhimurium (286GS) and a group similar to CTL group which was challenged with *S.* Typhimurium (SG) 5 days after the challenge, animals were euthanized by cervical dislocation for the analyses described below.

2.6.2 Translocation

Translocation of cultivable enterobacteria was determined according to Martins et al. (2010). Spleen and liver samples were excised from sacrificed animals, weighed, homogenized and then serially diluted in 0.85% (w/v) sterile saline solution. Aliquots of 0.1 ml of each dilution were pour-plated on MacConkey agar (Acumedia) and incubated under aerobic conditions (37 °C, 24 h) for counting total enterobacteria. The results were expressed as the $\log_{10}$ CFU per gram of organ.

2.6.3 IgA Analysis in the Intestinal Fluid

The level of secretory immunoglobulin A (S-IgA) in the intestinal fluid of conventional animals was evaluated by ELISA according to Pedroso et al. (2015). The small intestine was removed by incision of the gastroduodenal and ileocecal junctions. The intestinal content was removed, weighed and a protease inhibitor cocktail (1 μM of aprotinin; 25 μM of leupeptina; 1 μM of pepstatin and 1 mM of PMSF) was added at a rate of 2.0 ml of PBS (pH 7.2) containing the cocktail every 500 mg of intestinal content. Samples were centrifuged (5.000 x*g*, 30 min, 4 °C) and the supernatant was collected and frozen at −70 °C. For S-IgA determination, microplates coated with anti-IgA antibody were used (M-8769, Sigma Chemical Co., St. Louis, USA). The detection of S-IgA was performed with anti-IgA peroxidase (A-4789, Sigma). For the colour reaction OPD (o-Phenylenediamine dihydrochloride) was used and the reaction was stopped with sulfuric acid (H_2SO_4, 1:20). The concentration of total S-IgA was established using a purified IgA standard (0106–01, Southern Biotechnology Associates, Birmingham, USA). The readings were performed at 492 nm, on a microplate reader (Spectromax M3, Molecular Devices Inc., Sunnyvale, USA). The concentration of S-IgA was expressed in ng ml^{-1} of intestinal content.

2.6.4 Relative Expression of Cytokines

The cytokine analysis was performed according to the method of relative analysis of the Delta Ct gene expression. The ileus fragments collected and placed in RNAlater (Ambion, Austin, TX, U.S.A.), in proportions of 1:5 and stored at −70 °C. Total RNA was extracted according as described previously (Acurcio et al. 2017). Samples were stored at −20 ° C. Afterwards, the RNA was treated for DNA removal with "DNAse Turbo® I" Kit (Ambion by Life Technologies, Carlsbad, CA, USA), after, the sample concentration was adjusted to 100 μg per μl with ultrapure water (Sigma-Aldrich) and stored at −20 ° C. Then, the cDNA of the samples were obtained using the "High Capacity cDNA Reverse Transcription" Kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The relative amount of the gene expression of the following cytokines IL-10, IL-6, IFN-γ, TNF-α, TGF-β were assessed from the cDNA amplification by (RT-qPCR). For this purpose, the "Quantitect SYBR® Green PCR" Kit, by Qiagen (Hilden, Germany) was used according to the manufacturer's instructions at an "ABI Prism® 7900 HT Sequencing Detection System" (Applied Biosystems). The primers for detecting the cytokine genes mentioned above, as well as for the reference constitutive genes GAPDH and β-Actin have been proposed by Giulietti et al. (2001). The results were interpreted according to Hellemans et al. (2007).

2.6.5 Histopathological and Morphometric Analyzes

Samples collected from the ileum and liver were fixed in buffered formaldehyde (4%) and processed for inclusion and microtomy in paraffin. For histopathological analysis, the sections (3 and 5 μm) were stained with hematoxylin-eosin. The histopathological sections were coded and analyzed sequentially by a single pathologist who was unaware of the experimental conditions of the groups studied. The morphometry was assessed according to Gulbinowicz et al. (2004). In the ileum, the height of twenty villi was evaluated in three fields (10X magnification), for each animal of each experimental group, completing an average of 180 measurements per group. In the liver, the inflammatory foci were counted in ten fields of each animal, completing at least thirty fields per group. Inflammatory foci are defined as accumulations of inflammatory cells in a number greater than ten, accompanied or not by necrotic changes in the parenchyma (Mendonça et al. 2014).

2.7 Statistical Analysis

A completely randomized design was used. The results were expressed as the average ± standard deviation of at least two independent assays. The data were analyzed using the one-way ANOVA test, with Tukey's post-test of GraphPadPrism software version 6.0 (GraphPad Software Inc 2012). The data were considered significantly different when $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****\ p < 0.0001$. Other statistical analyzes were performed that included: Two-way RM ANOVA, with Tukey's post-test, for evaluation of ponderal development and weight, and unpaired T-test for analysis of translocation results.

3 Results and Discussion

The use of animal models for the assessment of new microbial strains with probiotic potential is a mandatory step between *in vitro* assays and human clinical trials (Papadimitriou et al. 2015). However, the correlation between *in vitro* outcomes with results obtained in animal and human trails may be uncertain (Vinderola et al. 2017). In a previous work, lactobacilli isolated from cocoa fermentation in the south of Bahia (Brazil) were screened *in vitro* for probiotic potential and some strains displayed functional potential for further *in vivo* assessment (Santos et al. 2016). In particular, *L. plantarum* 286 showed *in vitro* antagonistic activity against a series of food pathogens, including *S.* Typhimurium. In this work, germ-free and conventional mice were used to determine whether the *in vitro* antagonism would be verified *in vivo*.

3.1 Colonization of Germ-Free Mice and *Ex Vivo* Antagonism

In the mono-association assay in germ-free mice, the colonization of the intestine of gnotobiotic animals by *L. plantarum* 286 was observed, reaching population levels of $1 \times 10^8 \pm 0.17$ CFU per gram of feces on the fifth day after colonization. In the trial of *ex vivo* antagonism, an inhibiting halo (19.8 ± 3.2 mm) was observed for *S.* Typhimurium. Therefore, *L. plantarum* 286 was able to colonize the small intestine of germ-free mice and the potential antimicrobial compounds left in feces were able to inhibit *S.* Typhimurium.

3.2 Survival of *L. plantarum* 286-Fed Conventional Mice to *Salmonella* Infection

By the end of the assay, the control group displayed a grade 1 score in the general health scoring scale, whereas the group fed *L. plantarum* 286 and challenged with *S.* Typhimurium received a grade of 1.33. In relation to the accumulated mortality, there was no statistically significant difference between groups (Fig. 2). *L. plantarum* 286 was able to colonize the small intestine of germ-free mice and the potential

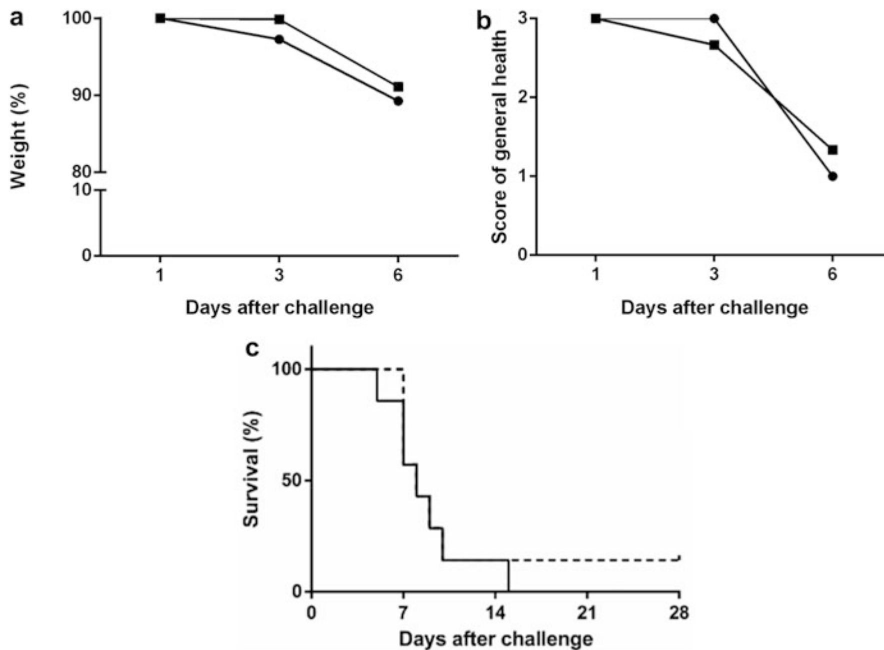

Fig. 2 Weight percentage (in relation to the initial weight) (**a**), evaluation of the general aspect of health (**b**) and accumulated mortality (**c**) of conventional BALB/c mice. Animal received *L. plantarum* 286 for 7 consecutive days (■ or dashed line) and challenged with *S.* Typhimurium, control animals (● or continuous line) were only challenged with *S.* Typhimurium

antimicrobial compounds left in feces were able to inhibit *S.* Typhimurium. In the accumulated mortality study (Fig. 2c) *L. plantarum* 286 failed to protect conventional mice from *S.* Typhimurium infection. A lack of correlation between *in vitro* and *in vivo* experiments for strains of *L. casei* and *L. plantarum* against *Salmonella* in mice were observed when animals were fed the probiotics for 6 days before the pathogenic challenge (Bujalance et al. 2014). However, when in the referenced study mice received the strains for 20 days, a significant reduction in the colonization of the spleen was observed for one of the strains, showing the importance of the administration period for triggering a successful outcome, a variable that is absent in *in vitro* experiments. Another factor that may have determined the lack of protection observed in this part of the study may has been that the *Salmonella* strain was particularly virulent when used, inducing the death of all animals in the control group (Fig. 2c). Zacarías and collaborators (2014) used the same *Salmonella* strain and the infection seemed to be less aggressive, as not all control animals died, as happened in our study, allowing the survival of 30–40% of the animals of the control group. It was reported that the intestinal microbiota in mice may vary among batches of the same mouse provider, affecting the reproducibility of rodent models (Franklin and Ericsson 2017) and it was also reported that the composition of the gut microbiota influences the resistance to *Salmonella* infection (Varmuzova et al. 2016). These factors together may explain the different colonization resistance of control mice when the *Salmonella* strain was used under the same conditions: this study and that of Zacarías et al. (2014).

3.3 Translocation Assay, S-IgA Production, Histopathological and Morphometric Analyzes in Conventional Mice and Germ-Free Mice

Translocation of enterobacteria followed *Salmonella* challenge in animals that had received

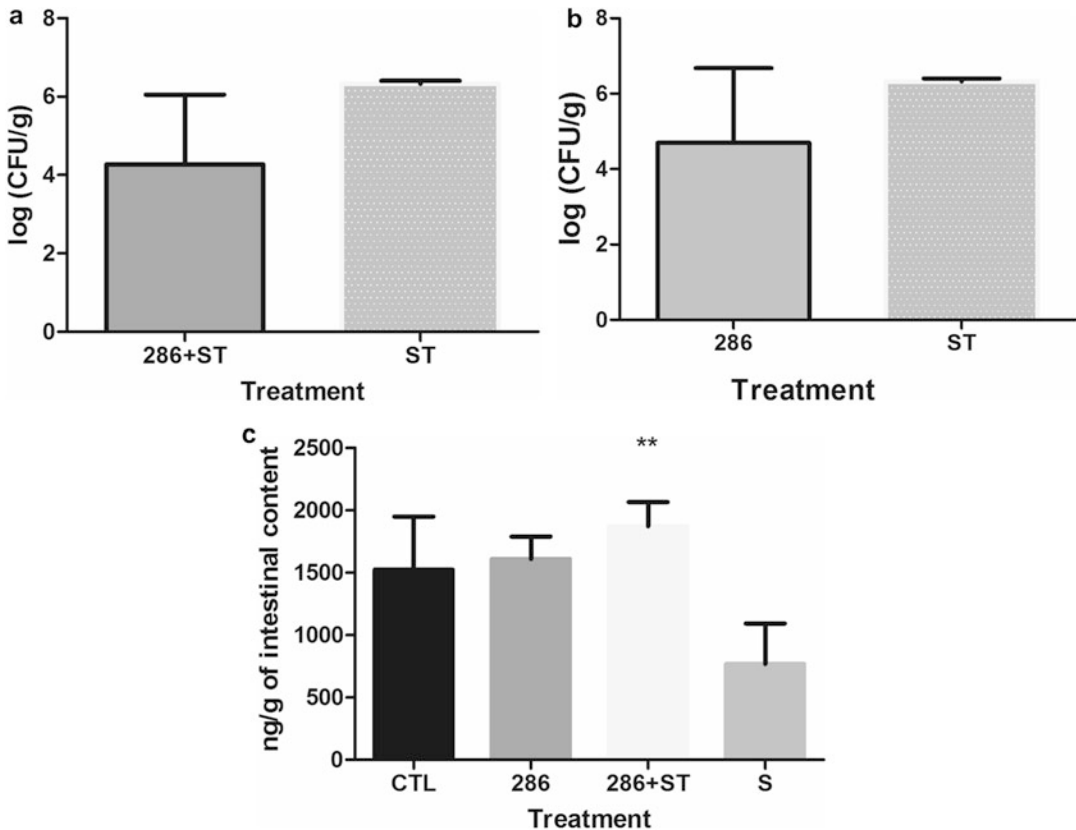

Fig. 3 Counts of enteric bacteria in liver (**a**) and spleen (**b**) in conventional BALB/c mice orally treated (286 + ST) or not (ST) with *L. plantarum* 286 for 7 consecutive days and challenged with *S.* Typhimurium. S-IgA in the intestinal fluid of conventional BALB/c mice (**c**). Group CTL received sterile saline solution, group 286 received *L. plantarum* 286 for 7 consecutive days, group 286 + ST received *L. plantarum* 286 for 7 consecutive days and then it was challenged with *S.* Typhimurium and group S was treated with sterile saline solution for 7 days and then challenged with *S.* Typhimurium

L. plantarum 286 was evaluated in liver (Fig. 3a) and spleen (Fig. 3b) in conventional animals. No translocation was observed in animals that received only *L. plantarum*. There was no statistically significant difference in total counts of enteric bacteria between the groups, but a trend towards a reduction in the counts in liver was observed in the animals that received *L. plantarum* 286 ($p = 0.1090$). No differences in the content of S-IgA were observed in the intestinal fluid among the groups CTL, 286 and 286 + ST (Fig. 3c). However, the group 286 + ST displayed a significantly higher concentration of S-IgA compared to the S group, indicating that the treatment with *L. plantarum* 286 induced an increased and significantly different response ($p < 0.01$), when there was the challenge with *S.* Typhimurium. S-IgA is involved in the resolution of the infection. The translocation of enteric bacteria to the liver (Fig. 4a) and spleen (Fig. 4b) of NIH/Swiss germ-free mice challenged with *S.* Typhimurium was evaluated for the different groups. No significant differences were found in liver. However, in the spleen a significant ($p < 0.05$) lower count of enteric bacteria was observed in the group challenged with the pathogen, compared to the group previously treated with *L. plantarum* 286.

The small intestine's histopathological aspects of conventional mice in the different groups are shown in Fig. 5. In the group that received *L. plantarum* 286 there was a slight, but not

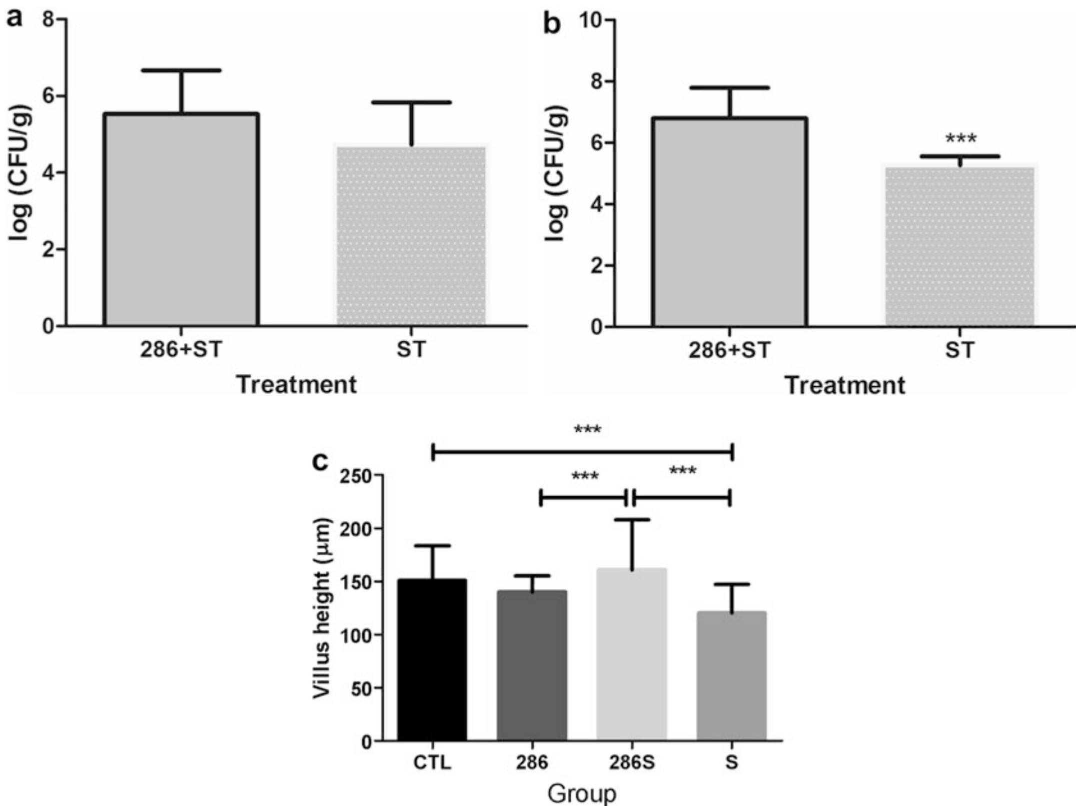

Fig. 4 Counts of enteric bacteria in liver (**a**) and spleen (**b**) in NIH/Swiss germ-free mice orally treated (286 + ST) or not (ST) with *L. plantarum* 286 for 7 consecutive days and challenged with *S*. Typhimurium. Height of intestinal villi of NIH/Swiss germ-free mice. (**c**) Group CTL received sterile saline solution, group 286 received *L. plantarum* 286 for 5 consecutive days, group 286S received *L. plantarum* 286 for 5 consecutive days and then it was challenged with *S*. Typhimurium and group SG was treated with sterile saline solution for 5 days and then challenged with *S*. Typhimurium

significant, reduction in the height of villi and increased cellularity, as well as an increase in the size of Peyer's patches, as consequence of the immunostimulation by the strain. The overall structure of the organ was preserved (Fig. 5c). In the ileum of animals that received *L. plantarum* 286 and that were further challenged with *Salmonella*, a discrete increase in cellularity of the villi was observed, but with a maintenance of the general structure (Fig. 5c). There was also a proliferation of caliciform cells, with a slight decrease, in general, of the villi height, but still preserving the overall structure of the organ. In mice only challenged with *S*. Typhimurium, mucosal necrosis, loss of villi and epithelium and inflammatory infiltrate were noticed. The liver's histopathological analyses showed that the group treated with *L. plantarum* 286 maintained its structure, except for a discrete degeneration around the lobular veins. In animals that were treated with *L. plantarum* 286 and challenged with *S*. Typhimurium, inflammatory foci were observed, with necrotic tissue associated, but with the parenchyma of the tissue and the organ structure well preserved. In the group that received only the pathogen, necrotic foci (Fig. 5b, d) and hydropic degeneration could be observed, showing clear damage to the liver tissue, beyond the inflammatory infiltrates that characterize the infection by the enteropathogen. The morphometric analysis of the small intestine confirmed the histopathological observations of

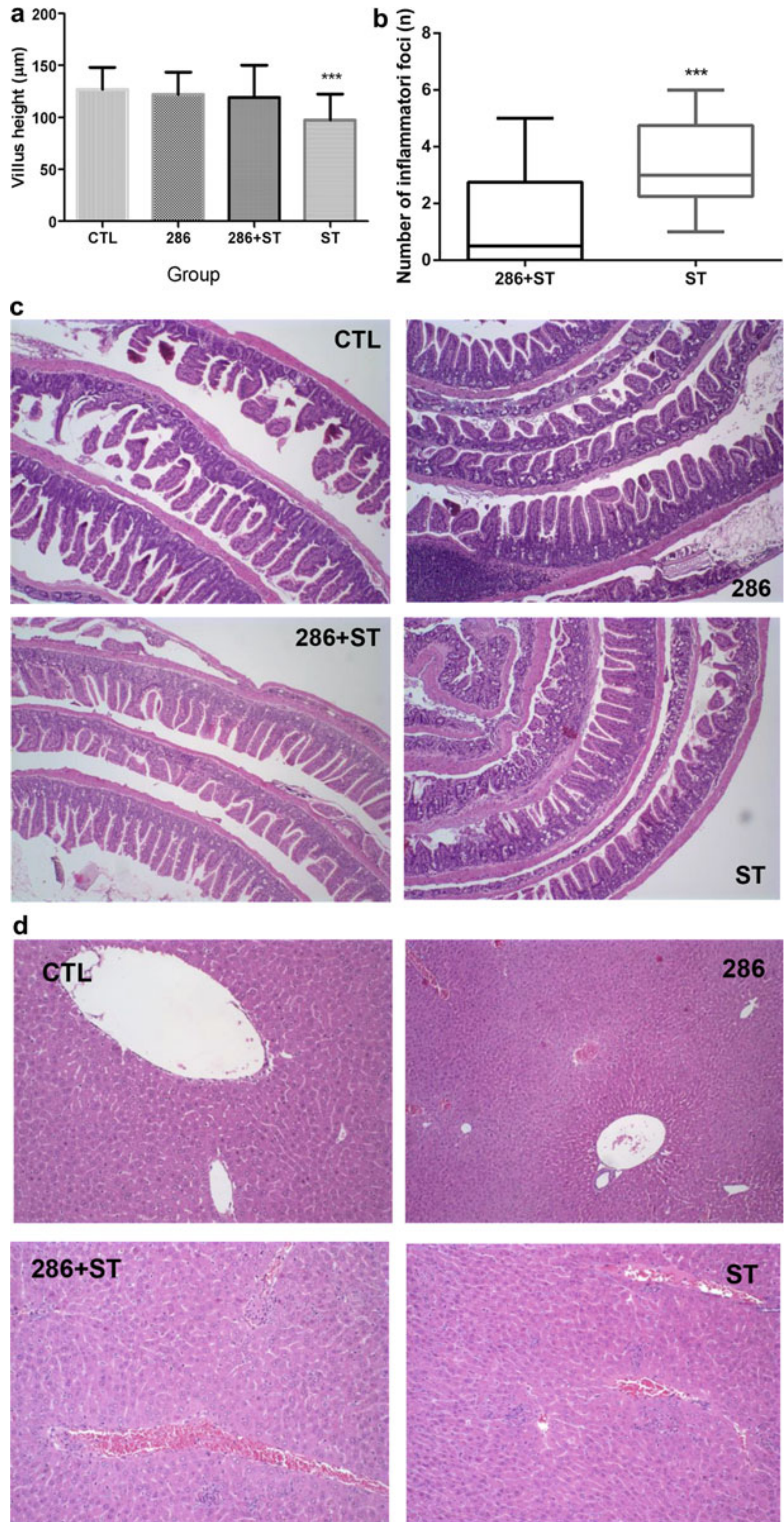

Fig. 5 Height of intestinal villi (**a**), number of inflammatory foci in the liver (**b**), histopathology of the ileum (**c**) (magnification 10x) and liver (**d**) (magnification 40x) of conventional mice. CTL: control group that received sterile saline solution, 286: group that received *L. plantarum* 286 for 7 consecutive days, 286 + ST: group that received *L. plantarum* 286 for 7 consecutive days and then challenged with *S.* Typhimurium, S: animals that received sterile saline solution for 7 days and then challenged with *S.* Typhimurium

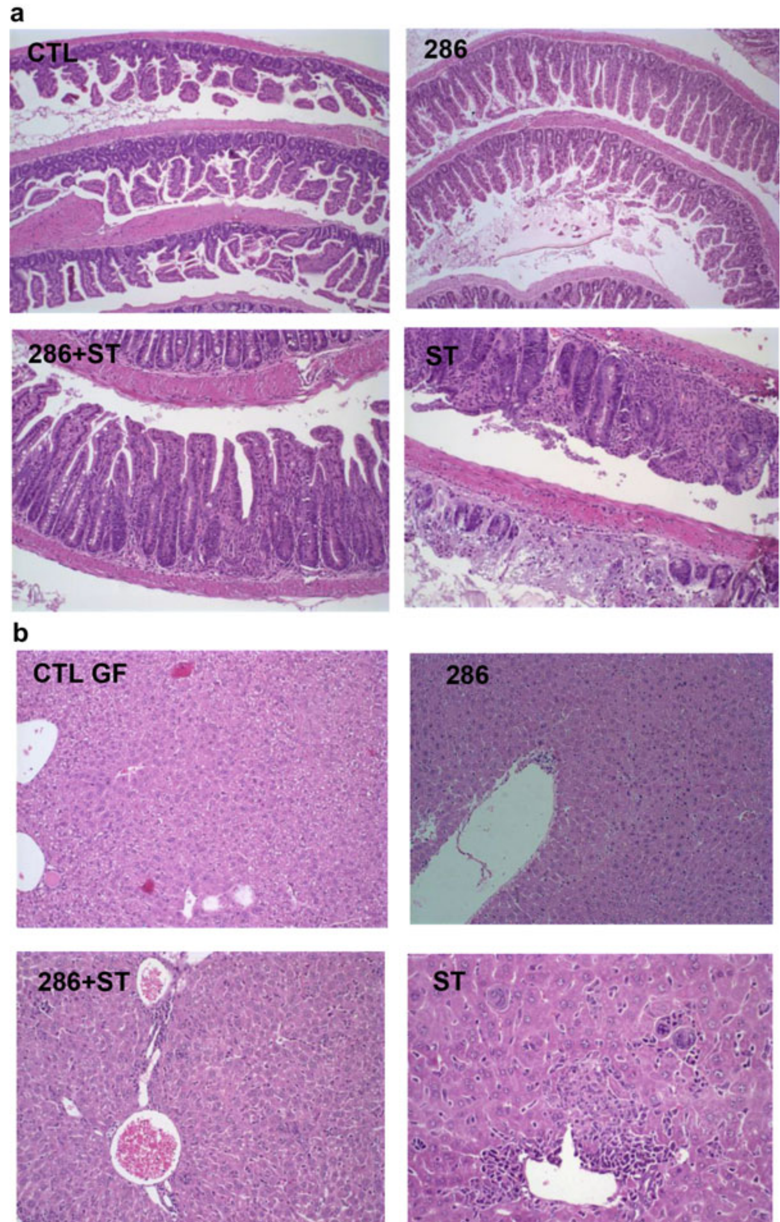

Fig. 6 Histopathology of the ileum (magnification 10x) and liver (magnification 40x) of NIH/Swiss germ-free mice. CTL: control group that received sterile saline solution, 286: group that received *L. plantarum* 286 for 7 consecutive days, 286 + ST: group that received *L. plantarum* 286 for 7 consecutive days and then challenged with *S.* Typhimurium, S: animals that received sterile saline solution for 7 days and then challenged with *S.* Typhimurium

the group that received only *S.* Typhimurium, with a significant reduction in the size of villi (Fig. 5a) when compared to the other groups. The liver morphometric analysis correlated with the histopathology findings of this organ, with a statistically significant reduction in inflammatory foci in the group treated with *L. plantarum* 286.

Germ-free animals colonized with *L. plantarum* 286 and challenged with *S.* Typhimurium presented villi sizes significantly larger than the group only challenged with the pathogen (Fig. 6a). In other words, the presence of *L. plantarum* 286 probably partially mitigated the tissue damage caused by the deleterious

action of *S*. Typhimurium. In the group colonized with *L. plantarum* 286 and challenged with *S*. Typhimurium, there was an increase of inflammatory infiltrates and a slight loss of the tissue structure, but in general, the integrity of the ileum was maintained. The group only challenged with *S*. Typhimurium displayed an intense inflammation and loss of mucosal integrity, showing an increase of inflammatory infiltrates in the submucosa and crypts, without associated villi. The presence of connective tissue indicates damage and tissue regeneration. The histological analyses of the liver in animals treated with *L. plantarum* 286 and challenged with *S*. Typhimurium showed no inflammatory foci, but a less full body structure (Fig. 6b). It was observed a widespread organ congestion, as well as a well diffused hydropic degeneration. The group only challenged with *S*. Typhimurium presented exacerbated inflammatory foci, perivascular, with associated necrotic tissue. The presence of megakaryocytes was noticed, which can be interpreted as an attempt of the animal's hematopoietic system to recompose himself, releasing immature cells to aid the fight against infection.

One of the pathogenicity mechanisms of *S*. Typhimurium involves the invasion of inner tissues by translocation from the intestinal lumen to organs such as spleen and liver. The translocation occurs because the pathogen's cells promote its proper phagocytosis by M cells located in the intestinal epithelium, from which they escape from phagosome. This provokes ruptures in the junctions of the epithelium and in the adjacent enterocytes access paving the way to the concomitant translocation of resident microbes. In this way, the pathogen can migrate to other organs, as liver and spleen, where it multiplies (Gill et al. 2001) until reaching levels that result lethal for animals in this murine model. The accumulated mortality assay is less laborious and demanding than the translocation assay, but it provides less information about the mechanisms of protection (or lack of protection) against infection. Therefore, the translocation assay was performed in conventional (Fig. 3a, b) and germ-free (Fig. 4) mice. Whereas a trend to less infection was observed in liver and spleen of conventional animals that received *L. plantarum* 286, a significant reduced colonization of spleen was observed in germ-free mice, but in those animals that did not received *L. plantarum* 286. The intestinal microbiota may have been involved in this apparent contradictive response to infection, as it was pointed as responsible for the different response of conventional and germ-free mice to a toxoplasmosis model (Nascimento et al. 2017). Yet, in line with previous work Zacarías et al. (2014), the treatment with the probiotic significantly increased the S-IgA response as a mechanism to fight against infection.

3.4 Relative Expression of Cytokines Analyzes in Conventional Mice and Germ-Free Mice

Further characterization of the mechanisms involved in the partial protection against *Salmonella* infection was performed by analyzing the relative expression of proinflammatory (IL-6, IFN-γ, TNF-α) and regulatory cytokines (IL-10, TGF-β) in conventional (Fig. 7) and germ-free (Fig. 8) mice fed *L. plantarum* 286 and challenged with *S*. Typhimurium.

The analysis of the levels of cytokines in the intestinal fluid of the different groups in conventional mice allowed to observe that an anti-inflammatory response was induced when *L. plantarum* 286 was administered prior to challenge (Fig. 7), with a significant reduction in the expression of proinflammatory cytokines (IFN-γ, TNFα and IL-6) and a significant increase for TGF-β and a positive (but not significant) trend for IL-10. The analysis of cytokine relative expression in the ileum of germ-free animals (Fig. 8) showed an increase in the expression of IFN-γ, 10 and IL-6 in the group associated with *L. plantarum* 286 and challenged with *S*. Typhimurium, in relation to the group only challenged with the pathogen. No significant differences were observed for TNFα and TGF-β.

In conventional mice, the administration of *L. plantarum* 286 prior to infection was able to downregulate the expression of pro-inflammatory cytokines such as IL-6, IFN-γ, and TNF-α. This

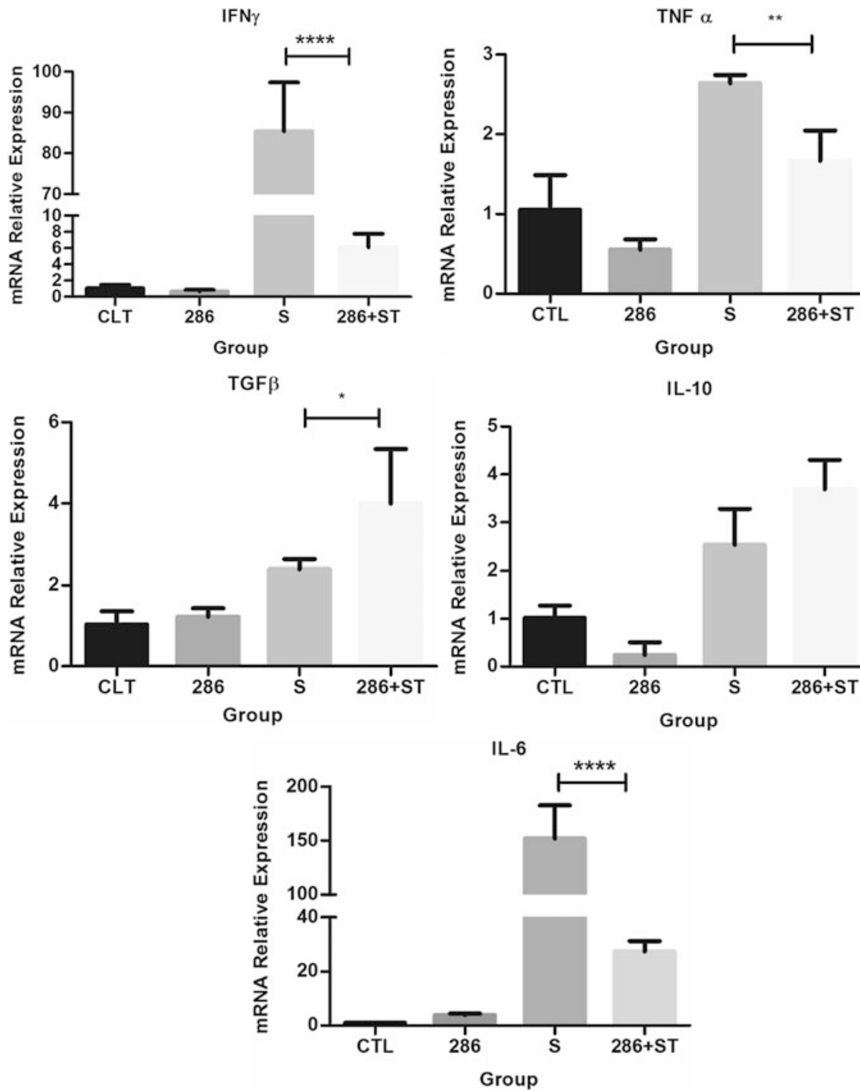

Fig. 7 Cytokines (IFN-γ, TNF-α, TGF-β, IL-10 and IL-6) expression in the ileum of conventional BALB/c mice. Group CTL received sterile saline solution, group 286 received *L. plantarum* 286 for 7 consecutive days, group 286 + ST received *L. plantarum* 286 for 7 consecutive days and then it was challenged with *S.* Typhimurium and group S was treated with sterile saline solution for 7 days and then challenged with *S.* Typhimurium

fact may have been the consequence of the upregulation of IL-10 (not significant trend) and TGF-β (Fig. 7). On the contrary, IL-6 and IFN-γ were significantly upregulated in germ-free mice in the same context, with a concomitant significant upregulation of IL-10 (Fig. 8). Yet a pro-inflammatory response in germ-free mice occurred, less tissue damage was observed in the small intestine and liver in animals fed *L. plantarum* 286. This fact was also observed in conventional mice.

The anti-inflammatory profile observed in conventional animals suggests a probiotic potential of the strain *L. plantarum* 286. When the infection initiates, *S.* Typhimurium interacts with the host's intestinal cells and gut immune system unchaining an inflammatory response, with the release of pro-inflammatory cytokines, in an

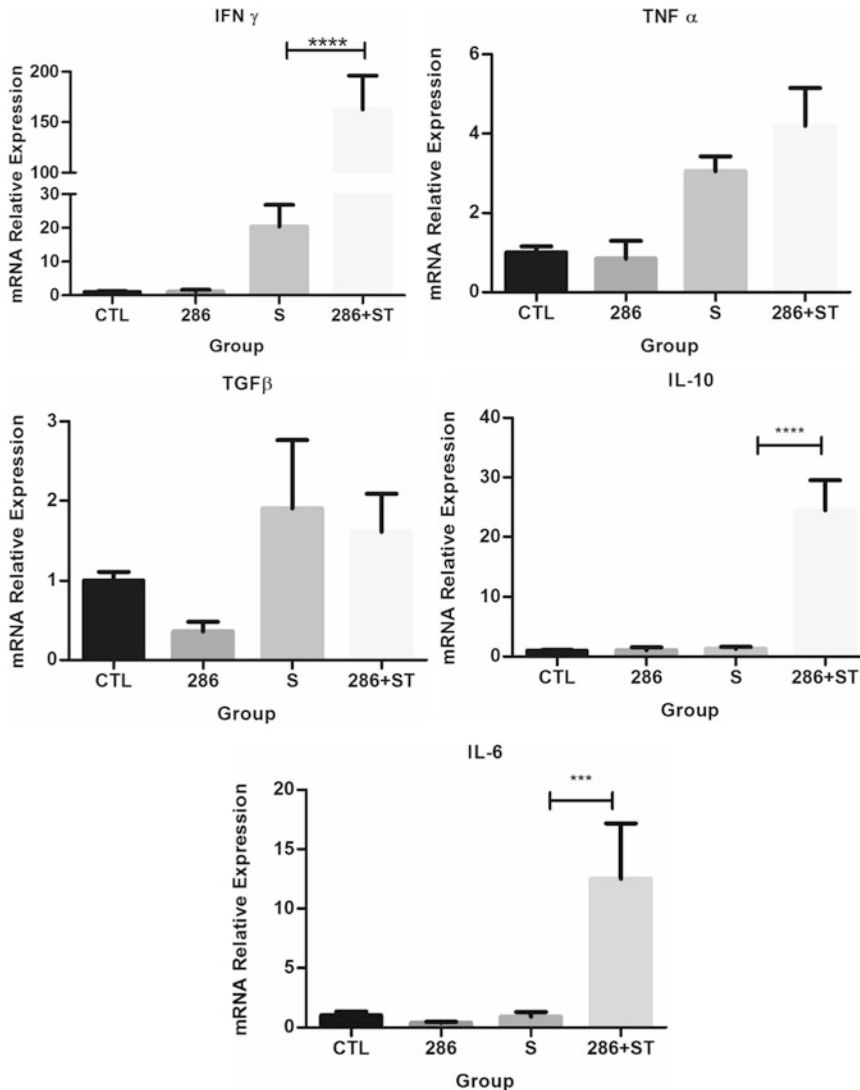

Fig. 8 Cytokines (IFN-γ, TNF-α, TGF-β, IL-10 and IL-6) expression in the ileum of NIH/Swiss germ-free mice. Group CTL received sterile saline solution, group 286 received *L. plantarum* 286 for 7 consecutive days, group 286S received *L. plantarum* 286 for 7 consecutive days and then it was challenged with *S.* Typhimurium and group S was treated with sterile saline solution for 7 days and then challenged with *S.* Typhimurium

attempt to stop the invasion (Mastroeni and Grant 2013). However, an exacerbated inflammatory response may aggravate the infection by promoting bigger tissue damage and further systemic invasion of the pathogen (Castillo et al. 2012). The concomitant regulatory response induced by *L. plantarum* 286 was important for the reduction of ulcerations and inflammatory foci in the organs of animals that received the strain.

Germ-free animals have an immature immune system, with belated responses. The immune response in germ-free animals may be deficient in comparison to conventional mice. Round and Mazmanian (2009) suggested that the immune response of gnotobiotic animals isn’t strongly influenced by the interaction with microorganism, partially impaired probably by the malformation of some intestinal structures that seem to be

related to the absence of microbiota. The disease model caused by *S.* Typhimurium promotes a fast and acute infection. In conventional animals, *S.* Typhimurium requires a change in the microbiota composition to establish the disease (Stecher et al. 2007). In germ-free animals, the absence of microbiota favors a more serious infection (Nardi et al. 1991), or a more exacerbated pro-inflammatory response, as it was observed in this study. In germ-free mice, the increased INF-γ and IL-6 expression in the group treated with *L. plantarum* 286 and challenged with S. Typhimurium (Fig. 8) could be related to the absence of the previous regulatory action of intestinal microbiota that takes place when it colonizes the gut. The colonization process that occurs in conventional animals from birth onwards promotes a symbiotic relationship with the microbiota, which leads to the establishment of tolerance against common microbes, without initiation of an overreacted inflammatory response in adults exposed to those microbes (Hooper and Gordon 2001). Gnotobiotic studies revealed how the microbiota influences oral tolerance to dietary and commensal bacterial antigens (Wagner 2008). As microbial colonization does not happen in gnotobiotic animals, the first contact with microbes (even not being pathogenics) can unchain an exacerbated inflammatory response, which combines with the induction of IFN-γ production associated to the infection with *S.* Typhimurium (Muotiala and Mäkelä 1990). In other words, the junction of the two factors mentioned above may have unchained an overreacted inflammatory response in the experimental group that received *L. plantarum* 286 and *Salmonella* (Fig. 8). However, the increase of the regulatory cytokine IL-10 may explain the absence of tissue damage, observed in the histopathological analyses of the ileum, even with an increase of pro-inflammatory cytokines (IFN-γ and IL-6).

Comparing the results obtained in the conventional and germ-free models, even the mechanisms were different, in both models it was possible to observe that the administration of *L. plantarum* 286 was able to, at least partially, protect the host against the tissue damages caused by the infection with *S.* Typhimurium. The differences observed in the profile of cytokines are probably related to the presence (or not) of intestinal microbiota between these two mouse models. However, the fact of having used different mice strains (germ-free NIH/Swiss and conventional BALB/c mice) should not be neglected as factor partially responsible for the differential response to *Salmonella* infection.

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

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Evaluation of Microbial Growth in Hospital Textiles Through Challenge Test

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Abstract

Introduction Ensuring the microbiological quality of textiles is an important requirement for health care facilities. The present study examines the way transport times and temperatures influence microbial growth in textiles. Therefore, the effectiveness of washing and disinfection processes has also been studied.

Methods Microbial Challenge Tests were set up through the artificial contamination of different dry and wet textiles which were stored at different temperatures.

The bacterial concentration was evaluated in well-defined time phases aimed at simulating the time it took for the textiles to be transported from the hospital facilities to the reconditioning unit. Three times were therefore considered from T = 0 inoculation moment to T = 72 h post inoculation.

At the end of each time, the increase in bacterial concentration was assessed by means of microbiological cultures, using selective media for the enumeration of each type of inoculated microorganism.

Results In all the contaminated textiles the bacterial concentration remained unchanged at a temperature of 4 °C, while at 22 °C and 37 °C there was a significant increase ($p < 0.05$) starting from 8 h of storage. In these textiles, the microorganism that showed the greatest growth capacity was *P. aeruginosa* with average initial concentration values of 10^4 CFU/cm^2 and a final concentration of 1.5×10^5 CFU/cm^2 at 22 °C and 1×10^5 CFU/cm^2 at 37 °C 72 h after inoculum.

Conclusion The data highlights the fact that the degree of contamination in textiles does not undergo an increase when transport takes place at a controlled temperature. Refrigerated transport of hospital textiles is thus a desirable preventive measure to keep microbiological risk under control.

Keywords

Challenge test · Hospital textiles · Industrial laundry · Microbiological quality

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1 Introduction

The industrial laundry sector is an important and continuously developing area of employment. The two markets in which industrial laundries record the greatest rate of expansion are the healthcare sector and the hotel tourism sector (EBLI 2009). The treated textiles come into daily contact with a large number of patients in a wide variety of work activities (Bloomfield 2011). This makes the textile sector a complex system that should not be underestimated in a risk management approach. Until about fifteen years ago (Fijan et al. 2005; Creamer and Humphreys 2008; Mitchell et al. 2015) the predominant orientation in this field was to provide products in which hygienic requirements coincided with people's sensory perceptions. Today, the customer has to be provided with a product that does not represent a biological risk (Lombardi et al. 2010; Brusaferro et al. 2018; Italian Law n. 24, 8 March 2017). That is why the industrial laundry sector is generating great interest especially in healthcare facilities where the management of biological risk is particularly relevant (Fijan et al. 2005; Fijan et al. 2006). Textiles are a common material in health facilities. It is therefore essential that they do not act as a vehicle for the transfer of pathogens to patients or hospital staff (Whyte 1988; Sehulster and Chinn 2003; Bureau-Chalot et al. 2004; Dancer 2014; Butler 2010). This is mainly because the patients who use hospital textiles are often individuals whose immune system is in some way debilitated. In immune-compromised patients, the onset of infections that are considered negligible in other individuals may have particularly negative outcomes and even lead to death in severe cases (Fijan and Turk 2012; WHO 2017).

The pathogenic microorganisms that are able to survive in hospital fabrics and which, in some cases, give rise to nosocomial infections include (Fijan and Turk 2012): *Staphylococcus aureus,* Methicillin resistant *S. aureus* (MRSA) (Ndawula and Brown 1991; Brunton 1995), *Streptococcus pyogenes* and vancomycin-resistant enterococci (Bonten et al. 1996). Moreover, as stressed in previous research studies, the survival of various microorganisms in textile materials was highlighted after washing in hospital laundries, where the following microorganisms were identified: aerobic bacteria, total coliforms, *Enterococcus faecium, S. aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes* and spores of *Clostridium difficile* (Fijan et al. 2014; Mitchell et al. 2015; Italian Law n. 24, 8 March 2017).

A survival time of microorganisms in textiles of 18 h was indicated in these studies.

In order to obtain microbiologically tested products, both in Europe and in the United States indications have been provided regarding some critical points of the washing process. Within the framework of the European Committee for Standardisation (CEN), the technical standard UNI EN 14065 – “Textiles treated in laundry – Biocontamination control system”(UNI EN ISO14065:2016 2016) has been developed. UNI EN 14065 develops a system of risk analysis and control of biocontamination (RABC, Risk Analysis Biocontamination Control System) based on principles of a preventive nature and on the identification of critical points in the production cycle (from receipt, storage, selection and washing of dirty textiles, up to the transport and delivery of treated fabrics) (Guidelines RABC 2016) to which the hospital linen is subjected the textile treatment site. The biocontamination control is carried out through a monitoring program that involves carrying out microbiological tests to verify the contamination degree of: surfaces in contact with the treated textiles (also during the transport phase), the washing water (in and out of the washing systems), operator's hands and clothes, dry and wet laundry after washing. The RABC system identifies the sampling methods (e. swab or contact plate for surfaces), the searched parameters and the tolerance limits. The monitoring program also includes the washing process validation (bioburden) according to the methods provided for by the UNI EN ISO 14698-1: 2004 standard. The effectiveness of the washing process is validated annually while biomonitoring is performed quarterly.

A proper hygienic management of hospital textiles could be achieved by implementing the minimum technical requirements for sorting, transport and reconditioning that have been identified as necessary. In order to continuously improve microbiological quality, more attention is often paid to neglected phases of the laundry process, that is, the collection of dirty linen and its transport to the laundry factory, which represents an important critical point.

A typical transport process involves the collection of dirty laundry from the use centers (hospitals, nursing homes, etc.) by vans specially designed for the textiles collection and their transport to the laundry. The transport phase has a very variable duration as it depends on the distances from the reconditioning stations, which in some cases can also be located in other cities or regions with respect to the centers of use. The potential for proliferation during the period of time of storage pre-collection, the environmental conditions, especially associated with the temperature and humidity, and the length of time it takes to transport "dirty" textiles from the hospital can play a fundamental role in determining the levels of contamination (Guidelines INAIL 2017) upon arrival at the plant and the effectiveness of the washing process.

The objective of the present study was to evaluate the way transport times and temperature of textiles can influence microbial growth and therefore affect the results of subsequent washing and disinfection process testing. This study has shown how it was possible to optimise and improve the effectiveness of the washing process and reduce microbial concentrations through a reduction in transport times and temperatures.

2 Methods

2.1 Microbiological Investigation

In the first phase of this study the microbiological evaluation of the degree of contamination of various types of dirty hospital textiles upon arrival at the reconditioning station was carried out. In the second phase an experimental challenge test to study the growth dynamics of microorganisms on the textile matrix during the transport process was conducted. The microbiological evaluation was performed before proceeding to the challenge test (EURL Lm 2014; Marras et al. 2019). The collection of analytical data took place during the years 2015–2016. The sampling was performed at an industrial laundry in the south of Sardinia. Out of a total of 126 textile samples from various hospitals in Sardinia, surface swabs (100 cm^2) were taken with seasonal frequency (autumn/winter, spring/summer) (Table 1).

The sampled textiles were divided into textiles with "not very visible" dirt and textiles with "visible dirt and presence of organic material". For each of the two categories of textiles, 4 types were examined (mattress covers, bedsheets, cotton pillowcases and the trilaminate drapes used in operating theatres).

Swab sampling was performed using a cotton swab moistened with buffered peptone water (BPW). This swab was swiped across a premarked surface (using a sterile $10 \times 10\ cm^2$ template) from left to right and from top to bottom using an even pressure and holding the swab flat against the surface. Used swabs were placed in a test tube containing 10 ml of diluent/neutraliser

Table 1 Number of samples from each hospital in each season

Variables		
Hospital	Swabs autumn/ winter	Swabs spring/ summer
1	6	9
2	6	9
3	6	9
4	3	6
5	3	6
6	3	6
7	3	6
8	3	6
9	3	6
10	3	6
11	3	6
12	3	6
Total 12	45	81

and stored at a controlled temperature. The initial suspension (10 ml of diluents), serial tenfold dilutions and viable plate counting (spread plating and pour plating) using the appropriate selective agar mediums noted below for each microorganism were utilised. The bacterial concentration was expressed as colony forming units (CFU)/cm^2.

All samples were collected and transported at controlled temperature to the Laboratory of Food Hygiene, Department of Medical Sciences and Public Health at the University of Cagliari, which operates in compliance with the UNI EN CEI ISO 17025: 2005 "General requirements for testing and calibration laboratories", and were analysed within 24 h (UNI EN ISO 7218: 2013). Table 2 shows the investigated parameters.

Selective agars were used as a medium for incubating each microorganisms. For *E. coli*, the TBX agar (Microbiol Diagnostici s.n.c.) (incubation 24 h at 44 °C), was used. For *S. aureus*, the Baird-Parker agar base (Microbiol Diagnostici s. n.c.), with added egg-yolk tellurite emulsion (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C), was used. Cetrimide agar base (Microbiol Diagnostici s.n.c.) with added glycerol (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C) was used for *P. aeruginosa* detection. Sabouraud Dextrose Agar (SDA) (Microbiol Diagnostici s.n.c.) (incubation 3–5 days at 25 °C) was utilised for moulds and yeast. Plate Count Agar (PCA) (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C) for Total mesophilic count (TMC) determination was used.

Table 2 Parameters and methods

Parameters	Methods
Total mesophilic count (TMC)	UNI EN ISO 14698-1:2004 + UNI EN ISO 4833:2013
Escherichia coli	UNI EN ISO 14698-1:2004 + ISO 16649-2:2001
Staphylococcus aureus	UNI EN ISO 14698-1:2004 + UNI EN ISO 6888-1:2004
Pseudomonas aeruginosa	UNI EN ISO 14698-1:2004 + UNI EN ISO 16266:2008
Moulds and yeast	UNI EN ISO 14698-1:2004+ Rapp.Istis.13/37

2.2 Textile Challenge Tests

Standard 10 × 10 (100 cm^2) samples of the different textiles (cotton mattress covers, trilaminate operating theatre drapes and cotton bedsheets) were artificially contaminated. Two experimental conditions were considered for each type of textile: dry textiles, in order to evaluate the growth/survival of microorganisms and wet textiles with the presence of nutrient material.

An inoculum composed of a mixture of reference ATCC strains and "wild type" microorganisms previously isolated from textile matrices (*Escherichia coli, P. aeruginosa, S. aureus, Saccharomyces cerevisiae* and *Candida albicans*) was used in both experimental models.

The reference strains used were: *E. coli* ATCC 10536, *P. aeruginosa* ATCC 9027, *S.aureus* ATCC 6538, *Saccharomyces cerevisiae* ATCC 3178 and *Candida albicans* ATCC 2091.

For the execution of the challenge test, the indications reported in the international experimental protocols were followed (EURL Lm 2014 and ISO 11930: 2012).

According to these guidelines, challenge tests are performed with a mixture of at least 2 strains to account for variations in growth among the strains. One of them has to be a strain with known growth characteristics (ATCC or NCTC strains). The other strains are freely chosen from environment, outbreak, collections; knowledge of the growth characteristics is not mandatory for these strains. Using an inoculum of multiple strains of a given pathogen is preferred, as it will help to encompass the variability among bacteria.

Each strain used for the preparation of the inoculum was stored in glycerol broth at a temperature of −80 °C ± 2 °C. It was then defrosted, transplanted onto nutrient agar and incubated at 37 °C ± 2 °C for the time needed to reach the start of the stationary phase. With the help of a nephelometer, the resulting colonies were used for the preparation of the known bacterial suspension with concentrations equal to 1 McFarland

(10^8 CFU/ml for bacteria and 10^7 CFU/ml for yeasts).

The suspensions employed for each strain were mixed in equal parts and scalar dilutions were set up to obtain a suspension with a microbial concentration of 10^4 CFU/ml. 1 ml of this suspension was used to contaminate the textile matrix.

For the second experimental condition, that is, wet cloth with nutrient material and inoculum, 4 ml of nutrient broth were added.

In both experimental conditions, textile samples were stored at three different temperatures (4 °C, 22 °C, 37 °C). This allowed the average environmental temperatures to which textiles were exposed during transport in the spring/summer and autumn/winter seasons to be simulated.

The bacterial concentration was evaluated in well-defined time phases aimed at simulating the time it took to transport the textiles from the hospital facilities to the reconditioning plant.

The experimental phases considered were: pre-harvest phase in hospital structures (T = 0) and the transport phase based on the variability of duration in relation to distances (T = 8 h, T = 24 h, T = 48 h and T = 72 h).

At the end of each time interval, the texile samples were suspended in a known volume (100 ml) of physiological solution (0.9% NaCl), using a stomacher for 2 min. The increase in bacterial concentration was assessed by means of viable plate counting (spread plating and pour plating) using selective media for each type of inoculated microorganism. For *E. coli*, the TBX agar (Microbiol Diagnostici s.n.c.) (incubation 24 h at 44 °C) was used. For *S. aureus*, the Baird-Parker agar base (Microbiol Diagnostici s. n.c.), with added egg-yolk tellurite emulsion (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C), was used. Cetrimide agar base (Microbiol Diagnostici s.n.c.) with added glycerol (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C) was utilised for *P. aeruginosa* detection. Sabouraud Dextrose Agar (SDA) (Microbiol Diagnostici s.n.c.) (incubation 3–5 days at 25 °C) was used for moulds and yeast. Plate Count Agar (PCA) (Microbiol Diagnostici s.n.c.) (incubation 48 h at 37 °C) for Total mesophilic count (TMC) determination was used.

The analytical examinations carried out were: T = 0 inoculation moment T = 8 h from inoculation, T = 72 h after inoculation.

The microbial challenge test carried out with the moulds underwent the same procedure described for artificial contamination with bacteria and yeasts. In this case too, an inoculum consisting of a mixture of reference strain of *Aspergillus brasiliensis* (ATCC 16404) and a "wild type" strain previously isolated in the textile matrix was used. A suspension with a microbial concentration of 10^3 CFU/ml was used to contaminate the textile matrix. Sabouraud Dextrose Agar (SDA) (Microbiol Diagnostici s.n.c.) (incubation 3–5 days at 25 °C) was used for *A. brasiliensis* detection.

In both experimental conditions, the contaminated specimens were stored at three different temperatures: 4 °C, 22 °C, 37 °C.

Cultural investigation was performed at the following time intervals: T = 0 inoculation moment, T = 24 h from inoculation, T = 48 h from inoculation, T = 72 h from inoculation. The experiment was repeated three times.

2.3 Statistical Analysis

Descriptive analysis of the data was carried out using average values and standard deviation (SD) for the quantitative variables. T-test was used to compare difference between average values and ANOVA test was used to compare difference between average values between groups. All analyses were performed using Excel (Microsoft®).

3 Results

3.1 Microbiological Investigations

From the microbiological investigations carried out on surface swabs during the spring-summer season, the average values of TMC were shown to

be equal to 260 (± 4) CFU/cm^2, 67 (± 2) CFU/cm^2, 51(± 3) CFU/cm^2 and 4 (± 1) CFU/cm^2 respectively in the textile types of trilaminate drapes, mattress covers, bedsheets and cotton pillowcases. Among the potentially pathogenic microorganisms, the presence of *S. aureus* was only detected in the mattress cover matrix with an average concentration of 30 (± 2) CFU/cm^2. All other parameters (*E. coli, P. aeruginosa*, moulds and yeasts) were absent in all the other types of textiles.

In the swabs taken during the autumn-winter season, the textile typology that proved to be the most contaminated was the mattress cover with average values of 3.6×10^4 (± 145) CFU/cm^2 for TMC, 7.5×10^3 (± 252) CFU/cm^2 for *E.coli*, 6.5×10^3 (± 577) CFU/cm^2 for *P.aeruginosa* and 10^3 (± 30) CFU/cm^2 for *S. aureus*. All the other types showed significantly lower ($p < 0.05$) average TMC values (from 1 CFU/cm^2 to 1.8×10^2 CFU/cm^2) and an absence of potentially pathogenic microorganisms except for the trilaminate drape matrix, where the presence of *S. aureus* was detected with an average concentration of 15 CFU/cm^2 (Table 3).

3.2 Textile Challenge Tests

Different results are obtained from the artificial contamination of the textile matrix depending on the type of textile considered and the experimental conditions used (dry textile and wet textile with or without nutrient material).

3.3 Mattress Cover

Cultural investigation of the dry mattress cover at 4 °C shows a more or less constant trend in all the parameters considered with a slight decrease in concentration values at time T = 72 h.

At 22 °C there is a noticeable increase ($p < 0.05$) in concentration starting from time T = 8 h until the end of the experiment. In particular, at T = 72 h, in addition to the high TMC values ($9.4 \times 10^4 \pm 10^3$ CFU/cm^2) there is a noticeable increase in the average concentration of *P. aeruginosa* and *E.coli* with values that ranged respectively from 3 ± 0.6 CFU/cm^2 at T = 8 h to $8.6 \times 10^3 \pm 2 \times 10^2$ CFU/cm^2 and from 3 ± 0.6 CFU/cm^2 at T = 8 h to $9.2 \times 10^3 \pm 2 \times 10^2$ CFU/cm^2. *S. aureus* is present with an average concentration of 98 ± 2 CFU/cm^2.

At 37 °C, high TMC values $3 \times 10^5 \pm 5.5 \times 10^3$ CFU/cm^2 and *S. Aureus* with an average concentration of 56 ± 3.5 CFU/cm^2 were found.

The microorganism that showed the greatest capacity for growth was *P. aeruginosa* reaching average T = 72 h concentration values of $8.6 \times 10^3 \pm 2 \times 10^2$ CFU/cm^2 and $9.5 \times 10^3 \pm 2 \times 10^2$ CFU/cm^2 at 22 °C and 37 °C respectively (Fig. 1).

Moreover, investigation of cultures from the wet mattress cover also showed more or less the same trend observed in dry conditions. A more or less constant trend in all the parameters considered is observed at 4 °C.

At 22 °C the trend resembles that found for the dry mattress cover at the same temperature with a concentration starting from T = 8 h until the end of the experiment. In addition to the high values of the TMC ($9.9 \times 10^4 \pm 5.8 \times 10^2$ CFU/cm^2), a significant increase was found ($p < 0.05$) in the average concentration of *P. aeruginosa* (Fig. 2) and *E. coli*, equal to $9 \times 10^3 \pm 5 \times 10^2$ CFU/cm^2 and $9.8 \times 10^3 \pm 2 \times 10^2$ CFU/cm^2 respectively. High values of TMC ($3 \times 10^5 \pm 5.5 \times 10^3$ CFU/cm^2) and of *P. aeruginosa* ($9.5 \times 10^3 \pm 5 \times 10^2$ CFU/cm^2) are found at 37 °C.

For the wet mattress cover, *P. aeruginosa* was again the microorganism that showed a greater capacity for growth reaching average T = 72 h concentration values of $9 \times 10^3 \pm 5 \times 10^2$ CFU/cm^2 and $6 \times 10^4 \pm 5 \times 10^3$ CFU/cm^2 at temperatures of 22 °C and 37 °C respectively (Fig. 2).

As far as the evolution of mould concentration is concerned, no growth was observed on the dry mattress cover. On the other hand, the only increase in concentration on the wet mattress cover occurred at a temperature of 37 °C, at T = 72 h from the inoculation with an average concentration of $1.48 \times 10^2 \pm 7$ CFU/cm^2 (Table 4).

Table 3 Microbiological investigations. CFU values are expressed as mean +/− SD

	Variables	**TMC**	***E. coli***	***S. aureus***	***P. aeruginosa***	**Moulds and yeasts**
	Bedsheets	1 ± 0.6	0	0	0	0
Autumn/winter	Trilaminate drapes	$1.2 \times 10^2 \pm 12$	10 ± 1	15 ± 2	0 ± 1	0 ± 1
	Cotton pillowcase	$1.80 \times 10^2 \pm 10$	0 ± 1	0 ± 1	0	0
	Mattres cover	$3.6 \times 10^4 \pm 1.45 \times 10^2$	$7.5 \times 10^3 \pm 2.52 \times 10^2$	$10^3 \pm 30$	$6.5 \times 10^3 \pm 5.7 \times 10^2$	0
	Variables	**TMC**	***E. coli***	***S. aureus***	***P. aeruginosa***	**Moulds and yeasts**
	Bedsheets	51 ± 3	0	0	0	0
Spring/summer	Trilaminate drapes	$2.6 \times 10^2 \pm 4$	0 ± 1	30 ± 2	0	0 ± 1
	Cotton pillowcase	4 ± 1	0	0 ± 1	0 ± 1	0
	Mattres cover	67 ± 2	1 ± 0	0	0	0

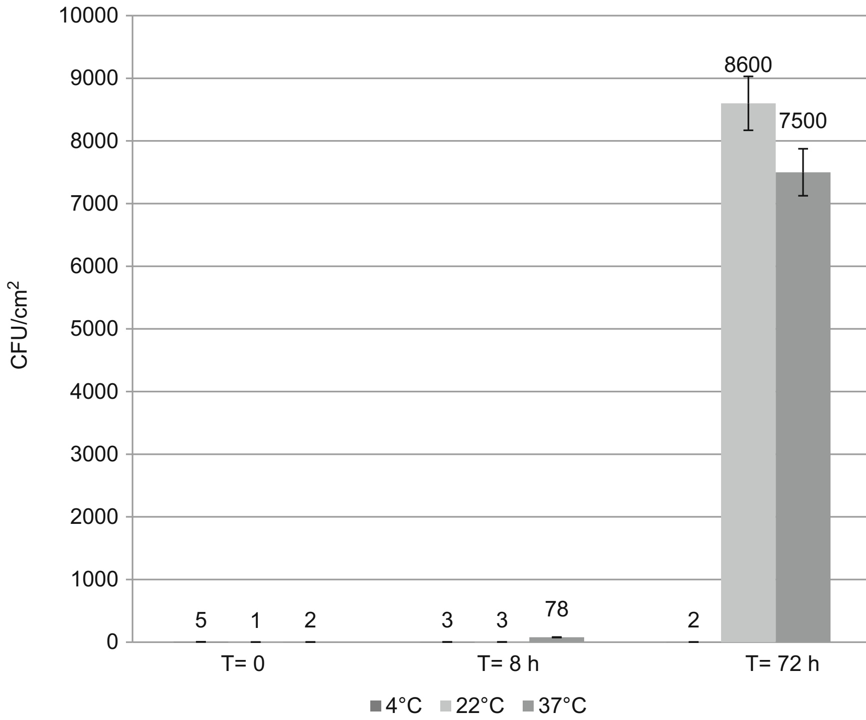

Fig. 1 Evolution of the concentration of *Pseudomonas aeruginosa* in the dry mattress covers. CFU values are expressed as mean +/− SD

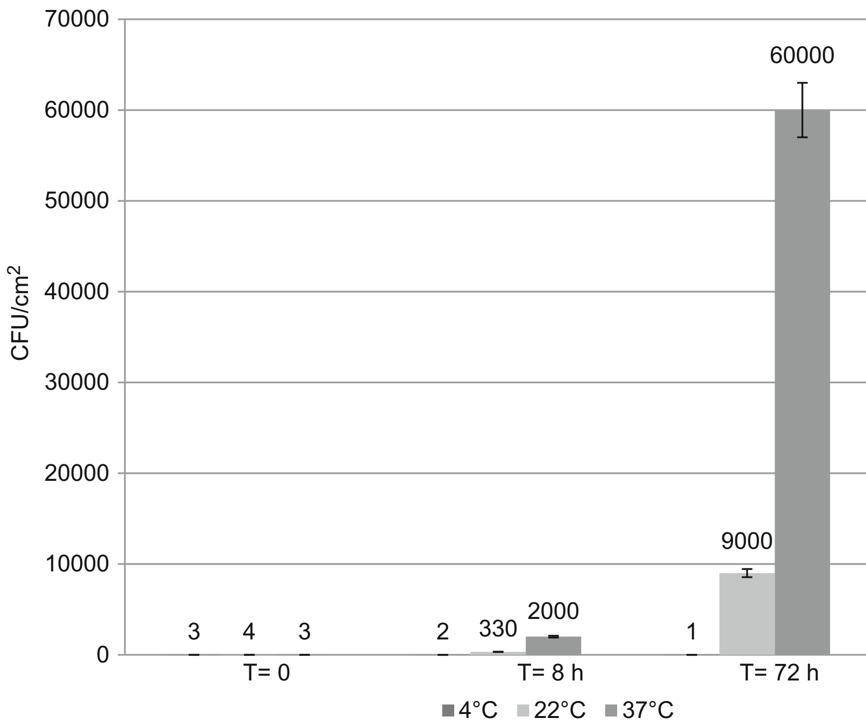

Fig. 2 Evolution of the concentration of *Pseudomonas aeruginosa* in the wet mattress covers. CFU values are expressed as mean +/− SD

Table 4 Textile challenge test results: mattress covers. CFU values are expressed as mean +/− SD

Dry mattress covers	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	12 ± 2.5	12 ± 1.5	11 ± 1		11 ± 1	12 ± 3.5	$9.4 \times 10^4 \pm 10^3$		11 ± 1	$3.4 \times 10^3 \pm 126$	$3 \times 10^5 \pm 5.5 \times 10^3$	
E. coli	2 ± 0.6	1 ± 0.6	1 ± 0		2 ± 0.6	3 ± 0.6	$9.2 \times 10^3 \pm 2 \times 10^2$		2 ± 0.6	$10^2 \pm 20$	2 ± 0.6	
S. aureus	6 ± 2.5	7 ± 1.5	4 ± 1		5 ± 2	7 ± 2	98 ± 2		4 ± 1.5	97 ± 2.5	56 ± 3.5	
P. aeruginosa	5 ± 2	3 ± 2	2 ± 1s		1 ± 0	3 ± 0.6	$8.6 \times 10^3 \pm 2 \times 10^2$		2 ± 0	78 ± 2.5	$9.5 \times 10^3 \pm 2 \times 10^2$	
Yeasts	2 ± 2	2 ± 2	1 ± 1		1 ± 0.6	3 ± 1	5 ± 1.5		2 ± 1	1 ± 0	7 ± 2.5	
Moulds	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
	2 ± 1	0	0 ± 1	0 ± 0	4 ± 0.6	0	0 ± 1	0	5 ± 1	0 ± 1	0	0 ± 1
Wet mattress covers	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	14 ± 2	11 ± 1.5	11 ± 3.5		20 ± 5	63 ± 5	$9.9 \times 10^4 \pm 5.8 \times 10^2$		25 ± 7	$3.4 \times 10^4 \pm 2 \times 10^3$	$8.2 \times 10^6 \pm 5.8 \times 10^3$	
E. coli	1 ± 0	2 ± 1	1 ± 1		1 ± 0.6	12 ± 25	$9.8 \times 10^3 \pm 10^2$		2 ± 0	$3.3 \times 10^3 \pm 10^2$	3 ± 1	
S. aureus	3 ± 1	4 ± 1.5	3 ± 2		6 ± 3	16 ± 3	1 ± 1		6 ± 3	$3.2 \times 10^2 \pm 10$	2 ± 0.6	
P. aeruginosa	3 ± 1.5	2 ± 1	1 ± 0.6		4 ± 2	$3.3 \times 10^2 \pm 10$	$9 \times 10^3 \pm 5 \times 10^2$		3 ± 1.5	$2 \times 10^3 \pm 2 \times 10^2$	$6 \times 10^4 \pm 5 \times 10^3$	
Yeasts	2 ± 0.6	3 ± 1	2 ± 1		1 ± 1	4 ± 3	$1.3 \times 10^2 \pm 20$		1 ± 0	$2.5 \times 10^2 \pm 30$	$8.7 \times 10^2 \pm 30$	
	T = 0	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
Moulds	4 ± 2	0 ± 1	0	0 ± 1	4 ± 1	0	0	0 ± 1	5 ± 1	0 ± 1	1 ± 0	$1.48 \times 10^2 \pm 7$

3.4 Trilaminate Operating Theatre Drape

Viable counts of the dry drape did not show any changes in microbial growth at 4 °C.

At 22 °C there is a noticeable increase ($p < 0.05$) in concentration starting from time T = 8 h until the end of the experiment. The following average values are found at T = 72 h: TMC $2.6 \times 10^4 \pm 9 \times 10^2$ CFU/cm^2, *P. aeruginosa* $3 \times 10^3 \pm 10^2$ CFU/cm^2, *E.coli* $5.2 \times 10^3 \pm 10^2$ CFU/cm^2 and yeasts $1.9 \times 10^3 \pm 10^2$ CFU/cm^2.

At 37 °C high values of TMC ($4.2 \times 10^4 \pm 10^2$ CFU/cm^2), *P. aeruginosa* ($2.7 \times 10^3 \pm 17.5$ CFU/cm^2) and *S. aureus* (100 ± 20 CFU/cm^2) are highlighted.

As regards the dry trilaminate drape, the microorganism that showed a greater capacity for growth was *P. aeruginosa* ($p < 0.05$) with an average concentration at T = 72 h of $3 \times 10^3 \pm 10^2$ CFU/cm^2 at 22 °C and $2.7 \times 10^3 \pm 17.5$ CFU/cm^2 at 37 °C (Fig. 3). A similar trend is found in the experimental "wet" condition at 4 °C as the one observed in the "dry" condition. Remarkable growth is observed at 22 °C from time T = 8 h with values not found in other tested textiles.

TMC shows an average value of $5.6 \times 10^6 \pm 5.8 \times 10^3$ CFU/cm^2 at T = 72 h while an average $3 \times 10^5 \pm 5.7 \times 10^2$ CFU/cm^2 of *P. aeruginosa* were found. *E. coli* show values of $5.4 \times 10^5 \pm 6.8 \times 10^3$ CFU/cm^2 and yeasts $9.6 \times 10^3 \pm 10^2$ CFU/cm^2. *S. aureus* was detected with a concentration of $1.1 \times 10^3 \pm 50$ CFU/cm^2.

High values of TMC ($1.5 \times 10^6 \pm 2 \times 10^4$ CFU/cm^2) and *P. aeruginosa* ($1.35 \times 10^5 \pm 5 \times 10^3$ CFU/cm^2) are highlighted at 37 °C. *S. aureus* was detected with a concentration of 170 CFU/cm^2 and yeasts at $4.8 \times 10^4 \pm 2 \times 10^3$ CFU/cm^2. *E. coli* was not found.

In the wet condition, the microorganisms which showed a greater capacity for growth were *P. aeruginosa* and yeasts. At T = 72 h *P. aeruginosa* reaches average concentration

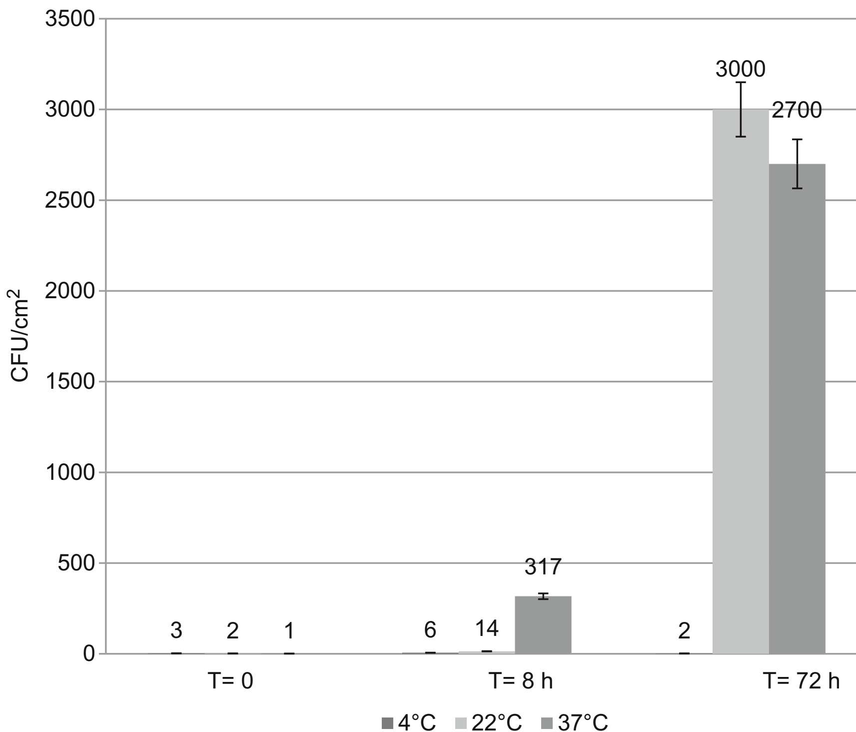

Fig. 3 Evolution of the concentration of *Pseudomonas aeruginosa* in the dry trilaminate operating theatre drapes. CFU values are expressed as mean +/− SD

values of $3 \times 10^5 \pm 5.7 \times 10^2$ CFU/cm^2 at 22 °C and $1.35 \times 10^5 \pm 5 \times 10^3$ CFU/cm^2 at 37 °C ($p < 0.05$). Yeasts showed values of $9.6 \times 10^3 \pm 10^2$ CFU/cm^2 and $4.8 \times 10^4 \pm 2 \times 10^3$ CFU/cm^2 at temperatures of 22 °C and 37 °C respectively (Fig. 4).

As far as the evolution of the mould concentration is concerned, there is no growth in the trilaminate textile matrix at temperatures of 4 °C, 22 °C and 37 °C in the dry experimental conditions. An increase in concentration is only observed in "damp wash cloths" at the temperature of 37 °C, at time T = 4 h and T = 6 h with an average concentration of 13 CFU/cm^2 and 245 CFU/cm^2 respectively (Table 5).

3.5 Bedsheets

Cultural investigation at 4 °C showed no growth variation and the concentration trend is constant over time.

The same can be seen at 22 °C and 37 °C up to time T = 72 h when there is a slight increase in both temperatures, even though this is more noticeable at 22 °C with TMC and *P. aeruginosa* values of $9.1 \times 10^4 \pm 5 \times 10^2$ CFU/cm^2 and $9.7 \times 10^3 \pm 10^2$ CFU/cm^2 respectively.

In the "wet" condition there is a growth at 22 °C and 37 °C for almost all the given parameters. At 22 °C, concentrations of *P. aeruginosa* equal to $1.4 \times 10^4 \pm 7.5 \times 10^2$ CFU/cm^2 and *E. coli* equal to $1.6 \times 10^4 \pm 4 \times 10^2$ CFU/cm^2 are found at T = 72 h. At 37 °C there are high values of TMC ($1.4 \times 10^6 \pm 5.7 \times 10^3$ CFU/cm^2) *P. aeruginosa* ($2.7 \times 10^4 \pm 10^3$ CFU/cm^2) and *S. aureus* ($1.8 \times 10^3 \pm 50$ CFU/cm^2).

The microorganism which showed the greatest growth capacity on the wet bed sheet was *P. aeruginosa* ($p < 0.05$), reaching average concentration values at T = 72 h equal to $1.4 \times 10^4 \pm 7.5 \times 10^2$ CFU/cm^2 and $2.7 \times 10^4 \pm 10^3$ CFU/cm^2 at 22 °C and 37 °C respectively (Fig. 5).

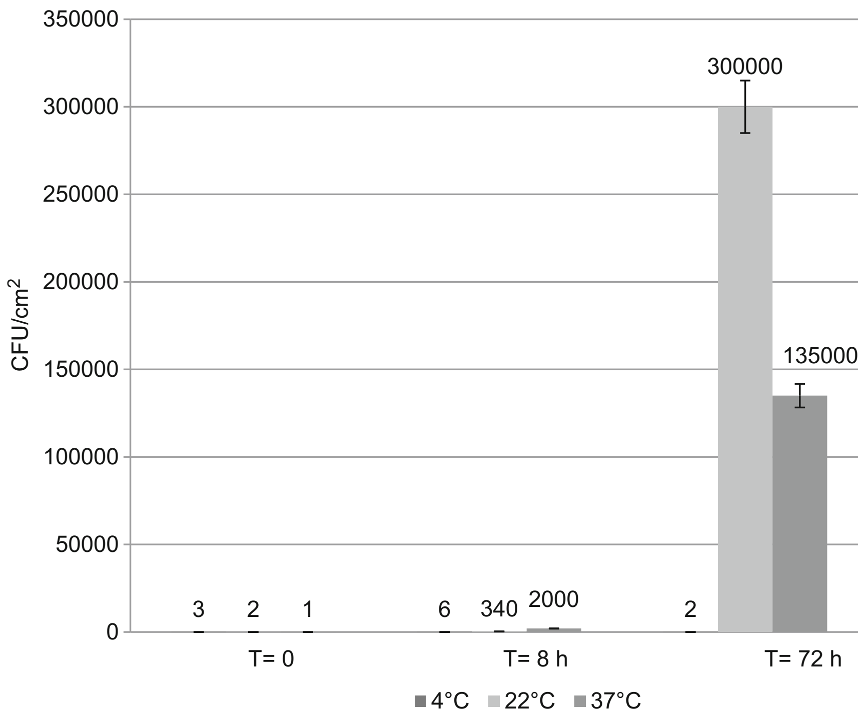

Fig. 4 Evolution of the concentration of *Pseudomonas aeruginosa* in the wet trilaminate operating theatre drapes. CFU values are expressed as mean +/− SD

Table 5 Textile challenge test results: trilaminate operating theatre drapes. CFU values are expressed as mean +/− SD

Dry trilaminate operating theatre drapes	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	14 ± 0.6	11 ± 0.6	12 ± 0.6		27 ± 1.2	57 ± 1.2	$2.6 \times 10^4 \pm 9 \times 10^2$		18 ± 4	$2.52 \times 10^2 \pm 7.5$	$4.2 \times 10^4 \pm 10^2$	
E. coli	3 ± 1	2 ± 1	1 ± 0.6		2 ± 1	1 ± 0	$5.2 \times 10^3 \pm 10^2$		2 ± 0.6	$2.75 \times 10^2 \pm 15$	2 ± 1	
S. aureus	4 ± 0.6	5 ± 1.5	4 ± 1.5		14 ± 1	7 ± 2	17 ± 2.5		10 ± 4	12 ± 2.5	$10^2 \pm 20$	
P. aeruginosa	3 ± 0.6	6 ± 1	2 ± 0.6		2 ± 1	14 ± 2.5	$3 \times 10^3 \pm 10^2$		1 ± 0.6	$2.7 \times 10^3 \pm 17.5$	$1.45 \times 10^3 \pm 10^2$	
Yeasts	2 ± 0	1 ± 0.6	1 ± 1		1 ± 0.6	3 ± 1	$1.9 \times 10^3 \pm 10^2$		2 ± 0	2 ± 0.6	$2.95 \times 10^2 \pm 25$	
	T = 0	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
Moulds	3 ± 0.6	0	0 ± 1	0	4 ± 1	0	0 ± 1	0	5 ± 2	0 ± 1	0	0 ± 1
Wet trilaminate operating theatre drapes	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	25 ± 5	22 ± 4	26 ± 4.5		33 ± 9	63 ± 8.5	$5.6 \times 10^6 \pm 5.8 \times 10^3$		19 ± 2.5	$3.4 \times 10^4 \pm 8.4 \times 10^2$	$1.5 \times 10^6 \pm 2 \times 10^4$	
E. coli	2 ± 0.6	1	1 ± 1		2 ± 0.6	12 ± 3.5	$5.4 \times 10^5 \pm 6.8 \times 10^3$		2 ± 1	$3.3 \times 10^3 \pm 10^2$	4 ± 2.5	
S. aureus	8 ± 2	5 ± 2.5	6 ± 2.5		34 ± 1	16 ± 25	$1.1 \times 10^3 \pm 50$		11 ± 1.5	$3.2 \times 10^2 \pm 10$	$1.7 \times 10^2 \pm 20$	
P. aeruginosa	3 ± 1.5	6 ± 2	2 ± 0.6		2 ± 0.6	$3.4 \times 10^2 \pm 20$	$3 \times 10^5 \pm 5.7 \times 10^2$		1 ± 1	$2 \times 10^3 \pm 10^2$	$1.35 \times 10^4 \pm 5 \times 10^3$	
Yeasts	2 ± 1.5	1 ± 0	3 ± 2		1 ± 0	4 ± 1.5	$9.6 \times 10^3 \pm 10^2$		2 ± 0.6	250 ± 10	$4.8 \times 10^4 \pm 2 \times 10^3$	
	T = 0	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
Moulds	2 ± 0.6	1 ± 0	1 ± 0	2 ± 1	4 ± 1	2 ± 06	1 ± 0	3 ± 1	4 ± 0.6	3 ± 1	13 ± 2	$2.45 \times 10^2 \pm 20$

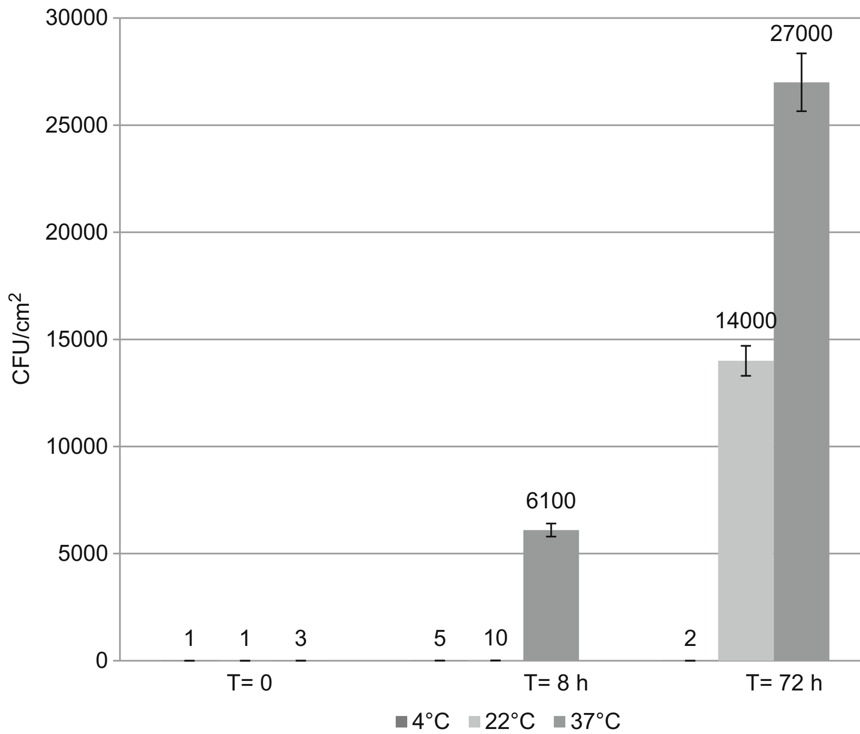

Fig. 5 Evolution of the concentration of *Pseudomonas aeruginosa* in the wet bedsheets. CFU values are expressed as mean +/− SD

As regards the evolution of mould concentration, as was demonstrated for the other matrices (mattress covers and trilaminate drapes), no growth occurs at 4 °C, 22 °C and 37 °C in dry conditions. The only increase in concentration on the "wet bedsheet" is at 37 °C, at time T = 4 h and T = 6 h with an average concentration of 15 ± 3 CFU/cm^2 and 85 ± 4 CFU/cm^2 respectively (Table 6).

4 Discussion and Conclusions

Hospital textile contamination has recently been the subject of several studies because many experts believe that they play an important role in the acquisition and transmission of pathogenic agents in health care environments (Bajpai et al. 2011; Fijan et al. 2014). Sanitation of sanitary textiles is generally entrusted to industrial laundries whose washing procedures are, for the most part, sufficient to return textiles free from microbial contamination (Lombardi et al. 2010; Brusaferro et al. 2018). However numerous factors such as pre-laundering practices, storage and transport of textiles from health facilities to reconditioning plants (industrial laundries) can contribute to the success of these industrial washing procedures (Mitchell et al. 2015). In fact, the quality of a textile is currently guaranteed by operating procedures that are strictly followed in industrial laundries. However, with the precise aim of trying to improve the washing process, following the criterion and goal of the concept of quality in a broader sense, the current study considers other aspects associated with phases of the same process that risk analysis suggests could be potentially associated with increased microbial concentration in textiles.

This study assessed how microbial growth is affected by the transport time and environmental factors (temperature, humidity, presence of organic material) involved in the transportation of hospital textiles from hospitals to the

Table 6 Textile challenge test results: bedsheets. CFU values are expressed as mean +/− SD

Dry bedsheets	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	14 ± 1	15 ± 1	13 ± 1.5		13 ± 1	970 ± 20	$9.1 \times 10^4 \pm 5 \times 10^2$		12 ± 1	90 ± 10	$9 \times 10^2 \pm 10^2$	
E. coli	2 ± 1	1 ± 0.6	1 ± 0.6		2 ± 1	1 ± 1	$1.5 \times 10^3 \pm 76$		12 ± 1	12 ± 2	47 ± 0.6	
S. aureus	5 ± 1	6 ± 1	3 ± 0.6		5 ± 1	2 ± 1	1 ± 0.6		4 ± 1	5 ± 1	2 ± 0.6	
P. aeruginosa	1 ± 0.6	1 ± 0	1 ± 0.6		1 ± 1	6 ± 1	$9.7 \times 10^3 \pm 10^2$		12 ± 2	11 ± 2	25 ± 1.5	
Yeasts	2 ± 0.6	2 ± 0.6	1 ± 0		1 ± 1	1 ± 1	5 ± 0.6		2 ± 1	2 ± 1	1 ± 0	
	T = 0	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
Moulds	2 ± 1	0 ± 1	0	0 ± 1	4 ± 0.6	0	0 ± 1	0	5 ± 2	0 ± 1	0	0 ± 1
Wet bedsheets	**4 °C**				**22 °C**				**37 °C**			
	T = 0	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**		**T = 0**	**T = 8 h**	**T = 72 h**	
TMC	14 ± 1	12 ± 2	13 ± 1.5		15 ± 3	16 ± 2	$9.7 \times 10^4 \pm 5 \times 10^2$		17 ± 1	$9.5 \times 10^4 \pm 10^2$	$1.4 \times 10^6 \pm 5.7 \times 10^3$	
E. coli	1 ± 0.6	2 ± 0.6	1 ± 0.6		1 ± 1	1 ± 0	$1.6 \times 10^4 \pm 4 \times 10^2$		2 ± 1	$9.6 \times 10^3 \pm 58$	3 ± 0.6	
S. aureus	5 ± 1	2 ± 1	3 ± 0.6		6 ± 1	12 ± 1	1 ± 0.6		6 ± 1	$8.5 \times 10^2 \pm 5$	$1.8 \times 10^3 \pm 50$	
P. aeruginosa	1 ± 0.6	5 ± 1.5	2 ± 0.6		1 ± 1	10 ± 1	$1.4 \times 10^4 \pm 7.5 \times 10^2$		3 ± 1	$6.1 \times 10^3 \pm 20$	$2.7 \times 10^4 \pm 10^3$	
Yeasts	2 ± 0.6	2 ± 0.6	1 ± 0.6		1 ± 0	7 ± 1	$7.6 \times 10^2 \pm 3$		1 ± 1	$2.15 \times 10^2 \pm 5$	$9.6 \times 10^2 \pm 20$	
	T = 0	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**	**T = 0**	**T = 24 h**	**T = 48 h**	**T = 72 h**
Moulds	2 ± 1	1 ± 0	1 ± 0.6	1 ± 0	4 ± 1	3 ± 0	1 ± 0.6	2 ± 1	4 ± 1.5	2 ± 1	15 ± 3	85 ± 4

reconditioning plant (industrial laundry) and, consequently, the extent to which these conditions impact the effectiveness of washing and disinfection processes. There is limited published research on the growth and survival of microorganisms on the types of textile fibres commonly found in hospital textiles (Honisch et al. 2014; Riley et al. 2017).

However, it is known that in a substratum that shows nourishment, microbial multiplication undergoes an increase that is significantly associated with an increase in temperature and extended time. For this reason the current study simulated transport at controlled temperatures and at different times which has led to results that call for reconsideration, in the light of RABC guidelines (Guidelines RABC 2010), for the optimisation of the industrial washing process. These results suggest that regardless of the season, the degree of contamination in textiles and in particular in draw sheets and trilaminate drapes, undergoes a significant increase ($p < 0.05$) after 8 h, at temperatures of 22 °C and 37 °C, whereas if transport takes place at a controlled temperature (4–8 °C) the increase is not significant within the maximum time interval considered in this study (72 h).

This trend was observed particularly with regard to *P. aeruginosa*, a microorganism not yet considered in the guidelines, but of considerable importance for opportunistic pathogens in a nosocomial environment (Takai et al. 2002; Fijan et al. 2014). Another aspect that emerged from the current study, in compliance with what has been reported in the literature (Honisch et al. 2014; Riley et al. 2017), relates to the fact that during the summer-spring season, the microbial concentration in textiles undergoes a significant decrease ($p < 0.05$) due to natural disinfection processes (light, drying) that even induce the death of labile bacteria such as *E.coli*. On the contrary, microorganisms survive more in textiles in the autumn and winter.

In literature, most of the work concerns the association between the contamination of hospital textiles and their possible role in the onset of nosocomial infections (Creamer and Humphreys 2008; Butler 2010; Fijan et al. 2014; Mitchell et al. 2015). On the contrary, very few studies have been developed on the dynamics and factors that may influence the growth of microorganisms in textiles (Riley et al. 2017). These data show that refrigerated transport and storage would block any growth of micro-organisms if transport and delivery to the laundry lasted longer than 8 h. Therefore, the refrigerated transport of hospital textiles represents an important and desirable preventive measure. In fact, in terms of the optimisation of the washing processes that currently provide and guarantee the abatement of concentrations even at high levels of contamination (10^8 CFU/ml), this condition would be minimised by further ensuring the safety of hospital textiles in light of the microbiological risk assessment.

Conflict of Interest Statement None declared.

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The Role of Gram-Negative Bacteria in Urinary Tract Infections: Current Concepts and Therapeutic Options

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Abstract

Urinary tract infections (UTIs) are some of the most common infections in human medicine worldwide, recognized as an important public health concern to healthcare systems around the globe. In addition, urine specimens are one of the most frequently submitted samples for culture to the clinical microbiology laboratory, exceeding the number of most of the other sample types. The epidemiology, species-distribution and susceptibility-patterns of uropathogens vary greatly in a geographical and time-dependent manner and it also strongly correlated with the reported patient population studied. Nevertheless, many studies highlight the fact that the etiological agents in UTIs have changed considerably, both in nosocomial and community settings, with a shift towards "less common" microorganisms having more pronounced roles. There is increasing demand for further research to advance diagnostics and treatment options, and to improve care of the patients. The aim of this review paper was to summarize current developments in the global burden of UTI, the diagnostic aspects of these infectious pathologies, the possible etiological agents and their virulence determinants (with a special focus on the members of the Enterobacterales order), current guidelines and quality indicators in the therapy of UTIs and the emergence of multidrug resistance in urinary pathogens.

Keywords

Antibiotics · Clinical microbiology' virulence · Epidemiology · *Escherichia coli* · Multidrug

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resistance · Pathogenomics · Therapeutic guidelines · Urinary tract infections

Abbreviations

ACSS	Acute Cystitis Symptom Score
AP	acute pyelonephritis
ASB	asymptomatic bacteriuria
AUC	acute uncomplicated cystitis
CAUTI	catheter-associated UTI
CDC	Centers for Disease Control
CFU	colony-forming units
CRGNB	carbapenem-resistant Gram-negative bacilli
cUTI	complicated UTI
ECDC	European Centre for Disease Prevention and Control
ESBL	extended-spectrum β-lactamase
ExPEC	extra-intestinal pathogenic *Escherichia coli*
GNB	Gram-negative bacteria
ICE	integrative and conjugative element
IDSA	Infectious Diseases Society of America
InCOM	intra-intestinal commensal
InPEC	intra-intestinal pathogenic *Escherichia coli*
LPS	lipopolysaccharide
m/z	mass-to-charge
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MDR	multidrug-resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus epidermidis*
MRSE	methicillin-resistant *Staphylococcus epidermidis*
MSSA	methicillin-sensitive *Staphylococcus aureus*
NACA	non-*albicans Candida*
NECE	non-*E. coli Enterobacterales*
NGS	next-generation sequencing
NICE	The National Institute for Health and Care Excellence
OMP	outer membrane proteins
OMV	outer membrane vesicles
PAI	pathogenicity islands
PCR	polymerase chain-reaction
PDR	pandrug-resistant
PROM	patient-reported outcome measures
QI	quality indicator
QoL	quality of life
rRNA	ribosomal RNA
RTX	repeats in toxin
rUTI	recurrent UTI
ShiToPInPEC	*Shigella* Toxin Producer InPEC
TMP/SMX	trimethoprim-sulfamethoxazole
UPCA	uropathogenic *Candida albicans*
UPEC	uropathogenic *Escherichia coli*
US	United States
UTI	urinary tract infections
VF	virulence factors
VRE	vankomycin-resistant *Enterococcus*
WHO	World Health Organization
XDR	extensively drug resistant

1 The Burden of Urinary Tract Infections

The global burden of diseases have shown considerable changes in the last century. In contrast to previous times of humanity, the introduction of appropriate sanitation and antibiotics has brought on an epidemiological transition, where the burden of diseases that was predominantly communicable, which has shifted towards one that is nowadays predominantly non-communicable (chronic) (Jamison et al. 2018). Nevertheless, infectious pathologies still constitute an important disease burden worldwide (Furuse 2019). Urinary tract infections (UTIs) are the second most common type of infections in human medicine (following respiratory tract infections) in the United States and Europe and the third most common infectious pathologies (following respiratory

tract infections and gastrointestinal infections) worldwide, recognized as an important public health concern to healthcare systems around the globe (Flores-Mireles et al. 2015; Sobel and Kaye 2015). In general, UTIs include infections of the urethra, bladder, ureter and the kidneys, most frequently due to bacteria originating from the alimentary tract (McLellan and Hunstad 2016). UTIs are multi-positional, multi-syndromal, multi-factorial and often multi-microbial infectious diseases occurring among different populations including men, women, adults, children, infants, aged and young people around the globe (Flores-Mireles et al. 2015; Tangdogdu and Wagenlehner 2016). UTIs should be considered as an important factor or morbidity and mortality, both among outpatients (representing 10–30% of infections) and hospitalized patients (Wiedemann et al. 2014). In fact, in the latter group, nosocomial UTIs are the most common infectious pathologies, responsible for 25–50% of infections overall (Stefaniuk et al. 2016; Wiedemann et al. 2014). The multi-positional clinical problem of UTIs is as follows: (i) UTIs cause considerable decrease in the quality of life (QoL) in the affected patients, especially in case of recurrence, complications and sequelae; (ii) the high number of patients with symptomatic UTIs will visit their primary case physicians or specialists, for which, considerable amount of human resources are required (globally, around 150–200 million people are diagnosed with UTI annually; UTIs are responsible for around 10 million GP visits, 1.5 million emergency room visits and 300,000 hospital admissions in the US alone); (iii) UTIs should be treated at the earliest convenience, as if therapy is not initiated or if the appropriate steps are not taken, it may lead to re-infection, ascending infections to the kidneys or other sequelae; (iv) the therapy of UTIs usually entails the administration of antibiotics (UTIs rank as the most common cause that leads to an antibiotic prescription after a GP visit), however, the adverse events associated with antibiotic use, *Clostridioides difficile* enterocolitis, and the emergence of antibiotic-resistant pathogens causing UTIs is a serious concern; (v) UTIs also have a substantial economic impact, including costs of pharmacotherapy, hospitalization and lost working days; the annual cost of UTIs in the US alone has been estimated to be more, than four billion US dollars, while the excess economic losses associated with UTIs to the global economy were shown to be around four billion US dollars (Bermingham and Ashe 2012; Flores-Mireles et al. 2015; Foxman 2003; McLellan and Hunstad 2016; Renald et al. 2015; Simmering et al. 2017; Stefaniuk et al. 2016; Tangdogdu and Wagenlehner 2016; Wiedemann et al. 2014). The Acute Cystitis Symptom Score (ACSS) has been recently developed for the diagnosis of acute cystitis and patient-reported outcome measures (PROMs), reporting on the typical symptoms of acute cystitis (frequency, urgency, dysuria, suprapubic pain, feeling of incomplete bladder emptying and visible blood in urine) (Alidjanov et al. 2016; Di Vico et al. 2020; Magyar et al. 2018). Recurrent UTIs (rUTI) are also frequently associated with psychiatric symptoms, such as reduced social activity, guilt (due to inability to perform various everyday tasks), anxiety (e.g., associated with incontinence in the elderly) and depression, which also major contributing factors to the QoL-decrease associated with these infections (Dason et al. 2011; Flower et al. 2014; Negus et al. 2020). As UTIs represent a major healthcare burden, there is increasing demand for further research to advance diagnostics and treatment options an to improve care of the patients (Jhang and Kuo 2017).

Under physiological conditions, urine was previously thought to be sterile; however, with the emergence of 16S rRNA PCR, metagenomics and the introduction of next-generation sequencing (NGS), the characterization of the urinary microbiome has begun and this dogma has been challenged (Bilen et al. 2018; Brubauker and Wolfe 2016; Govender et al. 2019). The threshold of microbial population for the definition of UTIs is usually reported as $\geq 10^5$ colony forming units (CFUs)/mL; however, this is subject to interpretation (going as low as 10^2 CFU/mL), depending on the studied patient population and the sample type submitted for microbial analysis (Chu and Lowder 2018; Roberts and Wald 2018; Schmiemann et al. 2010). UTIs and UTI-related

syndromes may be classified based on several characteristics: (i) based on the presence of symptoms: asymptomatic bacteriuria or symptomatic UTIs (mild/moderate/severe); (ii) based on the onset of the infections: acute or chronic/ recurrent infections (or rUTIs are defined as UTIs occurring more, than three times in a year), community-acquired and nosocomial infections; (iii) based on the anatomical region affected: lower urinary tract infections (i.e. cystitis), upper urinary tract infections (i.e. nephritis) or systemic (i.e. urosepsis); this terminology is more often used as uncomplicated and complicated urinary tract infections (cUTI) (Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010; Renald et al. 2015; Rizwan et al. 2018; Simmering et al. 2017; Stefaniuk et al. 2016; Tangdogdu and Wagenlehner 2016; Wiedemann et al. 2014). The emergence, symptomatology and outcome of these infections is highly dependent on the microbial composition of the microbiota of the surrounding anatomical regions (i.e., gut, genitalia), the pathogenic potential of the microorganisms in question, the duration of the infection and other attributes of the host (e.g., hygiene practices, immune status) (Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010; Wiedemann et al. 2014). In healthy individuals, physical and immunological barriers provide protection from urinary infections, and urothelial cells play a pivotal role in producing pro-inflammatory cytokines and other immunological responses (Abraham and Miao 2015; Hayes and Abraham 2017). Many predisposing factors have been described for the development of UTIs, including age, female gender (and corresponding anatomical characteristics), pregnancy, sexual intercourse (or multiple sexual partners), personal hygiene, disturbances in the vaginal microbiota (i.e. absence of vaginal lactobacilli, decreases in estrogen-levels, introduction of a diaphragm), use of spermicidal formulations, nutritional aspects and obesity, Type II diabetes, immunosuppression (caused by disease or pharmacotherapy), non-circumcision in males, introduction of urinary catheters, hospitalization, urinary retention, renal failure, paraplegia or other neurological disorders, developmental abnormalities of the urinary system (vesicoureteral reflux, obstruction), pelvic prolapse, surgeries in the genitourinary tract or genetic predisposition (e.g., blood group and stone formation) (Emiru et al. 2013; Hu et al. 2004; Scholes et al. 2000; Storme et al. 2019). Urinary catheterization (associated with hospitalization) is the main risk factor for nosocomial UTIs and subsequent secondary bacteremia; insertion of urinary catheters may lead to mucosal damage, which disrupts the natural barrier of the urinary tract, allowing for colonization and the aggregation of microbial pathogens in the form of a biofilm (extracellular matrix of polysaccharides and proteins) (Clarke et al. 2019; Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010; Nicolle 2014). This may facilitate the recurrence of UTIs in the host and the biofilm also provides protection for these pathogens against external noxa, such as the lethal effects of antibiotics (Clarke et al. 2019; Trautner and Darouiche 2004; Sabir et al. 2017). The catheter-associated pathogen may enter through the extra-luminal route (moving across the outer lumen of catheter) or the intra-luminal route (by directly entering the interior of catheter) (Clarke et al. 2019; Flores-Mireles et al. 2015; Trautner and Darouiche 2004; Sabir et al. 2017). It must also be noted that these pathogens may spread to the bloodstream from the urinary tract (if the pathogens cross the tubular epithelial barrier in the kidneys) causing secondary bacteremia and sepsis, which may occur in 30% of cases (Clarke et al. 2019; Conway et al. 2015; Flores-Mireles et al. 2015; Trautner and Darouiche 2004; Sabir et al. 2017).

UTIs have been described as an important infectious pathology in patient of both sexes and in all age groups (infants, children, adults and the elderly) (Stefaniuk et al. 2016; Wiedemann et al. 2014). Nevertheless, uncomplicated UTIs are most common between females over 18 years of age, with around two-thirds of women in the ages of 20–40 years experiencing a UTI at least once during their lifetime; in addition, rUTIs in adult females is present in 20–30% of cases, within 3–4 months of the initial infection (Clarke et al. 2019; Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010; Nicolle 2014).

Management of rUTI is of paramount importance as repeat courses of antibiotics to treat these infections often results in bacteria developing resistance to the mechanism of action of previously effective antibiotics (Negus et al. 2020; Wiedemann et al. 2014). UTI occurs in 25% of kidney transplant recipients within 1 year of their transplant, and this constitutes around half of infectious complications (Giessing 2012). UTIs in men occur significantly less frequently than in women, mainly in patients with structural abnormalities in the urinary system and in men with advanced age (with a lifetime prevalence of around 2–7%) (Harper and Fowlis 2007; Schaeffer and Nicolle 2016; Tan and Chlebicki 2016). In developed countries, 3–8% of girls and 0.2–1% of boys under 18 years of age are clinically diagnosed with a UTI (Clarke et al. 2019; Flores-Mireles et al. 2015; Hellerstein 1998; White 2011). As a general rule, the rate of asymptomatic or symptomatic bacteriuria increases in both men and women with advanced age (Clarke et al. 2019; Flores-Mireles et al. 2015; Harper and Fowlis 2007; Schaeffer and Nicolle 2016). In addition to advanced age, immunosuppression and catheterization, the occurrence of hospital-associated UTIs has several non-patient-specific risk factors, including poor hospital infrastructure (insufficient equipment, understaffing, inadequate training or poor knowledge/application of basic procedure, hygienic conditions), overcrowded healthcare-institutions and lack of local and national guidelines (Clarke et al. 2019; Flores-Mireles et al. 2015; Hooton et al. 2010). Asymptomatic bacteriuria (ASB; the presence of high numbers of bacteria without clinical symptoms) is usually not treated with antibiotics in any patient group, except for pregnant women. The definition of ASB varies based on methods of sample collection and the patient population in question (Cormican and Murphy 2011; Henderson et al. 2019; Imade et al. 2010; Nicolle et al. 2005; Wingert et al. 2019). However, in pregnant women (due to their altered immune status), untreated ASB may lead to manifest and usually severe UTIs, pyelonephritis, urosepsis and preterm delivery; therefore treating ASB in this patient population is a must (Cormican and Murphy 2011; Henderson et al. 2019; Imade et al. 2010; Nicolle et al. 2005; Wingert et al. 2019).

2 Diagnosis and Etiological Agents in Urinary Tract Infections

Main clinical signs and symptoms associated with UTIs include the strong and persistent urge to urinate, burning sensations, frequent urination with a small voided volume; in addition, voided urine may be cloudy, red, bright pink, bloody, and foul-smelling in character (Alidjanov et al. 2016; Chu and Lowder 2018; Di Vico et al. 2020; Magyar et al. 2018; Tan and Chlebicki 2016). Based on the severity of the infection, urinary incontinence, pelvic pain, fever, and nausea/vomiting may also occur (Alidjanov et al. 2016; Chu and Lowder 2018; Di Vico et al. 2020; Magyar et al. 2018; Tan and Chlebicki 2016). Urine specimens are one of the most frequently submitted samples for culture to the clinical microbiology laboratory, exceeding the number of most of the other sample types; therefore, the interpretation of culture results from urine samples provide little or no challenge to clinical microbiologists (Flores-Mireles et al. 2015; Gajdács 2020). The most common urine sample type submitted from adults is voided (midstream, clean-catch) urine, which mainly originates from outpatient settings (Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010). Clean-catch urine samples are inexpensive and non-invasive without the risk of complications. Contamination of the sample with bacteria from the normal flora or the distal urethrae is a risk, however, if the patients are instructed appropriately before sample collection and some hygienic considerations are complied with (Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010; Morris 2018). In contrast, collection of urine by the use of a single catheter („straight catheter technique") is a more appropriate method to use to avoid contamination, which is most frequently used in inpatient settings. In fact, one of the main indication for catheter-specimen urine is the monitoring of urinary catheters (Flores-Mireles et al.

2015; Gupta et al. 2011; Grahn et al. 1985; Hooton et al. 2010). However, it is not indicated for most patients, as it is not labour-intensive for non-inpatients, and the insertion of a catheter through the urethra is an invasive method, may also introduce bacteria into the bladder (Flores-Mireles et al. 2015; Hooton et al. 2010). To avoid contamination with bacteria from the distal urethra, subrapubic bladder aspiration (or "bladder tap") is the best method to use; in addition, urine collected through this methods is appropriate to be cultured anaerobically (Gajdács et al. 2019a, b, c, d; Guze and Beeson 1956; Rozenfeld et al. 2018). Nevertheless, subrapubic bladder aspiration is infrequently used (in special clinical circumstances), as it is invasive (leading to discomfort and bleeding), time and resource-dependent method (Guze and Beeson 1956; Ponka and Baddar 2013; Rozenfeld et al. 2018).

Urine samples are usually cultured on either non-selective culture media (mainly blood agar) or selective media for Gram-negative bacteria (eosin methylene blue, MacConkey etc.); however, nowadays, most laboratories use chromogenic media, which allow for the rapid, phenotypic differentiation of most urinary pathogens, which may be further verified by the use of biochemical tests, automated identification systems or other, more advanced identification methods (Chaux et al. 2002; Flores-Mireles et al. 2015; Gupta et al. 2011; Grahn et al. 1985; Hooton et al. 2010). The inoculation of selective media for Gram-positive bacteria is not necessary, especially from outpatient samples; however several reports highlighted enterococci as significant pathogens in nosocomial infections (Gupta et al. 2011; Grahn et al. 1985; Hooton et al. 2010). Introduction of molecular biological methods (e.g., polymerase chain reaction; PCR) and microarray technologies into clinical microbiology have definitely paved the way for more sharper identification, however, these non-cultures-based technologies are not widely used in the diagnosis of UTIs due to their price (Davenport et al. 2017; Zee et al. 2016). On the other hand, the introduction of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has revolutionized bacteriological diagnostics, allowing for rapid, reliable and easy identification for most common urinary pathogens, directly from the cultures of urine specimens; this technology allows for protein-based identification of microorganisms, based on the separation and measurement of smaller to larger fragments of highly conserved ribosomal proteins (which are small and basic in character) by their mass to charge (m/z) ratio (Gajdács et al. 2020a, b; Hou et al. 2019; Schubert and Kostrzewa 2017). In the MALDI-TOF MS measurements, the protein spectrum of the clinical isolate is compared with the protein spectrum of strains in the device-linked database and expressed as a log score (microFlex; Bruker Daltonics) or as a percentage (VITEK MS; bioMérieux), which provides information on the level of match and security of identification (Schubert and Kostrzewa 2017). Although the initial price of the mass spectrometer was a burden to diagnostic institutions, nowadays, more and more laboratories opt into purchasing such a machine.

Most pathogenic yeasts also grow well on agar plates, therefore it is not necessary to use selective media for the fungal culture (except if the pathogenic role of some fungi with fastidious growth requirements is suspected) (Behzadi et al. 2015; Dias 2020; Gajdács et al. 2019a, b, c, d). Cultivation of *Mycobacterium* spp. requires special media and preparation from the laboratory's side, therefore clinicians should always give notice is there is clinical suspicion of a mycobacterial infection of the urinary tract (Kulchavenya and Cherednichenko 2018). As mentioned previously, suprapubic bladder aspiration is the only suitable specimen type for anaerobic processing: these culture are usually limited to patients with anatomical abnormalities (e.g., in case of an enterovesicular fistula) or when sings on infection caused by anaerobes (e.g., due to foul smell) is suspected (Guze and Beeson 1956; Ponka and Baddar 2013; Rozenfeld et al. 2018). In addition to the culture of the samples on microbiological culture media, additional methods may taken into consideration for assessing the presence of clinical infection. The native microscopic analysis and/or Gram-staining of the urine samples

(looking for polymorphonuclear leukocytes with or without bacteria) is usually a good indicator of infections, however, this method is time-consuming and tedious, therefore it is not routinely used (Cantey et al. 2015). In laboratory medicine, the use of nitrite and leukocyte-esterase tests or a hemocytometer is also common in the diagnostics of UTIs (Young and Soper 2001; Alshareef et al. 2020).

Based on literature data, 50–70% of urine cultures are culture-negative, and out of the positive urine cultures, 40–50% of isolated bacteria are relevant urinary pathogens (the rest are contaminants and members of the normal flora) (Cantey et al. 2015; Flores-Mireles et al. 2015; Gupta et al. 2011; Hooton et al. 2010). A wide range of etiological agents have been described in UTIs: the predominant group constitutes the members of the Enterobacterales order (i.e. gut bacteria), noted as the group with having the most pathogenic potential in the urinary tract; however, there is substantial heterogeneity even among the members of this order (Adelou et al. 2016; Calzi et al. 2016; Sobel and Kaye 2015). *Escherichia coli* is the most common causative agent in both community-acquired and nosocomial UTIs (Gajdács et al. 2019a, b, c, d; Rizwan et al. 2018). Pathogenic strains of *E. coli* may be differentiated into distinct pathotypes, including intestinal pathogenic an extraintestinal pathogenic *E. coli* (ExPEC) (Miri et al. 2017). Enteric *E. coli* include seven major pathotypes (responsible for gastroenteritis and blood diarrhea), while among ExPEC strains, so-called uropathogenic *E. coli* (UPEC) are the most common (Bekal et al. 2003); UPEC strains are causative agents in 50–90% of community-acquired and 30–60% of nosocomial UTIs (Terlizzi et al. 2017). Other members of the Enterobacterales order are also represented (although to a lesser extent) in UTIs, namely uropathogenic *Klebsiella pneumoniae* (UPKP; outpatients: 5–10%, inpatients: 7–15%) (Anğ-Küçüker et al. 2002; Gajdács et al. 2019a, b, c, d; Rizwan et al. 2018), members of the Proteae tribe (i.e. *Proteus-Providencia-Morganella*; outpatients: 0.5–6%, inpatients: 2–10%) and the CES-group (i.e. *Citrobacter-Enterobacter-Serratia*; outpatients: 0.2–3%, inpatients: 0.5–5%) of pathogens (Barabás et al. 2015; Gajdács and Urbán 2019a, b; Jacobsen et al. 2008; Metri et al. 2013; Samonis et al. 2009; Stefaniuk et al. 2016; Yang et al. 2018). Generally speaking, the prevalence of so-called non-*E. coli Enterobacterales* (NECE) strains grows in proportion with the age of the patients; in addition, their relevance is much higher in hospitalized patients, they are more frequently isolated in complicated UTIs, pyelonephritis, from catheter-associated infections; they are also more often associated with recurrence and prolonged treatment (Amaretti et al. 2020; Jacobsen et al. 2008; Laupland et al. 2007; Maharjan et al. 2018; Mazzariol et al. 2017).

The potential pathogenic role of non-fermenting Gram-negative bacteria (*Pseudomonas aeruginosa* [outpatients: 1–5%, inpatients: 3–8%], *Acinetobacter* spp. [outpatients: 0.3–1%, inpatients: 0.5–3%] and *Stenotrophomonas maltophilia* [outpatients: 0.05–0.1%, inpatients: 0.05–0.8%]), Gram-positive cocci (*Enterococcus* spp. (including vancomycin-resistant [VRE] strains, *Staphylococcus aureus* (including methicillin-sensitive [MSSA] and methicillin-resistant [MRSA] strains), *S. epidermidis* (including methicillin-sensitive [MSSE] and methicillin-resistant [MRSE] strains), *S. saprophyticus* [also termed „honeymoon cystitis"] and *Streptococcus agalactiae* [or Group B streptococci]) or rods (*Corynebacterium urealyticum, C. pseudogenitalium, C. striatum*) and pathogenic yeasts (0.1–2% in outpatients and 1–7% in inpatients; including uropathogenic *Candida albicans* [UPCA] and non-*albicans Candida* [NACA] species) should also be taken into consideration (Adeghate et al. 2016; Baraboutis et al. 2010; Behzadi et al. 2015; Eriksson et al. 2012; Ferreiro et al. 2017; Dias 2020; Gajdács et al. 2019a, b, c, d; Gajdács 2019; Hegstad et al., 2010; Mittal et al. 2009; Nitzan et al. 2015; Shrestha et al. 2019; Swaminathan and Alangaden 2010; Ulett et al. 2009). The prevalence of Gram-positive cocci in UTIs ranges between 2–15% in outpatients and 5–25% in inpatient samples; the occurrence of *S. saprophyticus* is overwhelmingly seen in young, sexually-active females, while over time, the

epidemiology shift towards enterococci Adeghate et al. 2016; Eriksson et al. 2012; Ferreiro et al. 2017; Hegstad et al. 2010; Nitzan et al. 2015)

Other–although much more rarely occurring (<0.1%)–urinary pathogens include strict anaerobic bacteria (e.g., *Actinotignum schaali, A. urinale, Lactobacillus delbrueckii*), *Aerococcus* spp. (e.g., *A. urinae*), *Mycobacterium* spp., *Mycoplasma hominis, Ureaplasma urealyticum, Chlamydia trachomatis,* and *Trichomonas vaginalis* (Christofolini et al. 2012; Combaz-Söhnchen and Kuhn 2017; Darbro et al. 2009; Higgins and Garg 2017; Kulchavenya and Cherednichenko 2018; Lotte et al. 2016; Masha et al. 2018). The latter group of pathogens have a common characteristic in that they are usually found in specific, narrow patient populations, and their isolation and identification usually entails the use of some kind of specialized media, long incubation times, use of cell cultures or strict anaerobic conditions. For example, *Aerococcus* spp. are predominantly isolated from elderly males with benign prostatic hypertrophy, while *L. delbrueckii* has been reported as a urinary pathogen in elderly women (>70 years of age) (Darbro et al. 2009; Higgins and Garg 2017). Similarly, mycoplasmae and ureaplasmae are more frequently found in postmenopausal women as a causative agent in UTIs (Christofolini et al. 2012; Combaz-Söhnchen and Kuhn 2017; Masha et al. 2018).

The epidemiology and species-distribution of uropathogens varies greatly in a geographical and time-dependent manner and it also strongly correlated with the reported patient population studied (Gajdács et al. 2019a, b, c, d; Köves et al. 2017; Stefaniuk et al. 2016). Nevertheless, many studies highlight the fact that the etiological agents in UTIs have changed considerably, both in nosocomial and community settings, with a shift towards "less common" microorganisms having more pronounced roles. Similarly (as presented previously), in the elderly (and in immunocompromised persons), uncommon urinary pathogens are seen more often (Kumar et al. 2001). The local epidemiological characteristics and resistance trends of UTIs should be regularly surveyed to allow for appropriate choice of therapy (Abbo and Hooton 2014).

3 Virulence Factors of Various Urinary Pathogens in the Era of Molecular Biology and Bioinformatics

3.1 General Concepts

To understand the pathomechanisms of developing a UTI, one first need to establish the presence and relevance of various cell- and non-cell-associated virulence-determinants of individual pathogens. The sciences of molecular biology, immunology and bioinformatics are great supporters for detection, recognition and interpretation of molecular mechanisms belonging to both pathogenic bacteria and the hosts (Behzadi et al. 2016; Behzadi and Behzadi 2016, 2017; Behzadi 2020; Hozzari et al. 2020; Jahandeh et al. 2015). In this regard, this section focuses on microbial molecular treasures of virulence factors (microbial virulome) and the importance of bioinformatics in this regard. The microbial pathogenome and virulome are important factors in determining the severity of UTIs; some of these microbial virulence genes are located on plasmids, while others are integral part of the bacterial chromosomes (Behzadi et al. 2016; Behzadi and Behzadi 2016, 2017; Behzadi 2020; Hozzari et al. 2020; Jahandeh et al. 2015). Generally, adherence is a key step initiating UTI pathogenesis is adherence: initially, a urinary pathogen (most often residing in the gut) colonizes the periurethral region, followed by migration of these microorganisms upstream to the bladder (Flores-Mireles et al. 2015; Terlizzi et al. 2017). These bacteria need to withstand the strong hydrodynamic shear forces and the removal by the flow of urine. For these steps to occur, the presence of molecular appendages, such as flagella and pili are required. Bacterial adhesins bind to receptors (e.g., Type I fimbriae mediate binding to uroplanktins, which are D-mannosylated proteins) on the uroepithelium, mediating colonization and subsequent invasion (Issakhanian and Behzadi 2019; Behzadi et al. 2019). If these pathogens are present in sufficient amounts, they may overcome the host immune

response and ascend to the bladder and the kidneys. Uropathogens produce several tissue-damaging toxins and proteases (IgA protease, elastase, phospholipase, hemolysin, cytotoxins) to obtain nutrients from host cells, and siderophores to acquire iron, necessary for maintaining their biochemical processes (Behzadi et al. 2011; Behzadi et al. 2015; Behzadi and Behzadi 2008). In addition, urease-production (characteristic for *Proteus* spp., *S. saprophyticus, K. pneumoniae* and *P. aeruginosa* among others) is also important for colonization and persistence. Urease-production results in a shift in pH, leading to tissue destruction, scarring and stone formation, through the composition of struvite and apatite crystals via precipitation of Ca^{2+} and Mg^{2+} ions). Several species also produce pigments (pyoverdine, pyocyanine in case of *P. aeruginosa*, prodigiosin in case of *Serratia marcescens*), which may further cause tissue destruction (Behzadi 2018; Behzadi et al. 2011; Behzadi et al. 2015; Behzadi and Behzadi 2008).

3.2 Uropathogenic Escherichia coli (UPEC)

E. coli pathotypes are divided into three groups of Intra-intestinal pathogenic *E.coli* (InPEC), *Shigella* Toxin Producer InPEC (ShiToPInPEC) and Extra-intestinal pathogenic *E. coli* (Behzadi 2018; Jahandeh et al. 2015). The pangenomic studies indicate two genomic pools of flexible (movable genes for the cells' environmental adaption) and core (key genes, e.g., housekeeping genes) for the cells' survival) among commensal and pathogenic strains of *E. coli*. In accordance with pangenomic investigations, the *E. coli* strains encompass a high plasticity in their genomes. Due to this fact, the estimated genomic load of *E. coli* strains is from 4.5 Mb up to 5.5 Mb. In this regard, the lowest level of genomic volume belongs to Intra-intestinal commensal (InCOM) strains of *E. coli* while the highest level of genomic content (5.5 Mb) belongs to drug resistant ExPEC strains including UPEC. The InPEC and ExPEC (e.g., UPEC) drug-sensitive strains bear genomic contents more than 4.5 and lesser than 5.5 Mb. These properties make UPEC a pathogen with a strong virulome (Behzadi and Behzadi 2016, 2017; Behzadi et al. 2016; Behzadi 2018; Behzadi 2020; Hozzari et al. 2020; Jahandeh et al. 2015).

As mentioned, the flexible (or Supplementary, Accessory or Adaptive) genomic pool is consisted of different genes, gene clusters and gene cassettes belonging to plasmids, transposons, retrotransposons, pathogenicity islands (PAIs), integrons and phages. These mobile genes determine the virulencity and pathogenicity of *E.coli* pathogenic strains including UPEC (Behzadi and Behzadi 2017; Brockhurst et al. 2019; Jahandeh et al. 2015; Ranjbar et al. 2017). The presence of a wide range of genes and in particular those which constitute the supplementary genomic pools among *E. coli* strains, gives us a proper opportunity to have a phylogenetic classification with eight phylogroups of *Escherichia* cryptic clade I (*arpA*-, *chuA*-, *yjaA*+, TspE4.C2-)/(*arpA*+, *chuA*+, *yjaA*+, TspE4.C2-), A (*arpA*+, *chuA*-, *yjaA*-, TspE4.C2-)/(*arpA*+, *chuA*-, *yjaA*+, TspE4.C2-), B1 (*arpA*+, *chuA*-, *yjaA*-, TspE4.C2+), B2 (*arpA*-, *chuA*+, *yjaA*+, TspE4.C2-)/(*arpA*-, *chuA*+, *yjaA*-, TspE4.C2+)/(*arpA*-, *chuA*+, *yjaA*+, TspE4.C2+), C (*arpA*+, *chuA*-, *yjaA*+, TspE4.C2-), D (*arpA*+, *chuA*+, *yjaA*-, TspE4.C2-)/(*arpA*+, *chuA*+, *yjaA*-, TspE4.C2+), E (*arpA*+, *chuA*+, *yjaA*-, TspE4.C2-)/(*arpA*+, *chuA*+, *yjaA*-, TspE4.C2+)/(*arpA*+, *chuA*+, *yjaA*+, TspE4.C2-) and F (*arpA*-, *chuA*+, *yjaA*-, TspE4.C2-) (Clermont et al. 2000; Clermont et al. 2013). This phylogenetic categorization is based on PAIs markers (Clermont et al. 2000, 2013; Najafi et al. 2018). The PAIs genes and other virulence genes (e.g., fimbrial and afimbrial adhesins, chaperone-usher (CU) and non-chaperone-usher adhesins, capsule, LPS, flagella, toxins, outer membrane proteins (OMPs) and vesicles (OMVs), metal (mostly iron and zinc) acquisition systems and autotransporter proteins) which are located within the adaptive genomic pool for the most (Behzadi 2020; Bien et al. 2012; Jahandeh et al. 2015; Ko et al. 2019a, b; Subashchandrabose and Mobley 2017; Walters and Mobley 2009). Hence, *in toto* the virulence factors (VFs) of UPEC can be

divided into superficial virulome and secretome st (Behzadi 2020; Bien et al. 2012; Jahandeh et al. 2015; Ko et al. 2019a, b; Subashchandrabose and Mobley 2017). The virulome elements belonging to UPEC surface contribute in attachment, colonization and biofilm formation while the secretome including secreted VFs (mostly toxins), secretion machineries and the related receptors and components are not only involved in colonization and biofilm formation but also they contribute in bacterial invasion, bacterial internalization, the host immunologic responses etc. which directly are associated with bacterial survival (Behzadi 2020; Bien et al. 2012; Jahandeh et al. 2015; Ko et al. 2019a, b; Subashchandrabose and Mobley 2017; Walters and Mobley 2009).

The secretome is consisted of secreted toxins such as Cytotoxic Necrotising Factor-1 (Cnf-1), Autotransporter Toxin (AT/Secreted Autotransporter Toxin (Sat), α-Haemolysin (HlyA) known as a member of Repeats in Toxin (RTX) toxins family, and Cytolethal Distending Toxin (Cdt) (Behzadi 2020; Bien et al. 2012; Jahandeh et al. 2015; Ko et al. 2019a, b; Subashchandrabose and Mobley 2017; Walters and Mobley 2009); metal acquisition systems (such as autotransporter proteins), metal receptors and chelators (e.g. siderophores), OMPs, OMVs (Bien et al. 2012; Jahandeh et al. 2015; Ko et al. 2019a, b; Subashchandrabose and Mobley 2017); and different secretion machineries (Costa et al. 2015; Jahandeh et al. 2015; Sana et al. 2020). The secretion machineries are the main secretome components in both Gram-positive and Gram-negative bacteria. These machineries produce and secrete different types of molecules including DNA and proteins. The secreted molecules have direct effects on environmental factors and contribute in bacterial adaptation, bacterial adhesion and attachment, bacterial pathogenicity and virulencity and bacterial survival. Hence, the secreted molecules may have direct or indirect effects on exterior targeted cells (Costa et al. 2015; Jahandeh et al. 2015; Sana et al. 2020). Until now, we have recognized nine types of secretion systems (TSSs) including T1SS (type I secretion system) up to T9SS (Jahandeh et al. 2015). In accordance with transport procedures, the secretion machineries are categorized into two groups of single procedure or one-step process (just spanning the OM) and double-step procedure or two-step process (spanning the inner and outer membranes simultaneously) (Costa et al. 2015; Jahandeh et al. 2015; Navarro-García et al. 2016; Sana et al. 2020). T1SS, T3SS, T4SS (common between Gram-negative and Gram-positive bacteria) and T6SS are known as one-step and T2SS, T5SS, T7SS (recognized in mycobacteria), T8SS and T9SS are recognized as two-step secretion machineries (Abby et al. 2016; Jahandeh et al. 2015; Sana et al. 2020). In addition to T1SS-T9SS secretion machineries, Curli, CU and are recognized as important components of bacterial secretome (Abby et al. 2016; Hawthorne et al. 2016; Jahandeh et al. 2015; Konovalova and Silhavy 2015; Navarro-García et al. 2016; Sana et al. 2020).

3.3 Uropathogenic Klebsiella pneumoniae (UPKP)

UPKP is usually known as the second ranked Gram-negative bacterial agent of UTIs after UPEC (Behzadi et al. 2010; Terlizzi et al. 2017). Up to 5–10% of community-acquired and nosocomial UTIs are caused by UPKP (Paczosa and Mecsas 2016); while these percentages for UPEC are respectively, 95% and 50% (Behzadi et al. 2010; Terlizzi et al. 2017). Both of UPEC and UPKP belong to *the* Enterobacterales order and may cause community acquired and nosocomial UTIs (Behzadi et al. 2010; Paczosa and Mecsas 2016; Terlizzi et al. 2017). Besides Gram-negative bacteria such as UPEC and UPKP possess complicated cellular structure and therefore the effect of antimicrobial agents on them is tougher than Gram-positive bacteria. This property explains why the treatment procedure of UTIs caused by bacterial agents like UPEC and UPKP is harsher than UTIs caused by Gram-positive bacteria (Issakhanian and Behzadi 2019). *K. pneumoniae* resembling *E. coli* encompasses different types of strains from commensal strains to opportunistic pathogens and pathogens. The most common strains of *K. pneumoniae* belong to opportunistic pathogens

which may lead to classical (or health-care-associated) infections e.g. UTIs (Holt et al. 2015; Li et al. 2014; Paczosa and Mecsas 2016; Wyres et al. 2020). The main reservoirs for commensal strains of *K. pneumoniae* within human body are gastro-intestinal and respiratory tracts (Holt et al. 2015; Li et al. 2014; Paczosa and Mecsas 2016; Wyres et al. 2020). Those strains of *K. pneumoniae* which acquire antimicrobial resistance or hypervirulent (HV) genes are considered as serious problem regarding treatment of infectious diseases (Issakhanian and Behzadi 2019).

The pangenomic investigations confirm the presence of a genome with a size of five to six Mb in *K. pneumoniae*. It is estimated that this genome encodes about five to six thousands genes (Martin and Bachman 2018; Wyres et al. 2020). Among this number of genes, one thousand seven hundred genes belong to the core genomic pool of *K. pneumoniae* while the left belongs to supplementary (flexible/accessory/adaptive) genomic pool. The core genome is common between more than 95% of isolated strains of *K. pneumoniae* (Martin and Bachman 2018; Wyres et al. 2020). The adaptive genome usually includes a wide range of virulence and antimicrobial resistance genes which are gained from via horizontal gene transfer. They are mobile genes and genomic islands (Martin and Bachman 2018). Moreover, the total number of recognized sequences encoding proteins in different species of *Klebsiella* reaches more than hundred thousand proteins (Martin and Bachman 2018; Wyres et al. 2020). In accordance with bioinformatic and pangenomic surveys, *K. pneumoniae* involves a large number of genes with different origins of chromosomal, plasmids and phages. It seems that the diversity of recognized plasmids among *K. pneumoniae* is more than those that detected in ESKAPE members (Gajdács and Albericio 2019; Gajdács et al. 2020a, b; Wyres et al. 2020). In recent years, some bioinformatic databases have been established to determine the type and diversity of genomic elements like plasmid. The PlasmidFinder database is used for plasmid typing within *Enterobacteriaceae* pathogens (Carattoli et al. 2014; Carattoli and Hasman 2020). There is another database which can be used for molecular typing and microbial genome diversity (https://pubmlst.org/).

The VFs of UPKP are comparable with UPEC; because there is a wide range of virulence genes in UPKP and UPEC genomes. In this regard, capsule as a bacterial exopolysaccharide can be considered as an important VF in UPEC and UPKP (Holt et al. 2015; Li et al. 2014; Paczosa and Mecsas 2016; Wyres et al. 2020). Capsule is produced by *cps* gene clusters in classical UPKP (31). The HV strains possess thick capsules with significant content of mucoviscous polysaccharide increase the pathogenicity and virulencity of UPKP. The high content of capsule in HV strains can be supported by enhanced expression of plasmid borne genes of transcriptional regulators (*rmpA* and *rmpA2*). In the lack of *rmpA* and *rmpA2* genes in HV strains the *magA* gene contributes in hypercapsulation process. The *magA* gene is in association with invasion (Holt et al. 2015; Li et al. 2014; Paczosa and Mecsas 2016; Rastegar et al. 2019; Wyres et al. 2020). The lipopolysaccharide (LPS) is another VF which its genes are located on the core genome of *K. pneumoniae*. LPS is consisted of three structural sections including an O-antigen, a core oligosaccharide and the lipid A which are produced by the gene clusters of *wb*, *waa* and *lpx* (Holt et al. 2015; Paczosa and Mecsas 2016; Rastegar et al. 2019; Wyres et al. 2020). Siderophores are important metal-(iron) chelators which have significant role in UPKP infections throughout metal acquisition systems. The CU fimbrial adhesins of type 1 and type 3 fimbriae are produced by *fim* and *mrk* cluster genes, respectively which play significant role in bacterial attachment. Siderophores, type 1 and type 3 fimbriae are common between UPEC and UPKP strains (Holt et al. 2015; Li et al. 2014; Paczosa and Mecsas 2016; Rastegar et al. 2019; Wyres et al. 2020). The mentioned VFs together with efflux pumps and the secretion systems (e.g. T6SS) compose the main virulome of *K. pneumoniae* (UPKP) (Li et al. 2014; Martin and Bachman 2018).

3.4 Uropathogenic Proteus mirabilis (UPPM)

After UPEC and UPKP, Uropathogenic *P. mirabilis* (URPM) is recognized as the third bacterial causative agent of UTIs (Cestari et al. 2013). *P. mirabilis* is a well-known bacterial agent for blocking urine catheter and urolithiasis in urine bladder and kidneys by the help of its nickel metalloenzyme urease (encoded by the *ureDABCEFG* gene cluster/operon). The waste nitrogen within our urine (in the form of urea) is the main substrate for the urease which getting hydrolyzed into carbon dioxide (CO_2) and ammonia (NH_3). The released molecules of NH_3 within our urine may lead to alkalization of the pH of urine (Armbruster et al. 2018; Cestari et al. 2013; Ko et al. 2019a, b; Schaffer and Pearson 2017). The alkalized environment leads to crystallization of soluble anions and cationes and urolithiasis occurs by the mineral crystals of carbonate apatite [(pH $\geq$ 6.8) (calcium-phosphate/$Ca_{10}(PO_4)_6CO_3$)] and struvite [($\geq$7.2) (magnesium ammonium phosphate hexahydrate/$MgNH_4PO_4{\cdot}6H_2O$)]. The long-term UTIs caused by *P. mirabilis* can be lethal (Armbruster et al. 2018; Bichler et al. 2002; Cestari et al. 2013; Ko et al. 2019a, b; Prywer et al. 2012; Schaffer and Pearson 2017). *P. mirabilis* encompasses an abundance of VFs including capsule, LPS, Adhesins, fimbriae, flagellum, enzymes (urease), metal acquisition systems, different secretion systems, etc. which may lead to serious infections in patients with UTIs (Armbruster et al. 2018; Cestari et al. 2013; Ko et al. 2019a, b; Schaffer and Pearson 2017). In accordance with previous reports, up to 10% of UTIs is caused by *P. mirabilis* (Schaffer and Pearson 2017). This motile Gram-negative bacterium which is famous for its bull's-eye swarming pattern on agar, usually contributes in complicated UTIs and catheter-associated UTI (CAUTI) (Pearson et al. 2010; Schaffer and Pearson 2017). Moreover, *P. mirabilis* is a serious problem in elderly patients with CAUTI; because it can be the murderer of up to 50% of the old patients with long-term CAUTI (Schaffer and Pearson 2017).

Genomic investigations regarding *P. mirabilis* indicate a high diversity in genomic pool of this microorganism. Despite this significant diversity, a considerable part of chromosomal genome is conserved among different strains of *P. mirabilis* (Armbruster et al. 2018). All in all, *P. mirabilis* has a mosaic pangenome resembling *E. coli* which is obtained throughout horizontal gene transfer. The genomic island of integrative and conjugative element (ICE) which is known as ICEPm1 in *P. mirabilis* acts as a chromosomal transposon (Armbruster et al. 2018; Flannery et al. 2011). The ICE is common among bacterial microorganisms of *P. mirabilis*, *Morganella morganii* and *Providencia stuartii* and this PAI contains two virulence genes of *nrp* (encoding non-ribosomal peptide siderophore) and *pta* (*Proteus* Toxic Agglutinin) operons (Armbruster et al. 2018; Barker 2013; Flannery et al. 2011). The bioinformatic, molecular biological and genomic surveys have revealed a wide range of VFs in *P. mirabilis* including secretion systems (e.g. T1SS, T3SS, T4SS, T5SS and T6SS), toxins (such as hemolysin (HpmA-HpB), Pta, ZapA metalloprotease), fimbriae/fimbrial adhesins (like Mannose-resistant *Proteus*-like fimbriae (MR/P), "non-agglutinating fimbriae" (NAF/UCA), *P. mirabilis* fimbriae (PMF), Ambient temperature fimbriae (ATF), *P.mirabilis* P-like fimbriae (PMP), Fimbria 14), afimbrial adhesins (e.g. Uroepithelial cell adhesin (UCA/NAF)), metal acquisition systems (for iron, zinc, nickel and phosphate) and flagella (Armbruster et al. 2018; Barker 2013; Flannery et al. 2011).

In toto, uropathogenic bacteria and in particular the UPEC, UPKP and UPPM are "live treasures" of VFs which have their own properties and characteristics. Each VF has its own structure and molecular mechanism which can activate different molecules of the host's immune systems. Any defect within the host's immune system may lead to leak of uropathogenic bacteria into the host's urinary tract which results in different types of UTIs from mild and asymptomatic to severe infections. Due to this fact, the bioinformatics helps us to have conscious guess regarding the immunological, molecular biological

characteristics of bacterial virulence and pathogenicity.

4 Therapeutic Aspects of Urinary Tract Infections

In the following section, the therapeutic aspects of UTIs are summarized, based on the most recent international guidelines available in the published literature in English.

4.1 Indication of Antibiotic Therapy

Urinary tract infections (UTIs) have mainly bacterial etiologies, hence the crucial role of antibacterials in the treatment of UTIs is unquestionable. Even in the case of lower urinary tract infection (cystitis), antibacterial use is recommended as clinical cure is significantly higher compared to placebo (Bonkat et al. 2019). Despite this, some national guidelines recommend the watchful waiting approach for 48 h in case of acute cystitis in women if pregnancy is not present (NICE 2018), or the urinanalysis is negative (Kranz et al. 2018a, b) to allow spontaneous recovery of symptoms.

UTIs can be classified in many ways. Based on various patient characteristics, acute cystitis can be grouped into uncomplicated form and complicated form (Bonkat et al. 2019; Chapple and Mangera 2018). In the later case, the eradication of the pathogen is more difficult (Bonkat et al. 2019) and response rate to short-course antibiotic therapy is worse (Chapple and Mangera 2018).

4.2 Treatment of Acute Uncomplicated Cystitis (AUC)

Acute uncomplicated cystitis (sporadic or recurrent) pertains to pre-menopausal, non-pregnant women without relevant co-morbidities or anatomical/functional urinary tract abnormalities (Bonkat et al. 2019). The recommended empiric antibacterial agents in different guidelines are summarized in Table 1.

Most national guidelines recommend one dose (3 grams) fosfomycin as a first line treatment in AUC (Bonkat et al. 2019; Chapple and Mangera 2018; Gilbert et al. 2019; Kranz et al. 2018a, b; Melia 2017). Some American guideline recommends to reserve fosfomycin for suspected multidrug resistant (MDR) infections or when other first-line agents cannot be used (Hooton and Gupta 2019a, b). Nitrofurantoin is recommended as first line treatment of AUC in all of the identified guidelines (Table 1), with a dose varying from 150 mg to 400 mg daily, for 3–5 days (Bonkat et al. 2019; Chapple and Mangera 2018; Gilbert et al. 2019; Hooton and Gupta 2019a, b; Kranz et al. 2018a, b; Melia 2017; Melia and DeMaio 2017; Network SIG 2012; Wuorela 2018).

Trimethoprim and its combination with a sulphonamide derivative: trimethoprim-sulfamethoxazole (TMP/SMX) are often recommended in the treatment guidelines with the proviso that it should be used only if local resistance level among *E.coli* or *Enterobacteriaceae* is below 20% (Bonkat et al. 2019; Chapple and Mangera 2018; Gilbert et al. 2019; Kranz et al. 2018a, b; Melia 2017). Except pivmecillinam which is an extended spectrum penicillin (WHO 2019), all other beta-lactam agents are considered as second line treatment (see Table 1), with a longer course (usually 5–7 days).

The role of fluoroquinolones in the treatment of AUC is limited to special cases where other agents cannot be used due to adverse drug reactions (e.g. allergy, intolerance) (Melia 2017). Certain guidelines explicitly advise against the use of fluoroquinolones in AUC (Bonkat et al. 2019; Network SIG 2012; Wuorela 2018). This is in line with recent resolution of the both the American and European Medicine Authorities which recommend against the use of fluoroquinolones in any non-complicated infections (including AUC) as associated collateral damage and risk of severe permanent adverse effect clearly outweighs the potential benefits (Hooper 2019).

The evidence supporting the add-on effect of analgesics for symptomatic relief in

Table 1 The recommended empiric antibacterial agents for acute uncomplicated cystitis (women)

	Finnish-2017 (10)	Scottish – 2012 (11)	German-2017 (4)	UK-2018 (2)	BMJ Best Practise-UK 2018 (5)	John Hopkins-2017 (7)	EUA guideline-2019 – (1)	Sanford guide-2019 (6)	UpToDate 2018 (8)
Fosfomycin			First line	Second line	First line	First line	First line	First line	Second line
Nitrofurantoin	First line	First line	First line	First line and second line	First line	First line	First line	First line	First line
Nitroxolin			First line						
Trimethoprim[a]	First line	First line		First line	First line				First line
SMX-TMP			Second line		First line	First line	Second line	First line	First line
Pivmecillinam	First line		First line	Second line	First line	First line	First line	Second line	First line
Co-amoxiclav		Avoid				Second line	Avoid	Second line	Second line
Cephalexin		Avoid			Second line			Second line	Second line
Cefdinir		Avoid						Second line	Second line
Cefpodoxime-axetil		Avoid	Second line		Second line	Second line			Second line
Cefixime		Avoid				Second line			
Cefadroxil		Avoid							Second line
Ofloxacin	Second line	Avoid	Second line			Second line	Avoid		
Norfloxacin		Avoid	Second line		Third line	Second line	Avoid		
Ciprofloxacin	Second line	Avoid	Second line		Third line	Second line	Avoid	Second line	Third line
Levofloxacin	Second line	Avoid	Second line		Third line	Second line	Avoid	Second line	Third line

Bonkat et al. (2019), Chapple and Mangera (2018), ECDC (2018), Frassetto (2018), Gagyor et al. (2012), Gagyor et al. (2015), Gilbert et al. (2019) Hooton and Gupta (2019a, b), Hooper (2019), Kranz et al. (2018a, b), Kroneberg et al. (2017), Melia (2017), Melia and DeMaio (2017), NICE (2018), Network SIG (2012), WHO (2019), Wuorela (2018)
SMX-TMP sulphamethoxazole/trimethoprim
[a]Use in empiric therapy only if local resistance in *E. coli* is lower than 20% and if not used to treat UTI in past 3 months

uncomplicated UTIs is lacking. However some studies revealed that ibuprofen can reduce the rate of antibiotic prescribing (Gagyor et al. 2012, 2015). Consequently, some guidelines recommend the use of paracetamol (Bonkat et al. 2019; Chapple and Mangera 2018; Gilbert et al. 2019; Hooton and Gupta 2019a, b; Kranz et al. 2018a, b; Melia 2017; Melia and DeMaio 2017; Network SIG 2012; Wuorela 2018) or ibuprofen (Gagyor et al. 2012, 2015) or the urinary analgesic phenazopyridine (Bonkat et al. 2019; Chapple and Mangera 2018; Hooton and Gupta 2019a, b; Kranz et al. 2018a, b; Melia 2017) to relieve the discomfort (dysuria). Symptomatic treatment is primarily important in cases where patient refuse to take antibiotics or when the watchful-waiting approach for 48 h can be considered.

4.3 Treatment of Acute Cystitis in Men

The acute cystitis in men is often accompanied by prostate involvement. The choice of antibacterial is repetitive doses of fosfomycin (3 grams on consequent 2–3 days) or TMP/SMX, which should be revised based on microbiological results (Gilbert et al. 2019; Hooton 2018; Kroneberg et al. 2017). Nitrofurantoin and pivmecillinam should be avoided in the cystitis of men, due to the limited prostate penetration (Gilbert et al. 2019; Hooton 2018; Kroneberg et al. 2017).

4.4 Treatment of Acute Pyelonephritis (AP) and Complicated Urinary Tract Infections (cUTIs)

The definitions of upper urinary tract infections (acute pyelonephritis-AP) and complicated urinary tract infection (cUTIs) are smeared. Most guidelines differentiate between acute uncomplicated pyelonephritis where causative organisms are identical with AUC and complicated urinary tract infections- cUTIs (including pyelonephritis) with broad range of possible pathogen bacteria (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b). An other approach is to define all UTI cases that extend beyond the bladder as complicated infection, and do not automatically consider urological abnormalities, immuncompromising conditions, as complicating factors (Hooton and Gupta 2019a, b). The listed complicating factors (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b) also lack consensus and generally cover a wide variety of conditions. The heterogeneity of the patient population considered to have cUTIs preclude general approach to the initial empiric antibacterial therapy. The diversity in antibiotic resistance also makes the up-to-date knowledge of local resistance patterns critical.

The need of urine culture and sensitivity analysis concurs in different guidelines to tailor initial empiric therapy in cUTIs (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b). Duration of treatment can also range widely, but generally 7–14 days are needed to achieve clinical cure. Empiric antibiotic choice is typically based on individual assessment of disease severity, local bacterial susceptibilities, personal risk factors for antibiotic resistant bacteria, drug contraindications (e.g. allergies), and may include a wide range of antibacterial agents based on local availability of active agents (see Table 2).

Those patient that have mild-moderate symptoms, (hemodinamically) stable, laboratory parameters are essentially normal and lack any risk factors which predispose them to deteriorating can be treated as outpatients (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b). The recommended empirical agents typically include oral fluoroquinolones (if local resistance is below 10% and no personal risk for resistant bacteria), oral cephalosporins (mostly third generation agents) and in few guidelines with some restrictions: TMP/SMX (see Table 2). In many guidelines oral treatment should be preceded by one dose of ceftriaxone or aminoglycoside and in some guides ertapenem.

Patient who cannot take oral medication, volume depleted, has severe signs as early septic haemodynamic parameters or having relevant complicating factors should be admitted and

Table 2 The recommended empiric antibacterial agents for acute pyelonephritis (AP) and complicated urinary tract infections (cUTI)

	German	UK – 2018	USA BMJ-Best-2018	US – John Hopkins-2017	EUA-2019	Sanford guide-2019	UpToDate-2019
Levofloxacin	Uncomplicated pyelonephritis: First line iv/oral		Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease (if R < 10%): Second line oral	Complicated UTI, mild/ moderate ill: First line: Oral/iv	Uncomplicated pyelonephritis: First line oral/iv, only if R < 10% cUTI: Only if R < 10%, not severe infection and beta-lactam anaphylaxia	Pyelonephritis in women/men, with low risk for resistant bacteria: First line cUTI with low MDR GNB risk: First line	Outpatient, if personal risk of R isolate is low (if local R is above 10%, administer 1 dose of ceftriaxone, ertapenem or aminoglycoside first): First line Outpatient, high personal MDR risk AND no prior FQ use/resistance within 3 months Use only after one dose of ertapenem: First line Hospitalized, oral/iv, No risk for MDR GNB (if no FQ resistant isolate in prior 3 months and local *E. coli* R is below 10%): First line
Ciprofloxacin	Uncomplicated pyelonephritis: First line iv/oral	Acute pyelonephritis women/men: First line oral/iv based on severity	Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease (if R < 10%): First line oral AND Acute pyelonephritis, severe symptoms/ complicated disease: first line, iv	Complicated UTI, mild/ moderate ill: First line: Oral/iv	Uncomplicated pyelonephritis: First line oral/iv, only if R < 10% cUTI: Only if R < 10%, not severe infection and beta-lactam anaphylaxia	Pyelonephritis in women/men, with low risk for resistant bacteria: First line	Outpatient, if personal risk of R isolate is low (if local R is above 10%, administer 1 dose of ceftriaxone, ertapenem or aminoglycoside first): First line, Outpatient, if high personal MDR risk AND no prior FQ use/resistance within 3 months Use only after one dose

							of ertapenem: First line Hospitalized, oral/iv, No risk for MDR GNB (if no FQ resistant isolate in prior 3 months and local *E. coli* R is below 10%): First line
Ofloxacin			Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease (if R < 10%): First line oral Acute pyelonephritis, severe symptoms/ complicated disease: First line, iv				
Amikacin	Uncomplicated pyelonephritis: Severe infection: Second line	Acute pyelonephritis women/men, severe infection: First line i			Uncomplicated pyelonephritis: Second line, iv cUTI: First line, **with** amoxicillin/2nd gen cephalosporin		
Gentamycin	Uncomplicated pyelonephritis: Severe infection: Second line	Acute pyelonephritis women/men, severe infection: First line i	Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease: 1 initial dose before oral empiric treatment OR Acute pyelonephritis, severe symptoms/ complicated disease: First line (+/− ampicillin)		Uncomplicated pyelonephritis: Second line cUTI: First line, **with** amoxicillin/2nd gen cephalosporin	Pyelonephritis in women with low risk for resistant bacteria: Second line cUTI with low risk of MDR GNB: First line	Only as initial one dose before oral treatment (see above)

(continued)

Table 2 (continued)

	German	UK – 2018	USA BMJ-Best-2018	US – John Hopkins-2017	EUA-2019	Sanford guide-2019	UpToDate-2019
Plazomycin							Hospitalized: Critical illness and/or urinary tract obstruction AND selected cases of highly resistant infections (Outpatient, high MDR risk: Second line as initial one dose)
SMX/TMP			Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease: Second line oral (as many *E.coli* are resistant)		Uncomplicated pyelonephritis: First line oral, after a single dose parenteral ceftriaxone/ aminoglycoside cUTI: No indication		Outpatient, orally, if FQ is contraindicated AND low personal risk of MDR But only after 1 initial dose of ceftriaxone/ ertapenem/ aminoglycoside
Co-amoxiclav	Uncomplicated pyelonephritis: Severe infection: Second line,	Acute pyelonephritis women/men: First line, based on severity: Oral/iv, (only if sensitive on antibiogram)					Outpatient, orally, if FQ is contraindicated AND low risk of MDR But only after 1 initial dose of ceftriaxone/ ertapenem/ aminoglycoside
Ampicillin-sulbactam			Acute pyelonephritis, severe symptoms/ complicated disease: First line				

Piperacillin-tazobactam	Uncomplicated pyelonephritis: Severe infection: Second line		Acute pyelonephritis, severe symptoms/ complicated disease: Second line		Uncomplicated pyelonephritis: Second line	Acute pyelonephritis, severe: Second line only in pregnant women cUTI with low risk of MDR GNB: First line	Hospitalized, no risk for MDR GNB: First line
Cefalexin		Acute pyelonephritis women, men: Mild: First line					
Cefadroxil							Outpatient, mild if FQ is contraindicated AND low risk of MDR But only after 1 initial dose of ceftriaxone/ ertapenem/ aminoglycoside
Cefuroxim		Acute pyelonephritis women/men, severe: First line, iv			cUTI: First line in combination with aminoglycosides		
Cefixim			Acute pyelonephritis, mild/moderate symptoms & uncomplicated disease: First line as oral				
Ceftriaxone	Uncomplicated pyelonephritis: Severe infection: First line	Acute pyelonephritis women/men, severe: First line i	Acute pyelonephritis, mild/moderate: One initial dose before oral empiric treatment Acute pyelonephritis, severe symptoms or complicated disease: First line		Uncomplicated pyelonephritis: First line cUTI: First line	Pyelonephritis in women with low risk for resistant bacteria: First line cUTI with low risk of MDR GNB: First line	Hospitalized, no risk for MDR GNB: First line Outpatient: One initial dose before oral empiric treatment (see above)

(continued)

Table 2 (continued)

	German	UK – 2018	USA BMJ-Best-2018	US – John Hopkins-2017	EUA-2019	Sanford guide-2019	UpToDate-2019
Cefotaxime	Uncomplicated pyelonephritis: Severe infection: First line				Uncomplicated pyelonephritis: First line cUTI: First line		
Cefpodoxim OR cefdinir -3GEN	Uncomplicated pyelonephritis, mild-moderate: First line oral				Uncomplicated pyelonephritis: First line, after a single dose parenteral ceftriaxone/ aminoglycoside		Outpatient, mild if FQ is contraindicated AND low risk of MDR But only after 1 initial dose of ceftriaxone/ ertapenem/ aminoglycoside
Ceftibuten	Uncomplicated pyelonephritis, mild-moderate: First line oral				Uncomplicated pyelonephritis: First line, after a single dose parenteral ceftriaxone/ aminoglycoside		
Ceftazidime	Uncomplicated pyelonephritis: Severe infection: Second line			Complicated UTI, severe/ LTCF/prior FQ	cUTI: First line		
Ceftazidime/ avibactam	Uncomplicated pyelonephritis: Severe infection: Second line		Acute pyelonephritis, severe symptoms/ complicated disease: Second line	Complicated UTI, severe/ LTCF/prior FQ	Uncomplicated pyelonephritis: Second line	cUTI with high risk MDR GNB: First line	Critical illness/urinary tract obstruction OR hospitalized & MDR GNB risk AND selected cases of highly resistant infections

Ceftolozane/ tazobactam	Uncomplicated pyelonephritis: Severe infection: Second line			Complicated UTI, severe/ LTCF/prior FQ	Uncomplicated pyelonephritis: Second line	cUTI with high risk MDR GNB: First line	Critical illness/urinary tract obstruction OR hospitalized & MDR GNB risk AND selected cases of highly resistant infections
Cefepime	Uncomplicated pyelonephritis: Severe infection: Second line			Complicated UTI, severe/ LTCF/prior FQ	Uncomplicated pyelonephritis: Second line	Pyelonephritis in pregnant women: First line cUTI with low risk of MDR GNB: First line	
Ertapenem	Uncomplicated pyelonephritis: Severe infection: Second line					Pyelonephritis in women: First line if high MDR risk, second line if low MDR risk Pyelonephritis in men, high MDR GNB risk: First line	Outpatient, high risk of MDR: 1 dose followed by FQ (if no contraindication or no prior use/resistance) or continue as OPAT
Imipenem	Uncomplicated pyelonephritis: Severe infection: Second line		Acute pyelonephritis, severe symptoms/ complicated disease: Second line	Complicated UTI, severe/ LTCF/prior FQ	Uncomplicated pyelonephritis: Third line (only if early culture result indicate MDR)		Critical illness and/or urinary tract obstruction (+vancomycin) OR Hospitalized and risk for MDR GNB
Meropenem	Uncomplicated pyelonephritis: Severe infection: Second line			Complicated UTI, severe/ LTCF/prior FQ	Uncomplicated pyelonephritis: Third line (only if early culture result indicate MDR)	Acute pyelonephritis in women/men AND high risk for MDR: First line cUTI with high MDR GNB risk: First line	Critical illness and/or urinary tract obstruction (+vancomycin) OR Hospitalized and risk for MDR GNB

(continued)

Table 2 (continued)

	German	UK – 2018	USA BMJ-Best-2018	US – John Hopkins-2017	EUA-2019	Sanford guide-2019	UpToDate-2019
Doripenem				Complicated UTI, severe/ LTCF/prior FQ			Critical illness and/or urinary tract obstruction (+vancomycin) OR Hospitalized and risk for MDR GNB
Meropenem-avorbactam						cUTI with high risk MDR GNB: First line	Critical illness/urinary tract obstruction OR hospitalized & MDR GNB risk AND selected cases of highly resistant infections
Aztreonam						Pyelonephritis in pregnant women, moderately ill: Second line (if penicillin allergic) cUTI with low risk of MDR GNB: Second line (if penicillin allergic)	

Bonkat et al. (2019), Chapple and Mangera (2018), ECDC (2018), Frassetto (2018), Gagyor et al. (2012), Gagyor et al. (2015), Gilbert et al. (2019), Hooton and Gupta (2019a, b), Hooper (2019), Kranz et al. (2018a, b), Kroneberg et al. (2017), Melia (2017), Melia and DeMaio (2017), NICE (2018), Network SIG (2012), WHO (2019), Wuorela (2018)

treated with parenteral regimen (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b). Possible regimens include extended-spectrum cephalosporins, aminoglycosides with or without ampicillin (if enterococcus is being considered), fluoroquinolones, aminopenicillins with beta-lactamase inhibitors, antipseudomonal penicillins, and in special cases carbapenems. With clinical improvement, the patient can be switched to an oral antimicrobial to which the organism is susceptive to complete the course of therapy (Bonkat et al. 2019; Frassetto 2018; Hooton and Gupta 2019a, b).

The recommended therapeutic agents of AUC: fosfomycin, nitrofurantoin, pivmecillinam cannot be used in infections involving the kidneys, due to the low tissue penetration (Bonkat et al. 2019; Hooton and Gupta 2019a, b).

Despite the broad spectrum of antimicrobial activity against most uropathogen and achievement of high drug levels in the urinary tract, fluoroquinolones losted importance due to high resistance level (ECDC 2018) and can be recommended as first choice agent only in uncomplicated acute pyelonephritis (see Table 2). According to the guideline of the European Association of Urology fluoroquinolones can only be used in cUTIs if local resistance levels are below 10%, the patient is not severely ill and initial therapy cannot be started with a beta-lactam due to anaphylactic reaction (Bonkat et al. 2019). Other guidelines also make restrictions on the use of fluoroquinolones in cUTIs and even in uncomplicated pyelonephritis (see Table 2).

Aminoglycosides have a reinassance in treating UTIs: they can be used as an initial one dose before switching to oral regimen in mild/moderate cases, and is used widely in severe infections, usually in combination with amoxicillin or second generation cephalosporins.

The parenteral form of second generation cephalosporins has indication in AP (NICE 2018) or cUTIs in combination with aminoglycosides (Bonkat et al. 2019). Third generation parenteral cephalosporins, most often ceftriaxon, are the gold standard in the treatment of cUTIs and AP. Ceftriaxone is also recommended as an initial first dose before starting oral treatment in mild/moderate AP. Carbapenems have limited indications in UTIs: in uncomplicated AP they are used only if early results indicate MDR bacteria (so basically used only as targeted therapy), while in other guides they are used in severely ill patients or who are hospitalized and have a high risk of infections due to MDR gram negative bacteria (GNB) (i.e. extended spectrum β-lactamase-producing Enterobacterales), for example nursing home residents, or who had prior antibiotic exposure, etc. Aminopenicillins with beta-lactamase inhibitors have indications in few guidelines, while the antipseudomonal piperacilin-tazobactam is widely recommended, usually as second line agent in severe infection or in complicated disease, when there is low risk for MDR GNB.

4.5 Assessing the Quality of Antibiotic Use

The utilisation of antibiotics are often appraised by the so-called quality indicators (QIs). QIs are defined as a specific and measurable elements of practice performance for which there is evidence or consensus that can be used to assess and hence, change the quality of care (Campbell et al. 2003; Donabedian 1998). Most often, QIs are categorised as structure (reflecting the organizational issues), process (reflecting diagnostic and treatment related decisions) or outcome indicators (focusing on the consequences) (Donabedian 1998).

Recently two systematic reviews followed by an international, multidisciplinary consensus procedure have been published on quality indicators of antibiotic use: one on ambulatory care and one on hospital care (Le Marechal et al. 2018; Monnier et al. 2018). These internationally developed quality indicators are purposely generic (i.e. not specific to certain infections), can be applied worldwide by different stakeholders and provide a comprehensive evaluation of antibiotic use. The existence and elements of antibiotic stewardship programme, regular audits,

availability of antibiotic treatment guidelines, essential antibiotic list/antibiotic formulary and the continuous availability of essential antibacterial agents are examples of structure QIs that can be generally applied (Le Marechal et al. 2018; Monnier et al. 2018; Pollack et al. 2016). Nosocomial *C. difficile* infection rate is typical example for outcome indicator, but due to its limitations (potentially influenced by many concurrent factors) it provide only indirect evidence of the provided care (Monnier et al. 2018). The prescribing of antibiotics for acute bacterial infections, the compliance with different elements of treatment guidelines, rare prescription of certain antibiotics and acknowledgement of contraindications are some of those generic process QI that can be applied for urinary tract infections (Le Marechal et al. 2018; Monnier et al. 2018; Pollack et al. 2016).

Process indicators enable a direct evaluation of the provided treatment hence their use is widespread. In Table 3 we summarized those quality indicators that pertain specifically to urinary tract infections. No validated indicator exist for the diagnostic process of urinary tract infection, except the need for urine culture in complicated UTIs (Pollack et al. 2016). As urinary tract infections –with few exceptions- have bacterial aetiology, the use of antibiotics is acceptable in every case (quality indicator 1.). The European quality indicator and those from Norway and Sweden target to use the first line agents in minimum 80% or 90% of women with afebrile urinary tract infection (quality indicator 3 and 4). Moreover, the avoidance of fluoroquinolones in the ambulatory care treatment of UTI is evident, the most permissive target is that maximum 10% of women below the age of 80 years should be treated with fluoroquinolones (quality indicator 5,6,7) (Hermanides et al. 2008; Adriaenssens et al. 2011; Pollack et al. 2016). Due to the higher rate of trimethoprim resistance in the elderly population, in UK, they try to reduce its prescribing in the elderly (quality indicator 8, 9) (D'Atri et al. 2019; Norwegian Ministries 2015).

It is important to note that quality indicators cannot provide a definitive judgement of quality, but they generate reflection, debate and allow benchmarking across different practices and such comparison has been proven to be an important stimulus for quality improvement (D'Atri et al. 2019; Drivsholm 2014; Norwegian Ministries 2015). In summary, the assessment of the quality of antibiotic use in frequent infections like UTIs is essential to evaluate the impact of antibiotic stewardship activities and to help health care providers and policy makers to set priorities for interventions to rationalise antibiotic use (Le Marechal et al. 2018; Saust et al. 2016).

4.6 Treatment of Urinary Tract Infections in the Context of the Emerging Multidrug Resistance

Although uncomplicated UTIs are often a self-resolving infections (with cure rates of 15–45%), almost all UTIs are treated with the administration of antibiotics (both as self-medication or doctor-prescribed) (Bonkat et al. 2019). In many primary care/outpatient settings, most patients experiencing UTIs are treated empirically, without establishing the exact etiological agents or their antibiotic susceptibilities (Bischoff et al. 2018). Current therapeutic recommendations emphasize the role of nitrofurantoin, fosfomycin, pivmecillinam and trimethoprim-sulfamethoxazole (TMP/SMX) as first-line treatments in (uncomplicated) UTIs as safe and effective therapeutic alternatives; in general, β-lactam antibiotics [extended-spectrum cephalosporins and carbapenems], aminoglycosides and fluoroquinolones should only be considered in complicated UTIs (e.g., pyelonephritis), in inpatients and in patients with an unmodifiable drug interaction, intolerance, hypersensitivity (-β-lactam-allergy, prolonged QT interval or other risk factors for torsades de pointes) to the abovementioned agents (see previous subsections). The chosen antimicrobial drug should achieve adequate concentrations in the urine or in the respective anatomical region and should be effective in shorter courses. Additionally, the adverse events associated with inappropriate antibiotic use, the overuse of fluoroquinolones and the concept of

Table 3 Use of quality indicators related to urinary tract infections

No	Quality indicator	Acceptable range (%)	QI type
1	Percentage of female patients older than 18 years with cystitis/other urinary infection (ICPC-2-R: U71) prescribed antibacterials for systemic use (ATC: J01) (European indicator)	80–100	Decision to prescribe
2	Female patients older than 18 years with cystitis/other urinary infection receiving the recommended antibacterials (ATC: J01XE or J01EA or J01XX) (European indicator)	80–100	Decision on antibiotic choice: (preferring first line agents)
3	More than 80% of women and more than 50% of men with afebrile urinary tract infection should receive first-line treatment. (Sweden, ambulatory care)	80–100 (female) 50–100 (male)	Decision on antibiotic choice: (preferring first line agents)
4	More than 90% of patients with afebrile urinary tract infection should receive first-line treatment. (Sweden, hospital care)	90–100	Decision on antibiotic choice: (preferring first line agents)
5	Female patients older than 18 years with cystitis/other urinary infection receiving quinolones (ATC: J01M) (European indicator)	0–5	Decision on antibiotic choice (avoidance of certain agents)
6	To reduce the prescription rate of fluoroquinolones (and, in particular, ciprofloxacin) for treating uncomplicated urinary tract infections in women aged 20–79 years to less than 8% of all antibiotics prescribed for urinary tract infections in the same patient group. (Norway, ambulatory care)	0–8	Decision on antibiotic choice (avoidance of certain agents)
7	Maximum of 10% of all antibiotics used to treat urinary tract infections in women aged 18–79 years should be fluoroquinolones (Sweden, ambulatory care)	0–10	Decision on antibiotic choice (avoidance of certain agents)
8	Reduction of inappropriate antibiotic prescribing for urinary tract infections minimum 10% reduction in the trimethoprim/nitrofurantoin prescribing ratio prescribed to patients aged $\geq$70 years due to the higher rates of trimethoprim non-susceptibility in this age group (UK, ambulatory care)	At least a 10% reduction within 2 years	Decision on antibiotic choice (avoidance of certain agents)
9	Reduction of inappropriate antibiotic prescribing for urinary tract infections at least a 10% reduction in trimethoprim items prescribed to patients aged $\geq$70 years due to the higher rates of trimethoprim non-susceptibility in this age group (UK, ambulatory care)	At least a 10% reduction within 2 years	Decision on antibiotic choice (avoidance of certain agents)

Adriaenssens et al. (2011), Campbell et al. (2003), Donabedian (1998), D'Atri et al. (2019), Hermanides et al. (2008), Le Marechal et al. (2018), Monnier et al. (2018), Norwegian Ministries (2015), Pollack et al. (2016)
J01 Systemic antibacterials, J01EA Trimethoprim and derivatives, J01XX Other antibacterials (e.g. fosfomycin, methenamine), J01XE nitrofuran derivatives (e.g. nitrofurantoin), J01M Quinolones, ATC Anatomical, therapeutic and chemical classification, ICPC-2-R International Classification of Primary Care, Second edition

„collateral damage" (affecting the gastro-intestinal and vaginal flora) has taken center stage in the recent years, when it comes to the therapy of UTIs (Gajdács et al. 2019a; Looft and Allen 2012; Tanne 2008; Weber 2006). These empirical regimens (based on Infectious Diseases Society of America [IDSA] guidelines) should be guided by local susceptibility trends, e.g., TMP/SMX is recommended if resistance rates are lower, than 20%, while this rate is <10% for fluoroquinolones (see previous subsections). Nevertheless, if susceptibility data is available, pharmacotherapy should be tailored to these results (Bischoff et al. 2018). The treatment of UTIs is an increasingly complex challenge for clinicians, due to the plethora of intrinsic and acquired resistance mechanisms they possess; these mechanism should all be taken into consideration when selecting antibiotic therapy

(Doi et al. 2017; Pallett and Hand 2010; Rodríguez-Baño et al. 2018). The mechanism of antibiotic resistance may include porin loss and mutations affecting outer membrane permeability (β-lactam antibiotics), alterations in target sites (aminoglycosides, fluoroquinolones, tetracyclines), energy-dependent efflux pumps (a wide variety of antibiotics), in addition to the production of drug-inactivating enzymes (e.g., AmpC-β-lactamases, carbapenemases, aminoglycoside-inactivating enzymes) (Sanjait and Indrawattana 2016). In some cases, these resistance mechanisms affect the susceptibility of individual antibiotics differently (even in the same group); this is the reason why some isolates may be resistant to meropenem, but not imipenem, or resistant to amikacin, but not tobramycin (this is especially common in non-fermenters) (Ko et al. 2019a, b). CES bacteria are all intrinsically resistant to penicillins, several β-lactam/β-lactamase combinations (e.g., ampicillin/sulbactam, amoxicillin/clavulanic acid), first–second generation cephalosporins, and cephamycins (i.e., cefoxitin), due to their penicillinases and AmpC-β-lactamases (Gajdács et al. 2019a, b, c, d). Additionally, nitrofurantoin, doxycycline, colistin and most of the aminoglycosides (with the exception of streptomycin and amikacin) are also ineffective against *Serratia* spp. (Gupta et al. 2014). Members of the Proteae tribe have similar intrinsic resistance mechanism (nitrofurantoin, tetracyclines, and colistin are ineffective), they produce various β-lactamases (penicillinases, AmpC-β-lactamases) and they also have an intrinsic reduced susceptibility to imipenem (Barnaud et al. 1997; Gajdács et al. 2019a, b, c, d). In fact, due to their clinical significance, and their common AmpC-β-lactamase-production, these pathogens are a part of the “SPICE” group (*Serratia*, *Pseudomonas*, indole-positive *Proteus*, *Citrobacter*, and *Enterobacter*) of bacteria (Gajdács et al. 2019a, b, c, d; Moy and Sharma 2017).

Since the beginning of the twenty-first century, several national and global (e.g., the SENTRY Antimicrobial Surveillance Program or the Study for Monitoring Antimicrobial Resistance Trends; SMART) surveillance reports have evaluated and published the resistance trends of various Gram-positive and Gram-negative bacteria; these reports unanimously confirmed the increase in the resistance-levels among common UTI-causing pathogens (both community-associated and hospital-acquired), and the emergence of multidrug resistant strains (MDR), extensively drug resistant (XDR) and even pandrug-resistant (PDR) strains of bacteria (Gajdács et al. 2020a, b; Chen et al. 2015; Morissey et al. 2013; Sader et al. 2014; Ponce-de-Leon et al. 2018). These strains (in addition to their intrinsic resistance mechanisms), express plasmid-encoded (transmissible) resistance determinants, which is both a therapeutic and infection control concern. The increased resistance is these pathogens is one of the main risk factor for a poor prognosis, therapeutic failure and even increased mortality rate in the hospitalized patient population. Conversely, resistance seriously limits the therapeutic options in outpatient settings, which may force clinicians to utilize more expensive antibiotics with a disadvantageous side effect-profile (Gajdács et al. 2019a, b, c, d). Thus, in the current era of high-resistance rates, the knowledge regarding the epidemiological information becomes much more important than ever before.

Resistance to β-lactam antibiotics (which may be mediated by a variety of mechanisms, the most common ones in Gram-negative bacteria being the production of β-lactamases [AmpC β-lactamases, ESBLs, carbapenemases] is a severe therapeutic issue in general, especially in case of vulnerable patient groups (e.g., pregnant women, children), where some other therapeutic alternatives are inappropriate due to their toxicity and teratogenicity (Abbo and Hooton 2014; Abraham 2016; Cantón et al. 2019; Gondim et al. 2018; Ulett et al. 2009; Meier et al. 2011). One of the most important developments in resistance was the emergence of strains expressing extended-spectrum β-lactamases (ESBLs), which have become a worldwide public health concern (Dhillon and Clark 2012). ESBLs produced by members of the Enterobacterales order are capable of hydrolyzing amino and ureido penicillins, oxyimino cephalosporins, and monobactams, but not to 7-α-substituted β-lactams (Rupp and Fey 2003). The spread of

ESBLs depends on bacterial conjugation, during which plasmids carrying ESBL genes are transferred. The proximity of bacteria is ensured in case of extensive biofilm-production (where the load of bacteria embedded in biofilm is considerably high), which creates a favourable environment for the exchange of genetic material, especially by conjugative transfer (Dhillon and Clark 2012; Rupp and Fey 2003; Paterson and Bonomo 2005). This is especially true for In nosocomial settings, where the production of biofilm by these species is an important factor for their survival. ESBL-positivity rate is highest in *Klebsiella* spp., due to its pronounced genetic plasticity and heightened ability of taking up plasmids, while it is the lowest in Proteae. (*Klebsiella* > *Escherichia* > *Enterobacter* > *Citrobacter* > *Serratia* > *Proteus* > *Morganella* > *Providencia*) (Bonkat et al. 2011). Since the twenty-first century, the most prevalent (>95%) type of ESBL-enzymes are the *bla*$_{\text{CTX-M}}$-type β-lactamases (Cantón et al. 2012). Nonetheless, ESBL-producing strains usually also carry resistance-determinants to other antibiotic groups (e.g., aminoglycosides, quinolones, fosfomycin), which significantly reduced treatment options to a limited number of antibiotics (Dhillon and Clark 2012; Rupp and Fey 2003; Paterson and Bonomo 2005). If the local epidemiology suggests that a patient has a high risk for an MDR UTI, ertapenem is one of the suggested drugs as the first line-agent to be used in several therapeutic guidelines (see previous subsections). Carbapenems have been considered safe and effective therapeutic choices in case of ESBL-positive Gram-negative bacteria; however, their extensive use has lead to the development of carbapenem-resistant Gram-negative strains, both among non-fermenters and gut bacteria (Papp-Wallace et al. 2011; El-Gamal et al. 2017). Carbapenem-resistant Gram-negative bacilli (CRGNB) are an important therapeutic problem, as there are limited number of safe and effective therapeutic alternatives available (van Duin et al. 2013). The most prevalent mechanism of carbapenem-resistance is through the production of specific, plasmid-borne β-lactamases called carbapenemases (Meletis 2016). The differentiation of carbapenemase-producing carbapenem resistant strains from non-carbapenemase producers is of utmost importance, as these resistance-determinants are readily transferable on plasmids or integrons, with pivotal roles in nosocomial outbreaks and global dissemination (Karlowsky et al. 2017). Based on their amino-acid sequences, carbapenemase enzymes are classified into Ambler Class A (e.g. KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC and IBC), Class D (e.g. OXA-23 group, OXA-48-group) and Class B (e.g. VIM, GIM, SIM, NDM, IMP, IND, AIM, DIM and SPM) enzymes. Class A and D enzymes are serine-β-lactamases, while the members of Class B are exclusively metallo-β-lactamases. In infections caused by carbapenemase-producing strains, clinicians are left with very few therapeutic alternatives, some of which are toxic (e.g., colistin; nephrotoxicity and neurotoxicity), have disadvantageous pharmacokinetic properties (e.g., tigecycline) or expensive (e.g., ceftazidime/avibactam, meropenem/vaborbactam) (Gajdács et al. 2019a, b, c, d; Gajdács et al. 2020a, b). Carbapenem-resistant Enterobacterales strains have been designated as one of the most important threats by both the Centers for Disease Control (CDC) and the World Health Organization (WHO) (Cantón et al. 2012; David et al. 2019).

5 Conclusions

Urinary tract infections (UTIs) are one of the most common reasons for patients to a visit a physician and to receive antibiotics. The aim of this review paper was to summarize current developments in the global burden of UTI, the diagnostic aspects of these infectious pathologies, the possible etiological agents and their virulence determinants (with a special focus on the members of the Enterobacterales order), current guidelines and quality indicators in the therapy of UTIs. Members of the Enterobacterales order are the most common urinary pathogens; however, many studies have also highlighted that the etiological agents in UTIs are broadening, both in nosocomial and community settings. The emergence of drug resistance in Gram-negative bacteria should be closely monitored, due to their

proclivity to becoming MDR and their plasticity in drug resistance mechanisms. As the therapeutic armametarium of clinicians is largely limited in the current antibiotic resistance climate, energies should also be put into the prudent use of antibiotics. The use of modern diagnostic modalities will definitely improve the quality of patient-care around the globe.

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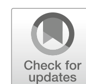

Diffusion and Characterization of *Pseudomonas aeruginosa* Aminoglycoside Resistance in an Italian Regional Cystic Fibrosis Centre

Gianmarco Mangiaterra, Nicholas Cedraro, Barbara Citterio, Serena Simoni, Carla Vignaroli, and Francesca Biavasco

Abstract

Aims Extensively-drug-resistant *Pseudomonas aeruginosa* constitutes a serious threat to patients suffering from Cystic Fibrosis (CF). In these patients, *P. aeruginosa* lung infection is commonly treated with aminoglycosides, but treatments are largely unsuccessful due a variety of resistance mechanisms. Here we investigate the prevalence of resistance to gentamicin, amikacin and tobramycin and the main aminoglycoside resistance genes found in *P. aeruginosa* strains isolated at a regional CF centre.

Results A total number of 147 randomly selected *P. aeruginosa* strains isolated from respiratory samples sent by the Marche regional Cystic Fibrosis Centre to the Microbiology lab, were included in this study. Of these, 78 (53%) were resistant to at least one of the three aminoglycosides tested and 27% were resistant to all three antibiotics, suggesting a major involvement of a chromosome-encoded mechanism, likely MexXY-OprM efflux pump overexpression. A specific pathogenic clone (found in 7/78 of the aminoglycoside resistant strains) carrying *ant(2″)-Ia* was isolated over time from the same patient, suggesting a role for this additional resistance gene in the antibiotic unresponsiveness of CF patients.

Conclusions The MexXY-OprM efflux pump is confirmed as the resistance determinant involved most frequently in *P. aeruginosa* aminoglycoside resistance of CF lung infections, followed by the *ant(2″)-Ia*-encoded adenylyltransferase. The latter may prove to be a novel target for new antimicrobial combinations against *P. aeruginosa*.

Keywords

Acquired resistance determinants · Aminoglycoside resistance · Cystic fibrosis · Efflux pumps · *Pseudomonas aeruginosa*

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1 Introduction

Extensively drug-resistant (XDR) *Pseudomonas aeruginosa* is a major cause of mortality in immunocompromised subjects, particularly in Cystic Fibrosis (CF) patients, whose chronic lung infections are mainly caused by this microorganism and are commonly treated with antimicrobial combinations that include tobramycin. Aminoglycosides (AMGs) are included in the gold standard treatment for *P. aeruginosa* lung infections and several studies have confirmed the efficacy of tobramycin (Stanojevic et al. 2014; Buttini et al. 2018). However, the development of AMG resistance is a growing threat hampering infection eradication (Costello et al. 2018).

AMG resistance relies on a broad variety of mechanisms (Poole 2005). First of all, the MexXY-OprM efflux pump (EP) is the main cause of the failure of routine antibiotic treatments against *P. aeruginosa* lung infection; in particular, it is involved in adaptive resistance (Morita et al. 2012), which enables pathogen survival in the presence of AMGs. The proneness of *P. aeruginosa* to accumulate mutations in the *mexXY* regulatory gene *mexZ,* results in EP overexpression and heightened resistance (Frimodt-Møller et al. 2018). Biofilm production hampers antibiotic action, further contributing to AMG resistance (Müller et al. 2018); notably, the genes involved in resistance development are upregulated in biofilm-embedded cells (Soto 2013; Hall et al. 2018), as reported for *ndvB*, encoding for cyclic glucans responsible of AMGs sequestration in the periplasmic space (Mah et al. 2003*).* Another recently described AMG resistance mechanism involves mutations in the *fusA1* gene, leading to single amino acid substitutions in the elongation factor G (EF-G1A), resulting in a lower drug affinity for the ribosome (Bolard et al. 2018).

Moreover, *P. aeruginosa* acquires new resistance determinants through horizontal gene transfer, which induces the formation of gene mosaics; the great variability of its genome results in a marked heterogeneity of AMG-resistant clones (Partridge et al. 2018). Enzymes that modify both the drug and its target are involved in the spread of high-level resistance (Poole 2011).

In the past few years, the search for new compounds capable of counteracting antibiotic resistance has mainly been directed at the modulation of EPs, a constitutive mechanism conferring resistance against several different antibiotics, which are often upregulated in *P. aeruginosa* (Mangiaterra et al. 2017; Laudadio et al. 2019). Another key area of investigation are other resistance mechanisms characterizing XDR *P. aeruginosa.*

We studied the prevalence of AMG resistance among the *P. aeruginosa* strains isolated from the CF patients managed by a regional referral centre in Marche (central Italy), by analysing the level of resistance to gentamycin, amikacin and tobramycin – the antibiotics routinely tested in the microbiology lab of the CF centre – and the resistance genes involved most frequently. In particular, we studied the frequency of transferable chromosome-independent resistance determinants and compared it with the frequency of chromosome-encoded resistances not involved in horizontal gene transfer events. We also compared the spread of the resistance mechanisms specific to each antibiotic with those providing resistance to all AMGs.

2 Material and Methods

2.1 Bacterial Strains, Media and Antibiotics

A total number of 147 randomly selected anonymized *P. aeruginosa* strains, sent for isolation by the Marche regional Cystic Fibrosis Centre to the Microbiology lab of “Ospedali Riuniti” Hospital (Ancona, IT), were included in the study. The strains had been collected from April 2014 to March 2015 (*P. aeruginosa* C1-C51) and from October 2015 to January 2016 (*P. aeruginosa* AR1-AR96) (Supplementary Table 1).

Additional strains included in the study were *P. aeruginosa* PAO1 and PA14, kindly provided by Prof. Olivier Jousson of the Integrated Biology Centre, University of Trento (Trento, IT), *P. aeruginosa* PAO1, carrying plasmid pHERD30T, kindly provided by Prof. Paul Williams of the Centre of Biomolecular Sciences,

University of Nottingham (UK), and *P. aeruginosa* ATCC 27853, from the collection of the Microbiology section of the Department of Life and Environmental Sciences, Polytechnic University of Marche (Ancona, IT). All strains were cultured in Luria Bertani (LB) broth and *Pseudomonas* cetrimide agar plates and stored as stock cultures in LB broth supplemented with 20% glycerol at −80 °C.

All media were purchased from Oxoid (Oxoid S.p.A., Rodano, Milano, IT); the antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2 Antibiotic Susceptibility Tests

The *P. aeruginosa* strains were screened for their resistance to tobramycin (TOB), gentamicin (GEN) and amikacin (AMK) by a routine antibiotic susceptibility test (Sensititre™ Complete Automated AST System, Thermo Fisher Scientific, Waltham, MA, USA). The resistant phenotype was confirmed by determination of the Minimal Inhibitory Concentration (MIC) either by agar dilution or by broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2003) using *P. aeruginosa* ATCC 27853 as the reference strain. The results were interpreted according to the EUCAST epidemiological cut-off (ECOFF) values (2016). Strains showing intermediate susceptibility were considered resistant. The following antibiotic concentration ranges were used: GEN, 0.5–32 μg/ml; AMK, 1–64 μg/ml; TOB, 0.25–16 μg/ml.

2.3 Detection of Aminoglycoside Resistance Genes

Two chromosome-encoded resistance determinants (*mexY* and *ndvB*) and 4 additional AMG resistance determinants (*aac(3)-Ia, aph(3′)-IIa, ant(2″)-Ia* and *rmtA*) were sought by PCR assays. Bacterial DNA was obtained from crude cell lysates (Hynes et al. 1992) and 5 μl were used in each PCR reaction together with 1.25 U Dream-Taq Polymerase (Thermo Fisher Scientific), 1x PCR Buffer, 0.2 mM dNTPs and 0.5 μM of each primer. The target genes and the specific primer pairs used are reported in Table 1. *P. aeruginosa* PAO1 and PA14 DNA was used as positive control in PCRs targeting *mexY* and *ndvB*, respectively; *P. aeruginosa* PAO1 harbouring the plasmid pHERD30T, which carries the *aac(3)-Ia* gene, was used as a positive control in the relevant PCR assays. Amplicons *rmtA*, *aph(3′)-IIa* and *ant(2″)-Ia* of the right size were purified (Gene Elute PCR Cleanup kit, Sigma-Aldrich) and sequenced using BigDye Terminator v.1.1 Cycle Sequencing kit according to the manufacturer's instructions. The sequences were analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and used as positive controls in

Table 1 Target genes, primer pairs, and amplicon size of the PCR assays used to detect six different AMG resistance genes

Target gene	Primer pair (5′-3′)	Amplicon size (bp)	References
mexY	mexY-F TGGAAGTGCAGAACCGCCTG	270	Oh et al. (2003)
	mexY-R AGGTCAGCTTGGCCGGGTC		
ndvB	ndvB JB-F GGCCTGAACATCTTCTTCACC	138	Beaudoin et al. (2012)
	ndvB JB-R GATCTTGCCGACCTTGAAGAC		
rmtA	rmtA-F CTAGCGTCCATCCTTTCCTC	635	Yamane et al. (2004)
	rmtA-R TTTGCTTCCATGCCCTTGCC		
aac(3)-Ia	aac3-F GGCTCAAGTATGGGCATCAT	389	Michalska et al. (2014)
	aac3-R TCACCGTAATCTGCTTGCAC		
aph(3′)-IIa	npt2-F GATCTCCTGTCATCTCACCTTGCT	129	Woegerbauer et al. (2004)
	npt2-R TCGCTCGATGCGATGTTTC		
ant(2″)-Ia	ant2bi-F GACACAACGCAGGTCACATT	500	Michalska et al. (2014)
	ant2bi-R CGCAAGACCTCAACCTTTTC		

PCRs targeting the corresponding gene. RNase-free water (Thermo Fisher Scientific) was used as the negative control. The PCR products were checked by 1.5% agarose gel electrophoresis.

2.4 Pulsed Field Gel Electrophoresis Typing

Pulsed Field Gel Electrophoresis (PFGE) was performed as described by Seifert et al. (2005), with some modifications. Briefly, agarose plugs (low-melting 1.6% agarose) were digested with 30 U of *SpeI* (Thermo Fisher Scientific) and the restriction fragments were separated using a CHEF MAPPER system (BioRad, Hercules, CA, USA). Running conditions were as follows: switch angle 120°, voltage 6 V/cm, temperature 14 °C, run time 18 h, initial and final switching time 5.8 s and 30 s, respectively. The low-range PFG marker (Amersham Biosciences, Little Chalfont, UK) was used as a molecular weight marker and PFGE patterns were analysed as described previously (Biavasco et al. 2007). Dendrograms were drawn using the TreeCon software.

2.5 Statistical Analysis

The frequency of *P. aeruginosa* strains resistant to each of the three antibiotics was evaluated by the chi square test. Values were considered statistically significant when p was <0.05.

3 Results

3.1 Detection of Aminoglycoside Resistance in CF *P. aeruginosa* Strains

The screening by Sensititre™ of 147 CF *P. aeruginosa* strains for resistance to gentamicin, amikacin and tobramycin by routine susceptibility tests found that 78 (53%) were resistant to at least one antibiotic. These 78 strains were subjected to further analyses. Testing of their resistance level by agar dilution (Supplementary Table 1) demonstrated that 66 were resistant to gentamicin, 66 were resistant to amikacin (each accounting for 84.62%) and 27 were resistant to tobramycin (34.62%). The frequency of strains resistant to gentamicin or amikacin was significantly ($p < 0.01$) higher than that of tobramycin-resistant isolates (Fig. 1).

In particular, resistance to all 3 antibiotics (26.92%) was statistically more common than resistance to gentamicin (7.69%) or tobramycin (2.56%) alone ($p < 0.05$ and $p < 0.01$, respectively), whereas resistance to gentamicin +amikacin was significantly ($p < 0.01$) more frequent (44.87%) than tobramycin resistance (2.56%) (Fig. S1). There were no strains resistant to amikacin+tobramycin.

3.2 Detection of Aminoglycoside Resistance Genes

The 78 strains resistant to gentamicin, amikacin and/or tobramycin were analysed for their AMG resistance genes by PCR assays targeting two chromosome-encoded (*mexY* and *ndvB)* genes and four additional (*rmtA, aac(3)-Ia, ant(2″)-Ia* and *aph(3′)-IIa*) genes (Supplementary Table 1).

mexY was detected in all strains and *ndvB* in all strains but one (C36), *aac(3)-Ia* and *ant(2″)-Ia* were detected respectively in 4 (C40, AR15, AR39 and AR66) and 7 (C44, C45, AR45, AR49, AR86, AR88 and AR89) isolates, whereas all strains were negative for *aph(3′)-IIa* and *rmtA*. Sequencing of *ant(2″)-Ia* amplicon of *P. aeruginosa* AR86 demonstrated a similarity of 99% to the sequence of *P. aeruginosa* strain PB350 (Accession no. CP025055.1). There were 11 strains carrying *aac(3)-Ia* or *ant(2″)-Ia* (Table 2). Their frequency is reported in Fig. 2.

As expected, all *P. aeruginosa* isolates carrying *aac(3)-Ia* were resistant to gentamicin alone (16.7%) or to gentamicin+amikacin (8.57%). *ant (2″)-Ia* was detected in 50% of strains resistant to tobramycin or tobramycin+gentamicin and in 10% of strains resistant to amikacin, in 9.52% of those resistant to tobramycin+amikacin+gentamicin and in 2.86% of those resistant to gentamicin +amikacin.

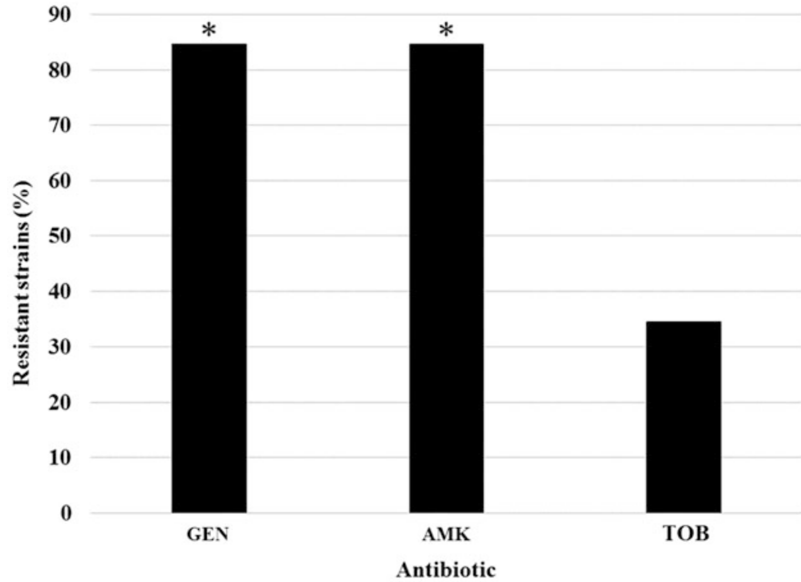

Fig. 1 Percent frequency of the CF *P. aeruginosa* strains resistant to GEN, AMK and/or TOB among the 78 aminoglycoside resistant isolates included in the study. *GEN* gentamicin, *AMK* amikacin, *TOB* tobramycin. * p < 0.01

Table 2 Minimal inhibitory concentrations of gentamicin (GEN), amikacin (AMK) and tobramycin (TOB) against the 11 aminoglycoside-resistant *P. aeruginosa* strains carrying the acquired resistance genes *aac(3)-Ia* or *ant(2″)-Ia*

Strain	Patient	MIC (μg/ml)			Resistance gene
		GEN	AMK	TOB	
C40	A	8	32	≤4	***aac(3)-Ia***
C44	B	>2048	16	256	***ant(2″)-Ia***
C45	C	16	32	16	***ant(2″)-Ia***
AR15	D	8	≤8	≤4	***aac(3)-Ia***
AR39	E	8	16	≤4	***aac(3)-Ia***
AR45	B	8	≤8	8	***ant(2″)-Ia***
AR49	B	8	≤8	8	***ant(2″)-Ia***
AR66	A	16	32	≤4	***aac(3)-Ia***
AR86	B	≤4	≤8	256	***ant(2″)-Ia***
AR88	A	≤4	32	≤4	***ant(2″)-Ia***
AR89	F	8	16	≤4	***ant(2″)-Ia***

GEN and TOB, S ≤ 4 R > 4; AN, S ≤ 8, R > 16 (EUCAST 2016)
GEN gentamicin, *AMK* amikacin, *TOB* tobramycin

The gentamicin, amikacin and tobramycin MIC values of the 11 strains carrying the additional resistance genes *aac(3)-Ia* or *ant(2″)-Ia* were further investigated by broth microdilution using a wider range of antibiotic concentrations and the specific resistance phenotype checked against the presence of *aac(3)-Ia* or *ant(2″)-Ia* (Table 2).

Notably, the two *P. aeruginosa* strains (C44 and AR86) with the highest tobramycin MIC (256 μg/ml) harboured *ant(2″)-Ia,* as well as the strain with the highest gentamycin MIC (>2048 μg/ml).

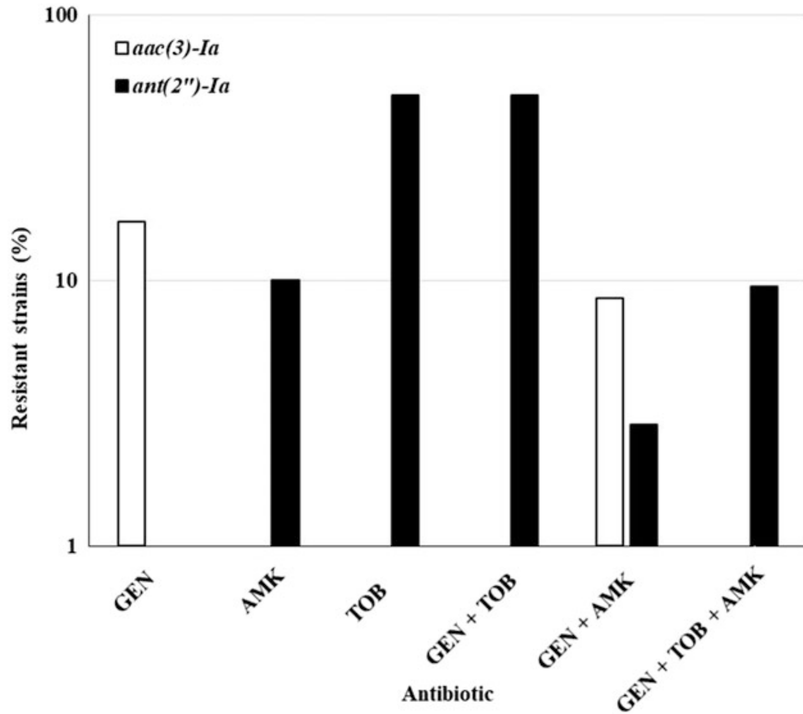

Fig. 2 Frequency (log scale) of the aminoglycoside resistance determinants *aac(3)-Ia* and *ant(2″)-Ia* in the 78 CF *P. aeruginosa* strains resistant to one or more aminoglycosides. The number of strains carrying each resistance gene is reported as a proportion of the *P. aeruginosa* isolates resistant to the same antibiotic(s). *GEN* gentamicin, *AMK* amikacin, *TOB* tobramycin

3.3 PFGE Typing of the Aminoglycoside-Resistant *P. aeruginosa* Strains Carrying *aac(3)-Ia* or *ant(2″)-Ia*

The 11 *P. aeruginosa* strains carrying *aac(3)-Ia* or *ant(2″)-Ia* were typed by PFGE (Fig. 3), which highlighted two main clusters with a similarity <55%. One cluster encompassed the seven isolates encoding *ant(2″)-Ia* (top) and the other the four isolates encoding *aac(3)-Ia* (bottom). Within the former cluster, five isolates, of which four were from the same patient (Table 2), belonged to the same clone (100% similarity) and displayed a similarity <65% to the other 2 isolates. The 4 strains of the second cluster belonged to 4 different pulsotypes and shared a low similarity (about 60%) although 2 of them, represented by C40 and AR39, seem more related each other showing a similarity of 75%.

4 Discussion

A major issue in the control and management of CF *P. aeruginosa* lung infection is bacterial unresponsiveness to antibiotic treatment. This is due to a variety of causes, including chromosome-encoded resistance, biofilm development and the increasing diffusion of XDR strains (Colque et al. 2020). AMGs are frontline antibiotics, especially tobramycin, which is included in the eradication protocols of chronic lung infections (Ratjen et al. 2019). This work aimed to investigate the prevalence of resistance to tobramycin, gentamicin and amikacin (the three AMGs routinely tested in CF samples) and of the additional resistance genes *rmtA*, *ant(2″)-Ia*, *aac(3)-Ia* and *aph(3′)-IIa* in the CF *P. aeruginosa* strains isolated from sputum samples collected at the Marche Cystic Fibrosis centre. The high prevalence (53%) of

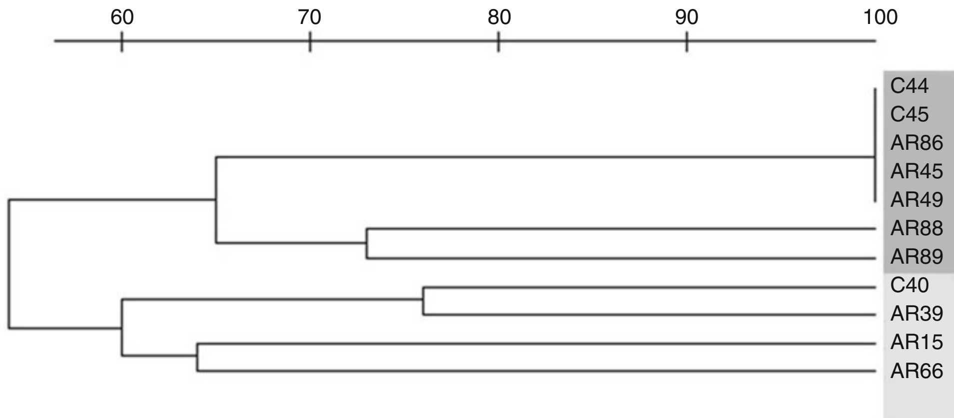

Fig. 3 Dendrogram showing the similarity index among the 11 aminoglycoside-resistant *P. aeruginosa* isolates carrying the *ant(2″)-Ia* (black square) or *aac(3)-Ia* (gray square) gene. Strain similarity is expressed as percentage (%). Strains sharing ≥80% similarity are considered as related

AMG-resistant strains among these isolates highlights the need for measures to contain the spread of *P. aeruginosa* antibiotic resistance and for novel therapeutic approaches. Resistance to gentamicin and amikacin was the most frequent (44.87%) AMG resistance phenotype and was followed by resistance to all three AMGs (26.92%), to amikacin (12.82%), to gentamicin (7.69%), to gentamicin and tobramycin (5.13%) and to tobramycin alone (2.56%); therefore the proportion of strains resistant to two or all three AMGs was higher (76.92%) than that of strains resistant to a single antibiotic (23.08%). These findings suggest the major involvement of a cross-resistance mechanism capable of counteracting different AMGs. The most likely seems to be MexXY-OprM EP upregulation; this has been described as the main AMG resistance mechanism in *P. aeruginosa* (López-Causapé et al. 2018) through mutations of its regulatory gene *mexZ*, although mutations of hot-spot genes, like *fusA1*, could also be responsible for the multi-resistant phenotype. The lack of additional resistance genes in the strains showing multi-AMG resistance in our study, supports these views. Notably, the susceptibility to tobramycin of 44.87% of strains resistant to gentamicin and amikacin and lacking additional AMG resistance genes, could be explained by mutations in the MexY inner membrane channel not affecting specifically the tobramycin binding site; this would be in line with our modelling results, which suggest that these polymorphisms can occur as a consequence of *mexY* single nucleotide substitutions (unpublished data). None of our 147 strains was resistant to amikacin and tobramycin and susceptible to gentamicin, reflecting the absence among them of resistance mechanisms capable of counteracting the antibacterial activity of amikacin and tobramycin without affecting gentamicin effectiveness.

Tobramycin has proved to be a better therapeutic option than gentamicin and amikacin, with 34.62% of resistant strains compared to 84.62% of the other two AMGs (Fig. 1). These data are in agreement with those reported by Mustafa et al. (2016) and support the use of tobramycin, rather than other AMGs, in the eradication protocols of early *P. aeruginosa* CF lung infection (Mostofian et al. 2019).

This study involved the examination of six genes to evaluate the chromosome-dependent (*mexY* and *ndvB*) or -independent (*rmtA*, *ant (2″)-Ia*, *aac(3)-Ia* and *aph(3′)-IIa*) nature of AMG resistance in our strains, and the assessment of the prevalence of antibiotic specific (*ant (2)″-Ia*, *aac(3)-Ia* and *aph(3′)-IIa*) or aspecific (*mexY*, *ndvB* and *rmtA*) resistance mechanisms. As expected, *mexY* and *ndvB* were detected in all AMG-resistant strains, the only exception being

P. aeruginosa C36, which showed no *ndvB* amplification, likely due to mutations in the sequences targeted by the ndvB primer pair. Since *ndvB* is involved in resistance of biofilm-growing *P. aeruginosa* (Mah et al. 2003) and we used planktonic cultures in the antibiotic susceptibility assays, the overexpression of the *mexXY-OprM* gene cluster seems responsible for the AMG resistance of *P. aeruginosa* isolates lacking additional resistance genes and is consistent with the above considerations about the prevalence of multi-AMG resistance phenotypes. MexXY-OprM is involved in the adaptive low-level resistance of CF *P. aeruginosa* (Morita et al. 2012); high-level resistance may evolve as a consequence of the gradual accrual of mutations in the *mexXY-oprM* regulator *mexZ*, which has often been reported in patients in chronic *P. aeruginosa* infections (Prickett et al. 2017). In such patients, the expression of the *P. aeruginosa mexXY-oprM* gene cluster progressively increases, also as a consequence of adaptation to the stressful conditions of the CF lung environment (Martin et al. 2018). This can explain the variable resistant phenotypes observed in the *P. aeruginosa* strains lacking the additional resistance determinants *aac(3)-Ia* and/or *ant(2″)-Ia*. Further investigation of the *mexY* expression level of these strains is warranted. We are currently evaluating the correlation between these phenotypes and *mexY* expression level.

Of the additional AMG resistance genes sought in this study – *rmtA, aac(3)-Ia, aph(3′)-IIa* and *ant(2″)-Ia* – only *aac(3)-Ia* and *ant(2″)-Ia* (Poole 2011) were detected and were found in only 11/78 strains, in line with the notion that AMG resistance in CF *P. aeruginosa* isolates is mainly a consequence of mutational events (López-Causapé et al. 2018). As expected, the four strains harbouring *aac(3)-Ia* were all resistant to gentamicin and susceptible to tobramycin (Ramirez and Tolmasky 2010), whereas two (*P. aeruginosa* AR88 and AR89) of the seven strains carrying *ant(2″)-Ia*, which confers resistance both to gentamicin and tobramycin (Poole 2005), were susceptible to the latter (MIC <4 μg/ml), likely due to the lack or downregulation of *ant(2″)-Ia* expression. These results are consistent with the hypervariability of CF *P. aeruginosa* (Qin et al. 2018).

PFGE typing assays of the 11 strains carrying *aac(3)-Ia* or *ant(2″)-Ia* demonstrated that the four *aac(3)-Ia*-carrying strains were unrelated, thus suggesting the spread of the GEN resistance gene among different *P. aeruginosa* clones through horizontal gene transfer (Kiddee et al. 2013). In contrast, five of the seven tobramycin-resistant isolates carrying the *ant(2″)-Ia* gene showed 100% similarity, suggesting the spread of a single clone. Since the 147 *P. aeruginosa* isolates were collected randomly and anonymously, we retrospectively investigated whether they came from the same patient. Indeed, four of them had been collected from patient B over an eight-month period (Table 2), thus highlighting failed eradication of *P. aeruginosa*, which induced symptom relapse. On the other hand, recovery of the same clone from different patients suggests a clonal spread of the tobramycin-resistant strain. The selective pressure exerted by the repeated use of tobramycin to counteract *P. aeruginosa* lung infection in CF patients (Smith et al. 2017) can explain the persistence and spread of this clone among patients referring to the CF centre. The role of additional resistance genes in *P. aeruginosa* persistence has been described by Mózes et al. (2014), who suggested a relationship between endemic *P. aeruginosa* clones and their carriage of integron-borne AMG resistance determinants. Ostensibly the fitness cost due to the additional gene is largely offset by the favourable conditions found inside the host.

In conclusion, *P. aeruginosa* AMG resistance is a major threat for CF patients, whose lung infections are routinely treated with these drugs. Our results show the spread of AMG resistance among the patients managed by the Marche regional Cystic Fibrosis centre, who showed a high prevalence of strains resistant to gentamicin, amikacin and tobramycin. The origin of resistance seems to be largely mutational, probably related to the MexXY-OprM EP or to other chromosome-encoded determinants. This suggests that MexXY-OprM EP inhibitors suitable for synergistic combinations with AMGs

should urgently be developed to treat *P. aeruginosa* lung infections (Lamers et al. 2013; Aron and Opperman 2016; Laudadio et al. 2019). Our findings also suggest that monitoring additional AMG resistance genes, particularly *ant (2″)-Ia,* in early *P. aeruginosa* lung infection could contribute to a more effective management of CF patients.

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Synovial Fluid Mediated Aggregation of Clinical Strains of Four Enterobacterial Species

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Abstract

Septic arthritis and prosthetic joint infection (PJI) are conditions commonly associated with Gram-positive cocci, however, a drastic increase in cases derived from enterobacterial species has been observed. Recently it has been reported by multiple groups that staphylococci rapidly form free-floating aggregates in the presence of synovial fluid. These aggregates are comparatively more resistant to antimicrobial challenge than their planktonic counterparts, and thus may play a role in the pathogenesis of joint infection. While staphylococcal aggregates have been the primary focus of interest in the field, it is unclear just how widespread synovial fluid mediated aggregation (SFMA) is in Gram negative enterobacteria (GNE). Through this work we have evaluated SFMA in clinical GNE isolated from PJIs. Two PJI clinical strains each of *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis* strains representing a range of antibiotic susceptibilities were exposed to 10% bovine synovial fluid supernatant (BSF) using a relatively simple, quick semi-quantitative method using an imaging plate reader. BSF stimulated aggregation within 0.5 h both strains of *E. cloacae* and *P. mirabilis* and one strain of *E.coli*. In one strain of *P. mirabilis* and *E.coli,* the size of the aggregates significantly increased from 0.5 to

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2 h exposure. In contrast, neither *K. pneumoniae* strain aggregated in BSF. These preliminary findings show that aggregation can occur quickly in GNE, but the extent appears strain and species specific. Further work is required to assess the impact of SFMA on antibiotic tolerance, host innate immunity and the establishment of biofilms.

Keywords

Aggregates · Biofilm · Enterobacteria · Prosthetic joint infection · Rapid screen · Septic arthritis

1 Introduction

The frequency of both native and prosthetic joint infections is rapidly increasing (Nair et al. 2017). There are many factors contributing to this rise including an aging population, the growing prevalence of degenerative joint diseases, immunosuppressive treatments, and the use of invasive joint operations (Salar et al. 2014; Kaandorp et al. 1997). Septic arthritis (SA) is an invasion of a joint by an infectious agent resulting in localized inflammation. This pathology is infrequent, but it is considered a medical emergency associated with substantial morbidity and mortality (Margaretten et al. 2007). A recent retrospective study following the outcomes of 10,195 patients with septic arthritis stated that about 7% died within 90 days of receiving an arthroscopic knee washout as treatment, 1% required knee arthroplasty within 1 year and 9% within 15 years of the treatment (Abram et al. 2020). The development of SA can occur through a myriad of sources including hematogenous seeding, introduction of bacteria during joint surgery, or direct extension from a contiguous focus of the joint space by a pathogen microorganism (Shirtliff and Mader 2002; Goldenberg and Reed 1985). The most common microorganisms involved are Gram-positive cocci with *Staphylococcus aureus* making up 52% of all infections (Ross 2017) whilst Gram-negative bacilli represent about 10–20% of cases (Deesomchok and Tumrasvin 1990). SA events associated with Gram-negative bacilli often yield outcomes far more severe than those caused by Gram-positive bacteria (Chiu and Wang 2013). These infections may occur in both the native (non-prosthetic SA) and artificial joint, for example after a knee or hip replacement (Nair et al. 2017). Thus, patients who have artificial joints are at an increased risk of developing a joint infection (Del Pozo and Patel 2009). Recent studies suggest that although Gram-positive cocci represent the dominant source of prosthetic joint infections, the number of cases attributed to Gram-negative bacilli are continuously rising. (Benito et al. 2016). It has been suggested that GNE are the causative agents of 10–23% of all PJI (Rodriguez-Pardo et al. 2014).

PJIs are characteristically difficult to treat (Tande and Patel 2014) due to the ability of microorganisms to form biofilms on a variety of surfaces (Donlan and Costerton 2002). While the process of biofilm formation in the joint space is still being elucidated, multiple groups have shown that exposure to synovial fluid can stimulate bacteria to rapidly form macroscopic aggregates. It has been demonstrated that *S. aureus* aggregates formed in the presence of either human or bovine synovial fluid display an increased antimicrobial tolerance and resistance to immune system defenses (Gilbertie et al. 2019). Synovial fluid mediated aggregation (SFMA) has also been shown to occur in other bacterial species (*Streptococcus zooepidemicus*, *Pseudomonas aeruginosa* and *Escherichia coli*) stimulated by either equine or porcine synovial fluid (Gilbertie et al. 2019). Aggregates of *P. aeruginosa* biofilms have been observed in clinical specimens (Howlin et al. 2017) and have been shown to seed biofilm formation *in vitro* (Kragh et al. 2016). In view of these observations and evidence that biofilm-based infections related to Gram-negative enterobacteria are increasing, additional *in vitro* investigations are needed to better understand the interactions between these microorganisms and synovial fluid. The aim of the present study was to evaluate bovine SFMA in respect to time and aggregate size of four enterobacteria species isolated from PJI.

In order to semi-quantify the SFMA potential in the GNE clinical strains we first needed to develop a relatively quick, cheap, and simple

rapid screen method of assessment. To accomplish this, we based our assay on a multiwall plate platform using an imaging plate reader in which aggregation patterns in the wells could be captured and semi-quantified. Previously we found that the viscosity and heterogeneous composition of 100% bovine synovial fluid posed difficulties in pipetting and also contained fibrous and particulate material which caused optical interference in the plate reader. Therefore, we first evaluated the use of 10% bovine synovial fluid in Ringer's solution as a sufficient stimulus to demonstrate bacterial aggregation. Prior to testing our Gram-negative strains, conditions and usage of 10% bovine synovial fluid supernatant as an aggregating agent was demonstrated using a *S.aureus* 1276 strain known to aggregate in the presence of synovial fluid (Pestrak et al. 2020).

2 Materials and Methods

2.1 Flow Cytometry

A single colony of GFP-expressing *S. aureus* strain, AH1726, a constitutive GFP expressing derivative of the USA300 LAC strain (Chiu et al. 2013) which is known to form synovial fluid mediated aggregates (Pestrak et al. 2020; Ibberson et al. 2016), was used to inoculate 5 mL of Tryptic Soy Broth (BD, Germany). The culture was grown shaking in a 37 °C incubator overnight (Innova 44, New Brunswick Scientific). The optical density at 600 nm was measured and 0.75 OD_{600} of cells from stationary phase cultures was pelleted at 21,000 xg, washed twice and resuspended in 500 μL of Ringer's solution buffer (BR0052G, Fisher Scientific). After the washes, cells were pelleted and re-suspended in 500 μl of Ringer's solution or 1%, 5%, or 10% whole bovine synovial fluid or bovine synovial fluid supernatant (Lampire Biological Laboratories, Pipersville, PA, USA) in Ringer's solution. Cells were incubated in the treatment for 1 h prior to flow cytometry. After the incubation time, 100 μl of the cells was collected from the bottom of the microcentrifuge tube and transferred to a 5 ml round bottom polystyrene tube. As described elsewhere (Pestrak et al. 2020) bacterial aggregation can be quantified using a BD FACsCanto II flow cytometer (BD sciences). In order to exclude synovial fluid debris, only the forward and side scatter of the GFP+ population was quantified. Data collected from the flow cytometer was analyzed using FlowJo 9.0. The population of single cells was determined by gating a population of single bacterial cells in the negative control (no treatment). This population was confirmed previously by microscopy to be planktonic. Calculations to determine the percentage of the population existing as aggregates was accomplished by subtracting the single cell population from the total population. At least 10,000 events were measured during flow cytometry for 3 biological replicates per treatment. Additionally, the median fluorescence intensity (MFI-FSC) of the forward scatter was calculated to determine the relative aggregate size within the population. Statistical significance of variation in aggregation or MFI-FSC between whole bovine synovial fluid and bovine synovial fluid supernatant groups was determined by two-way ANOVA (GraphPad Prism version 5.0b software).

2.2 Antimicrobial Susceptibility Testing

Two bacterial strains of each of four enterobacterial species: *Enterobacter cloacae* (E.cl7 and E. cl8), *Escherichia coli* (E.c3 and E.c4), *Klebsiella pneumonia* (K.p1 and K.p2), and *Proteus mirabilis* (P.m5 and P.m6) were used in the study. All strains were isolated from samples of patients with PJI in the Clinical Microbiology Department of a metropolitan university hospital. They were frozen at −80 °C and subsequently sent to the reference laboratory. All PJI were diagnosed according to Infectious Diseases Society of America (IDSA) internationally accepted criteria (Osmon et al. 2012). Antimicrobial susceptibility testing was performed based on VITEK® 2 Systems (Biomerieux, France) and

Table 1 Details and antibiogram of GNE isolates

Species	*E.cloacae*		*E.coli*		*K. pneumoniae*		*P. mirabilis*	
Strain designation	E.cl8	E.cl7	E.c3	E.c4	K.p1	K.p2	P.m5	P.m6
Origin	KPI	HPI	HPI	HPI	KPI	EPI	HPI	HPI
Antibiogram								
AMK	≤2	≤2	**32**	≤2	4	≤2	≤2	≤2
AMC	≥**32**	≥**32**	≥**32**	4	≥**32**	≤2	8	4
CTX	≥**64**	≤1	≥**64**	≤1	≥**64**	≤1	≥**64**	≤1
CXM	≥**64**	16	≥**64**	4	≥**64**	≤1	≥**64**	≤1
CIP	≥**4**	≤0,25	≥**4**	≤0,25	≥**4**	≤0,25	≥**4**	≥**4**
GEN	≤1	≤1	≤1	≤1	≥**16**	≥**16**	≤1	≤1
IMP	2	≤0,25	≤0,25	≤0,25	2	≤0,25	≤0,25	≤0,25
SXT	≤20	≤20	≥**320**	≤20	40	≤20	≥**320**	≤20

Infection type (*EPI* Elbow prosthetic infection, *KPI* knee prosthetic infection, *HPI* hip prosthetic infection) and antibiogram of the clinical isolates. Susceptibility rates were interpreted according to EUCAST breakpoints. Abbreviations: *AMK* Amikacin, *GEN* Gentamicin, *CXM* Cefuroxime, *CTX* Cefotaxime, *AMC* Amoxicillin/clavulanic acid, *CIP* Ciprofloxacin, *SXT* Trimethoprim/sulfamethoxazole, *IMP* Imipenem; Bold values are considered resistant by EUCAST criteria

categorized according the EUCAST breakpoints (Table 1).

2.3 Plate Reader Imaging

First, the bovine synovial fluid was centrifuged, and the supernatant was taken. To assess bacterial aggregation formation, an overnight bacterial tryptic-soy broth culture (BD, Germany) was centrifuged and washed three times. 25 μL of the pellet were added to 100 μL of Ringer's Solution (Sigma Aldrich, Missouri, United States) with or without 10% bovine synovial fluid (Lampire Biological Laboratories, Pennsylvania, United States) following a methodology previous described (Pestrak et al. 2020) in a 96-well plate (ThermoFisher Scientific, Massachusetts, United State). After 0.5 and 2 h incubation at 37 °C and 5% CO_2, images of the aggregate formation were taken using an imaging plate-reader (SpectraMax i3x, Molecular Devices). The experiment was performed in triplicate. The images were analyzed using ImageJ (O'Brien et al. 2016). Data were statistically evaluated using non-parametric Wilcoxon test. The values were represented as median and interquartile range. As a positive control, the GFP-expressing *S. aureus* strain, AH1726, which has previously been shown to aggregate in synovial fluid, was utilized. Methods for imaging after 1 h exposure were followed as described above using 1%, 5%, or 10% whole bovine synovial fluid or bovine synovial fluid supernatant.

3 Results

3.1 Evaluation of Bovine Synovial Fluid Treatments

Regardless of whether whole synovial fluid or synovial fluid supernatant was utilized, a dose-dependent increase in aggregation was observed using plate reader imaging (Fig. 1a–f). Additionally, visually the aggregate size was comparable between the two treatments. These results were corroborated using flow cytometry to quantify the percentage of the population within aggregates after 1 h of exposure to either whole bovine synovial fluid (Fig. 2c) or bovine synovial fluid supernatant (Fig. 2d). There was no statistically significant variability in aggregation (Fig. 2a) or the median fluorescence intensity size of the forward scatter (MFI-FSC) (Fig. 2b) between the two treatment groups.

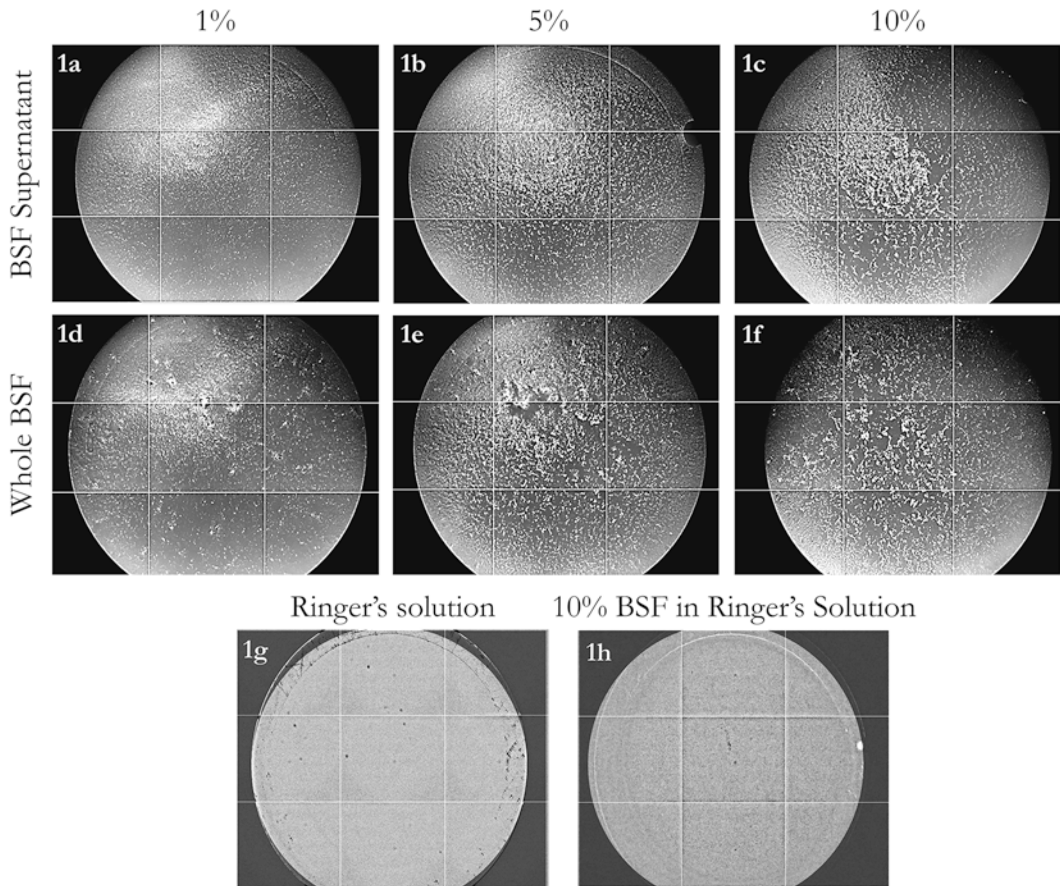

Fig. 1 Plate reader imaging of bovine synovial fluid induced aggregation. Plate reader imaging of AH1726 was used to observe aggregation 1-h post exposure to bovine synovial fluid supernatant (**a-c**) or whole bovine synovial fluid (**d-f**) diluted in Ringer's Solution. Control wells containing Ringer's Solution (**g**) or 10% bovine synovial fluid in Ringer's Solution (**h**) but no bacteria

3.2 Bacterial Aggregation of GNE

The use of 10% of bovine synovial fluid favored the bacterial aggregation of enterobacteria in 5 of our 8 strains tested and these aggregates were visible after 2 h as illustrated by data with *E. coli*. The presence of synovial fluid significantly increased the size of aggregates of both *E. cloacae* and *P. mirabilis* strains and one *E. coli* strain (E.c4), regardless of exposure time ($p < 0.05$) (Fig. 3). The size of aggregates significantly increased from 0.5 to 2 h of exposure in *P. mirabilis* P.m5 and P.m6 and *E. coli* E.co4 ($p < 0.05$). Neither of the *K. pneumoniae* strains showed evidence of aggregation patterns by our plate assay either with or without synovial fluid and there was no significant difference in aggregate size ($p < 0.05$) (Fig. 4).

4 Discussion

4.1 Treatment Evaluation

In order to increase consistency in our experiments and minimize the presence of artifact during image analysis, we opted for the use of synovial fluid supernatant over whole synovial fluid. As it has been speculated that aggregation is predominantly mediated by fibrinogen and fibronectin components within the synovial fluid (Pestrak et al. 2020), we hypothesized that the

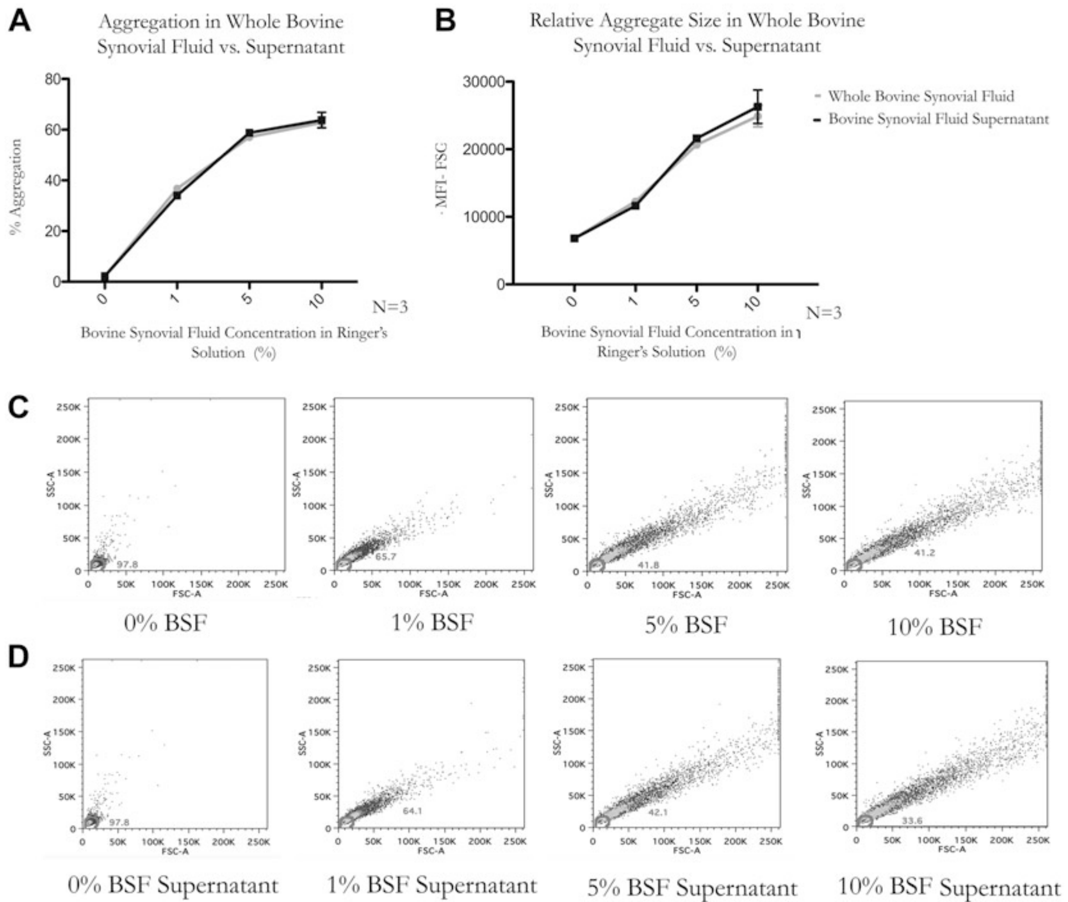

Fig. 2 Synovial fluid supernatant is sufficient to stimulate S. aureus aggregation. Flow cytometry was used to calculate percent of population within aggregates of AH1726 after 1-h exposure to whole bovine synovial fluid or bovine synovial fluid supernatant (**a**). Aggregation was calculated at 0%, 1%, 5%, and 10% of treatment in Ringer's Solution. The median fluorescence intensity of the forward scatter (MFI-FSC) was calculated in order to assess the average aggregate size within the sample (**b**). Flow cytometry outputs of whole bovine synovial fluid treatments (**c**) and bovine synovial fluid supernatant (**d**) 1-h post exposure. Gated area represents single cells within population while anything outside gate indicates aggregation. Error bars indicate mean ± SEM. Statistical significance was determined by two-way ANOVA. Statistical analysis indicates treatment is not a statistically significant source of variation (ns $p = 0.9935$) while treatment concentration is (*** $p < 0.0001$)

supernatant alone would yield comparable degrees of induced bacterial aggregation. *S. aureus* strain AH1726 was able to aggregate just as well when stimulated with bovine synovial fluid supernatant as it was with whole bovine synovial fluid. Not only will utilizing the supernatant increase the accuracy of image analysis for our multi-well plate reader assay, but it also allows for greater consistency in future experiments. Whole bovine synovial fluid contains macroscopic bits of floating tissue and particulates which are eliminated after centrifugation, creating a more homogenous media. Utilization of this refined synovial fluid in future *in vitro* studies will allow for a more accurate analysis of other contributing factors, such as strain specific discrepancies which may influence aggregation.

4.2 GNE Synovial Fluid Induced Aggregation

Five of the eight enterobacterial strains evaluated formed aggregates in presence of synovial fluid within a 2 h exposure. These results are consistent

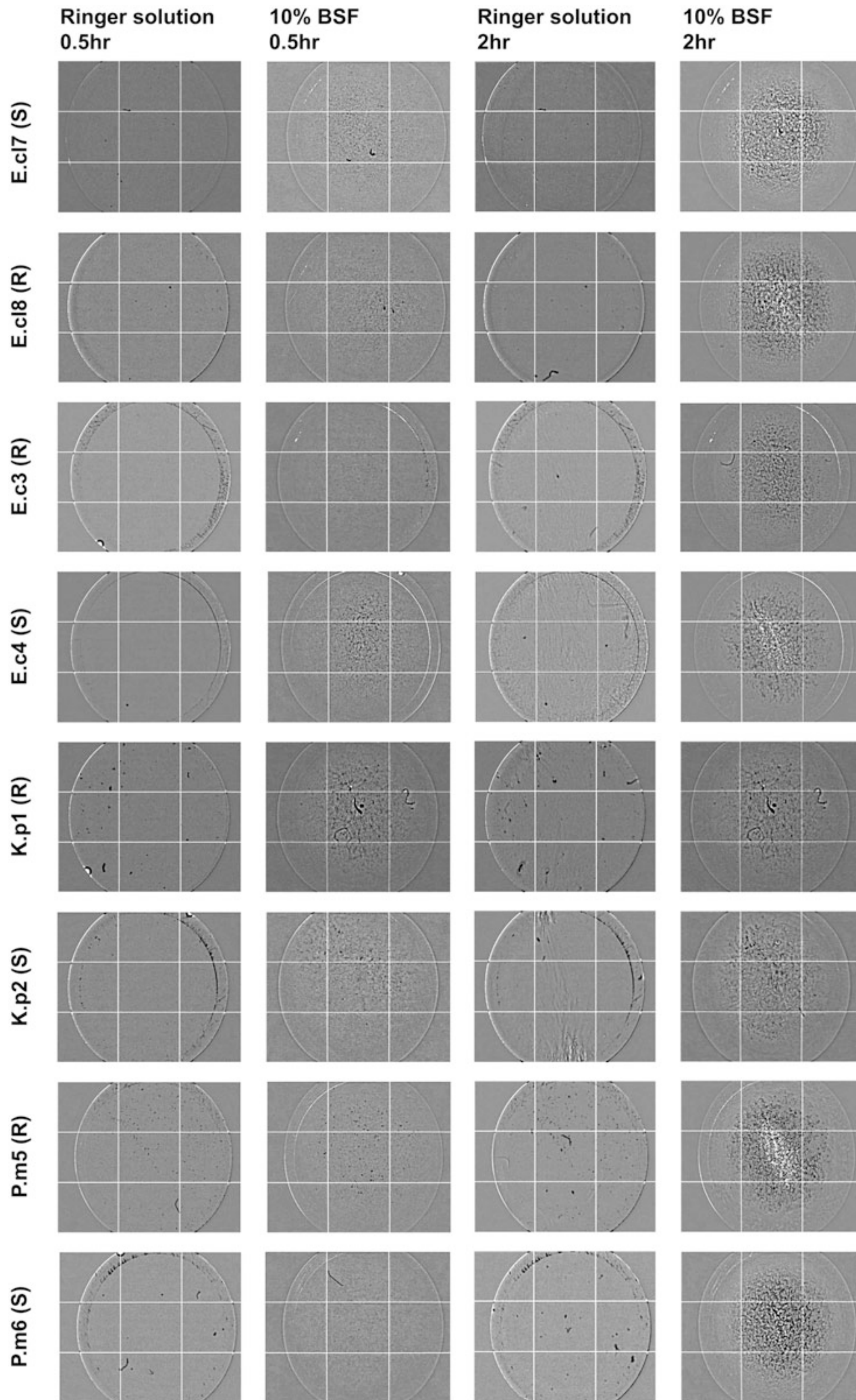

Fig. 3 GNE isolates visualized by an imaging plate reader (SpectraMax i3x, Molecular Devices, magnification x3) incubated 0.5 h and 2 h at 37 °C and 5% CO_2 in Ringer solution and with 10% bovine synovial fluid

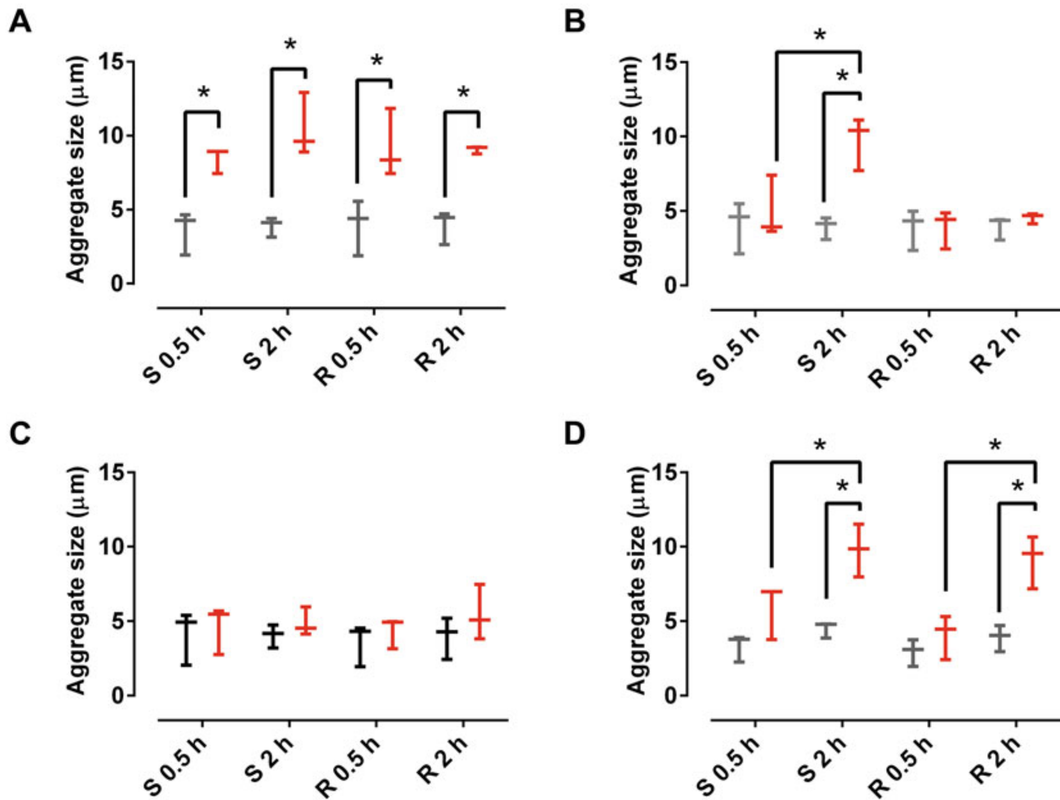

Fig. 4 Average size of enterobacterial aggregates in Ringer solution (black) and with 10% bovine synovial fluid of strains of *E. cloacae* E.cl8 and E.cl 7 (**a**), *E. coli* E.co3 and Eco.4 (**b**), *K. pneumoniae* K.p1 and K.p2 (**c**), and *P. mirabilis* P.m5 and P.m6 (**d**) at 0.5 and 2 h. *p-value<0.05

with previous work, where different grown patterns in synovial fluid were observed for different species (Dastgheyb et al. 2015; Gilbertie et al. 2019; Perez and Patel 2015). Gilbertie et al. observed that after 24 h incubation, non-staphylococcal aggregates were smaller than those formed by *S. aureus*. In our study, while 10% of synovial fluid was able to increase bacteria aggregation in at least one *E. cloacae*, *E. coli* and *P. mirabilis* strain, *K. pneumoniae* did not show this characteristic. SFMA reached steady state at 0.5 h for both *E. cloacae* strains and the antibiotic-susceptible *E. coli* strain, suggesting that the rate of formation was also strain dependent.

Aggregation of both *P. mirabilis* strains increased over time in presence of synovial fluid. This finding implicates the formation of bacterial aggregates as a potential virulence factor of *P. mirabilis* which may contribute to the difficulty associated with diagnosis and treatment of these infections. Early aggregation may explain how in some cases septic arthritis evolves into a chronic infection despite administration of antibiotic therapy. It remains to be seen how aggregate formation may vary with exposure to other host fluids (such as serum). Additionally, future studies are required to assess the influence on susceptibility to natural immunity and antimicrobials as well as the initiation of biofilm formation.

While we acknowledge that our findings that synovial fluid can mediate aggregation in GNE is somewhat confirmatory of other studies, our work expands on the range of species and strains and demonstrates that synovial fluid mediated aggregation is strain dependent. Another important aspect of our work is the development of a relatively simple and cheap rapid screening technique

for synovial fluid mediated aggregation which may be used to further characterize clinical strains allowing possible correlations to be found between aggregation and clinical outcomes.

5 Limitations

While this study displays the capacity of Gram-negative bacteria to aggregate when exposed to bovine synovial fluid, there are limitations to our methodologies which constrain the scope of these findings. First, this research was restricted to a limited number of clinical strains as well as a single type of inoculum growth media. In the future, additional strains need to be included to determine if there are any general correlations in aggregation within and between species and whether antibiotic resistance is related to this phenomenon. Second, we used only 10% bovine synovial fluid supernatant in order to avoid optical interference from fibrous and particulate matter in the synovial fluid. Along with our restriction to relatively short incubation times, it is possible that we may have underestimated the full degree of aggregation and potential size of the aggregates. Therefore, although our rapid screen appears capable of identifying aggregation differences in strains, this methodology may require further refinement. Finally, we used relatively low resolution imaging of the plate reader to semi-quantify SFMA potential through particle size analysis. While we were able to observe clear differences in aggregation patterns in the wells with and without synovial fluid, across 5 of the strains, a higher resolution technique such as confocal microscopy in conjunction with flow cytometry may be required to more precisely size the aggregates. In the future, these more in-depth techniques can be used to "calibrate" the imaging plate reader as a rapid screen method.

6 Conclusions

In conclusion, the ability to form biofilm-like aggregates by enterobacterial species which is mediated by synovial fluid appears to be dependent on strain and time of contact. Further work is required to study the aggregate phenotype and how aggregate formation might play a role in the establishment of GNE biofilm infection in the joint space. Development of a rapid screen assay to semi-quantify SFMA aggregation may be a useful tool to characterize this phenotype in clinical strains and to allow correlations be made between clinical outcomes and aggregation.

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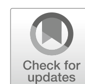

Keyboard Contamination in Intensive Care Unit: Is Cleaning Enough? Prospective Research of *In Situ* Effectiveness of a Tea Tree Oil (KTEO) Film

Gabriele Melegari, Ramona Iseppi, Martina Mariani, Enrico Giuliani, Valeria Caciagli, Elisabetta Bertellini, Patrizia Messi, and Alberto Barbieri

Abstract

After the SARS-CoV-2 pandemic, disinfection practices and microbial load reduction have become even more important and rigorous. To determine the contamination of keyboard surface and the relative risk to transfer healthcare-associated pathogens to susceptible patients, as it frequently happens in Intensive Care Unit (ICU), a standard keyboard (SK), a cleanable keyless keyboard (KK) with smooth surface and a standard keyboard coated with a 3 M Tegaderm® film added with active essential oil (tea tree oil) (KTEO) were tested. *S. aureus*, including MRSA strains, were detected in ICU, with values ranging from 15% to 57%. Gram negative strains belonging to the *Enterobacteriaceae* family were also found with values ranging from 14% to 71%. Similar Gram positive and Gram negative strains were found on all surfaces, but with low percentage, and only environmental bacteria were detected using the settling plates method. The Microbial Challenge Test performed on KTEO showed high rates of decrease for all the pathogens with statistical significance both at 24 and 48 h ($p = 0.003*$ and $p = 0.040*$, respectively). Our results suggest that the use of KTEO may be a feasible strategy for reducing the transmission of pathogens in health care setting and may be complementary to surface cleaning protocols.

Keywords

Challenge test · Essential oil · Intensive care unit · Keyboards · Microbial contamination · Pathogens

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Abbreviations

ATCC	American Type Culture Collection
CI	confidence intervals
CRE	carbapenem-resistant *Enterobacteriaceae*
EO	essential oils
ESBL	extended spectrum beta-lactamase
HAIs	health care-associated infections
ICU	Intensive Care Unit
KK	keyless keyboard
KTEO	standard keyboard plus a daily special cover with 3 M Tegaderm® and active essential oil
MRSA	methicillin-resistant *Staphylococcus aureus*
OR	odds ratio
PCA	plate count agar
SK	standard keyboard
VRE	vancomycin-resistant *Enterococci*

1 Introduction

In the last years, multi-drug-resistant bacteria have risen to be among the most serious threat worldwide, especially when they are implicated in a great variety of environments linked to human's health (Fernando et al. 2017). The One Health program of World Health Organization recognizes that the health of humans, animals and ecosystems are interconnected, with the circulation of antibiotic resistant bacteria not only in hospitals, but also in the community (Stefani et al. 2014; Messi et al. 2015). The environment's role on the spreading of multidrug resistant bacteria responsible of health care-associated infections (HAIs) is a cause of concern, especially for Intensive Care Unit (ICU), where immunocompromised and high susceptible patients are common, increasing the ICU workload (Giuliani et al. 2018). Health professionals and work environments are often implicated, unintentionally, in the transmission of pathogens to patients and the workstation is one of the most important reservoirs of pathogenic bacteria. A significant source of nosocomial bacteria transmission is represented by the computer keyboards, especially in the ICU, where such devices are usually handled by many health workers (Giuliani et al. 2018). Therefore, prevention in the form of a throughout disinfection of the workplace, is the best option to contain the spread of these nosocomial infections. After the SARS-CoV-2 pandemic, disinfection and reduction of microbial load have become central and common practices in everyday life (Bures et al. 2000; Melegari et al. 2020). Effectiveness of cleaning and disinfection practices depends on many factors, including type of pathogen, its microbial load/organization in biofilm, sensitivity to biocides, concentration and time of contact of chemical agent, and last but not least, staff compliance (Almatroudi et al. 2018; Assere et al. 2008; El-Azizi et al. 2016). However, these aspects are often not enough to guarantee hospitalized patients a significant risk reduction of acquiring an infection. Recently, the essential oils (EOs), endowed with strong antibacterial activity, are providing new ways to reduce bacterial contamination in many fields, without using chemical products (Sakkas et al. 2016; Iseppi et al. 2019; Valdivieso-Ugarte et al. 2019; Condò et al. 2020; MacGibeny and Wassef 2020). The removal of possible environmental sources of pathogens becomes even more important in all those situations in which, due to work dynamics, there are both a wide dissemination of pathogens and weaknesses in the contamination prevention system. Therefore, all chemical and physical changes to surfaces capable of reducing microbial contamination are important prevention tools (Adlhart et al. 2018).

The aim of the study was to determine the contamination rate of 4 different keyboard systems four different keyboard systems, a standard keyboard (SK), a cleanable keyless keyboard (KK) with smooth surface, a laser keyboard (LK), and a standard keyboard coated with a 3 M Tegaderm® film added with active essential oil (tea tree oil) (KTEO), and to verify the survival of six healthcare-associated pathogens artificially inoculated on the least contaminated device.

2 Materials and Methods

The research started in September 2018 in the ICU of an Italian hospital (Ospedale Civile Sant'Agostino Estense of Modena, Italy), after Ethical Committee approval (Protocol AUO 0022833/18 of 14/08/2018, Modena, Italy). The study design provided a preliminary phase with an accurate cleaning of ICU (see below), without patients inside. The experiment was divided into 2 parts. The first part was carried out to assess the bacterial load of keyboards surface, and to compare the contamination rates of each device. The second part was conducted on the best performing device emerged with the above study, using a controlled microbial contamination test (Microbial Challenge Test). In both cases the recovery of viable bacteria was performed by Contact Plate method.

2.1 Types of Devices

Three different keyboard systems, daily cleaned following the hospital's protocol (see below), were employed: a standard keyboard (SK), a keyless keyboard (KK) with smooth surface, and a standard keyboard, daily coated with a 3 M Tegaderm® soaked with an active essential oil (tea tree oil-TTO) (KTEO). The essential oil (EO) from *M. alternifolia* (tea tree oil) consists of more than 100 components and its composition has been regulated by the International Standard ISO 4730 (2017): 'EO of *Melaleuca*, terpinen-4-ol type (TTO)' (International Organization for Standardization 2017). TTO contains many sesquiterpenes and monoterpenes, of which terpinen-4-ol is the main component endowed with antimicrobial activity (May et al. 2000; Carson et al. 2006). The compounds in the tea tree essential oil (TTEO) used in this study were purchased in a local herbalist's shop, analyzed and identified by GC-FID and GC-MS. Monoterpenes were the most represented class of volatile compounds, in particular, the most abundant were terpinen-4-ol (43.29%), γ-terpinene (20.16%) e α-terpinene (8.89%). The special antibacterial cover KTEO was prepared by spraying the TTEO on the 3 M Tegaderm® surface and after drying the adhesive sterile film was employed.

2.2 Keyboard Surface Sampling

For the determination of the total microbial count on dry, sanitized surfaces, Contact Plate method was used. Sampling was performed with Plate Count Agar plates (PCA, Biolife, Milan, Italy), added with Lecithin and Polysorbate 80 for disinfectants inactivation. After the usual daily cleaning of ICU, the PCA plates, with a surface area of 23.76 cm^2, were pressed against the keyboard for a few seconds, so that the bacteria colonizing the surface could be transferred to the agar surface. The samples were taken in three different spots of the keyboards for 1 week, twice every day and at a given time of the day (at 8:00 am and 8:00 pm). The ICU workstation was cleaned with hospital's protocol that states a daily use of Taski Profi®, ECOLAB Incidin Oxyfoam® and other products with chlorhexidine. In addition, first thing in the morning, to have a direct assessment of the number of microorganisms hovering in the ICU that could settle on surfaces and objects, PCA settling plates for passive air sampling were exposed to room air for 1 h. Moreover, all types of bacteria isolated from admitted patients were recorded every day for 5 weeks, in order to define every possible correspondence and to further stress the possible role of keyboard as source of transmission. It was necessary just a single infection present in one patient to assign the presence of the bacteria in the ICU. After sampling, contact and settling plates were returned to the laboratory, incubated at 37 °C for 48 h and viable cells counted. All bacteria strains were identified based on gram staining, colony morphology and rapid identification kits (Liofilchem, Teramo, Italy). To detect the presence of antibiotic resistant strains, all *Staphylococcus*, *Enterococcus* and *Enterobacteriaceae* isolates were sub-cultured on the selective media Brilliance MRSA agar, Brilliance VRE agar (Oxoid LTD, Basingstoke, UK),

Brilliance CRE (carbapenem-resistant *Enterobacteriaceae*) agar and Brilliance ESBL (extended-spectrum b-lactamase) agar (Biolife, Milan, Italy), respectively.

2.3 Microbial Challenge Test

To determine the rate of microbial load reduction of KTEO, a controlled contamination test (Microbial Challenge Test) was performed, and the killing effect on the contaminated surface was determined against six microorganisms commonly encountered in health care environments. Both classified bacteria (ATCC – American Type Culture Collection) and antibiotic resistant strains of clinical source were used. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212 and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus faecalis* (VRE) were grown in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, MI), supplemented with 0.6% yeast extract (TSB-YE) (Difco), and kept at 30 °C for 18 h. The device was accurately cleaned and left at room temperature for 1 h to dry. Aliquots (1 mL) of serial dilutions of each strain cultures were sprinkled on the device surface. Firstly, the KTEO was contaminated once and the bacterial load was evaluated over time (48 h) at different intervals, as described above. In a subsequent study, to simulate the determination performed in ICU and to verify the activity for longer, the KTEO was contaminated every 24 h for 3 consecutive times and the bacterial load was evaluated after 5 and 13 h collecting the samples in three different spots of the keyboards.

2.4 Statistical Analysis

The statistical analysis was performed using the STATA 16® program (STATA Corp LP 4905 Lakeway Drive TX 77845 USA); the sample size was calculated with historical data of infections in ICU. The following tests were conducted: a Shapiro-Wilk test was used to verify the normal distribution of the continuous data, Student's t-tests were used for comparisons of normally distributed, continuous variables, and Wilcoxon signed-rank tests were used for not normally distributed variables. The microbial load was estimated with nonlinear regression test also. The associations between variables were calculated by chi-square tests with Fischer's correction applied. Confidence intervals (CIs) were calculated at 95% and were always expressed where the results were significant; in other cases, the data were reported as the mean ± standard error (std). Odds ratios were expressed as ORs and calculated with Fischer's exact test. The comparisons and correlations were considered significant when the applied test presented a p value <0.05.

3 Results

3.1 Keyboard Surface Microbial Load

From October to November 2018 data were collected from 3 different devices: SK, KK and KTEO. As shown in Table 1, different microbial loads among the tested keyboards have been observed, but the variance analysis does not reach the statistical differences. On the other hand, the KTEO device had a lower microbial load, with statistical difference compared to the SK and KK devices.

The microbial load during time is shown in Fig. 1. For the standard keyboard the maximum value recorded in 1 week was 145 UFC/cm^2 and the minimum 0 UFC/cm^2, with average of 16,88 UFC/cm^2; the cleaning keyboard registered a maximum value of 118.9 UFC/cm^2 and the minimum 0 UFC/cm^2, with average of 22.9 UFC/cm^2; the KTEO device recorded a maximum value of 36 UFC/cm^2 and 0 UFC/cm^2, as minimum, with average of 12.5 UFC/cm^2. KTEO device showed the lower microbial load in 1 week. Figure 2 shows the percentage of pathogens isolated in ICU and on the device surfaces used in the study. In the first week, during the determination

Table 1 Microbial load detected on the surface of each keyboard. The KTEO device shows a lower microbial load than standard and cleaning ones

Keyboard	Observations	Microbial load average (UFC/cm^2)	Standard deviations	Confidence interval 95%	p-value
SK	41	16.88	±3.79	9.22–24.54	**SK** vs **KK p 0.144**
KK	42	22.90	±4.04	14.74–31.07	**KK** vs **KTEO p 0.00***
KTEO	42	12.5	±1.99	8.48–16.52	**SK** vs **KTEO p 0.033***

Variance analysis p value 0.09

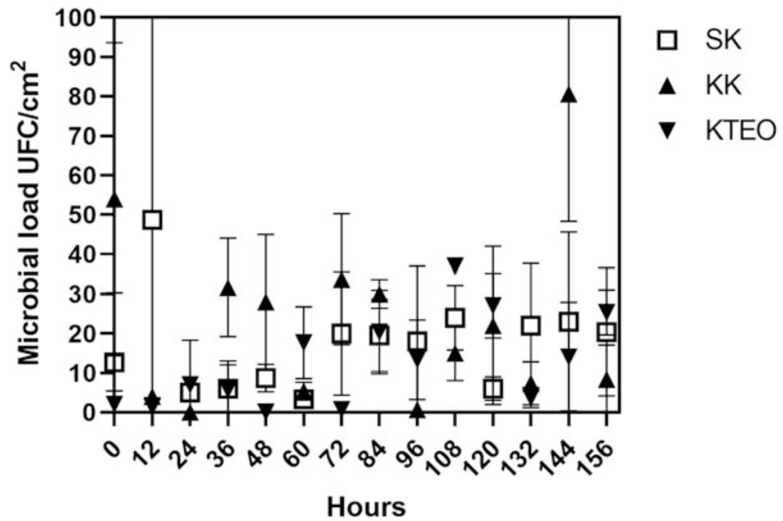

Fig. 1 Microbial load during time of experiments. For the SK the maximum value recorded in 1 week was 145 UFC/cm^2 and the minimum 0 UFC/cm^2, with average of 16.88 UFC/cm^2; the KK registered a maximum value of 118.9 UFC/cm^2 and the minimum 0 UFC/cm^2, with average of 22.9 UFC/cm^2; the KTEO device showed the lower microbial load, with a maximum value of 36 UFC/cm^2 and 0 UFC/cm^2 as minimum, and average of 12.5 UFC/cm^2

of the SK microbial load, the bacteria detected in the ICU was 29% MRSA, 14% *E. coli*, and in the remaining 57% non-pathogens were detected. At the same time, only environmental bacteria were detected in the settling plates, such as *Bacillus firmus*, *Bacillus megaterium* e *Brevibacillus laterosporus*, with average of 6.43 of UFC/m^3. Regarding the SK surface contamination, *S. aureus* (26%), *S. epidermidis* (7%), and the same percentage for *S. aureus* e/o *S. epidermidis* plus other pathogens (7%) were recovered. In the remaining 38% non-pathogens were found. The following week, during the observation of the KK, data collection in the ICU showed the presence of MRSA (15%), susceptible *S. aureus* (57%) and, among gram negative bacteria, *Proteus mirabilis* (71%) and *Serratia marcescens* (14%). In the remaining (14%) non-pathogens were detected. The environmental settling plates recorded bacteria without clinical relevance only, with average of 13 UFC/m^3, value similar to the previous week. Regarding the contamination of the KK surface, the following bacteria were isolated: *S. aureus* (19%), *S. epidermidis* (7%), *Enterobacteriaceae* (5%), *S. aureus* and *S. epidermidis* plus others 33%, and no pathogens in the remaining 36%. Lastly, during the week in which KTEO device was employed and valued, in the ICU the presence of MRSA (29%) and *C. difficile* (71%) was shown. The environmental settling plates recorded only bacteria without clinical significance, with average of 11.71 UFC/m^3. The device KTEO surface presented the lowest value in pathogens contamination, with *S. aureus*, *S. epidermidis*, *Enterobacteriaceae* and *S. aureus* and/or *S. epidermidis* detected in low percentage (12%, 2%, 12%; 5%, respectively), whereas in the remaining 69% no pathogen was isolated.

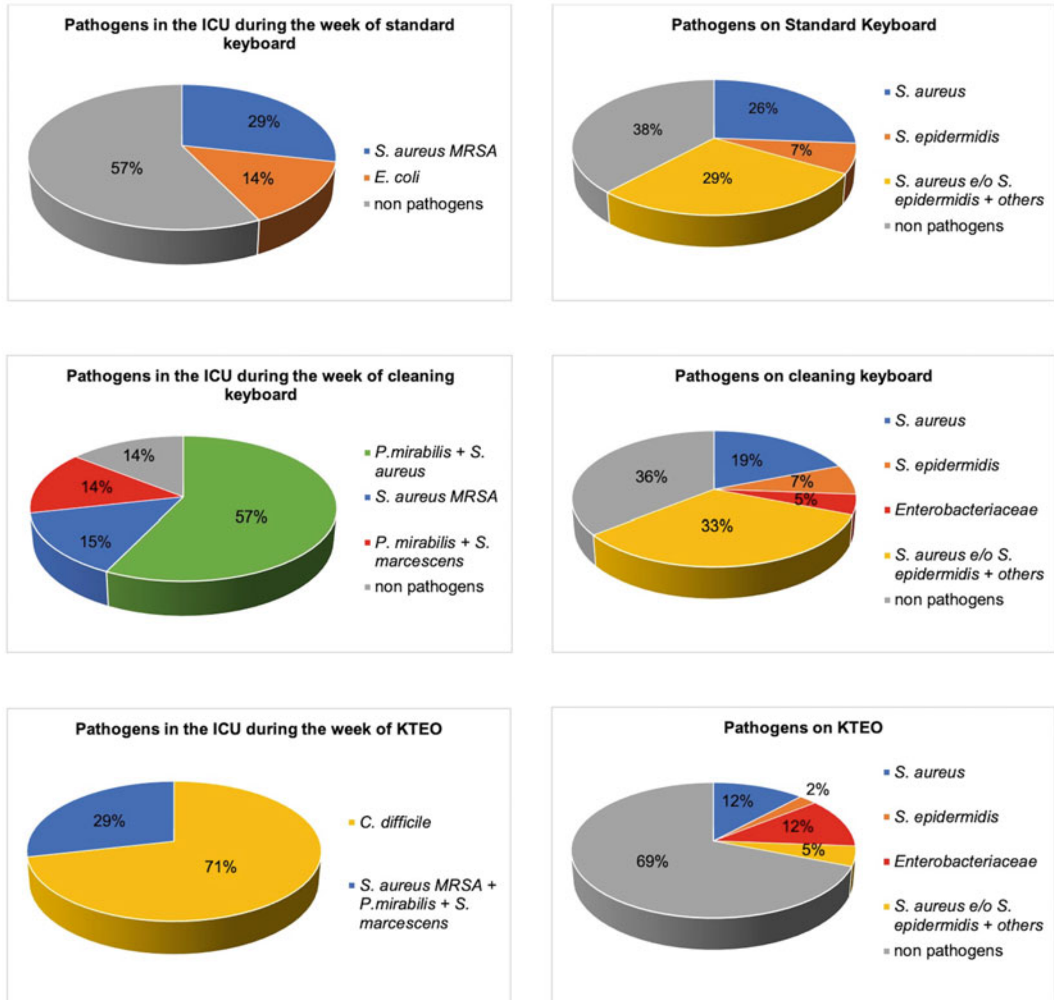

Fig. 2 Percentage of pathogens and non-pathogens found in ICU and on devices surface. The KTEO surface presented the lowest value in pathogens contamination

All microbial loads were also tested with non-linear regression, every time the fitting measured as R^2 was lower than 0.5 and p value higher than 0.05. The peak concentration reached was lower for KTEO than KK and SK. As shown above, the KTEO device demonstrated the lower presence of pathogens, measured such a presence of pathogens at the least in one bacterial plate, with p value of 0.007. Figure 3 shows the microbial load of pathogens on device surfaces and correspondence to infections found in ICU. The relationship between the presence of pathogens in the ICU and pathogens on the device was lower for the KTEO device, with p value of 0.004. The correspondence was measured such as finding of the same bacterial strain both on the device and in the ICU. In Fig. 4a–c some examples of bacterial plate count collected on SK, KK and KTEO device, respectively. The KTEO device was again the least contaminated.

3.2 Microbial Challenge Test

Microbiological challenge investigation is useful in determining the ability of microorganisms to grow in an artificially contaminated surface. The study was performed on the KTEO device

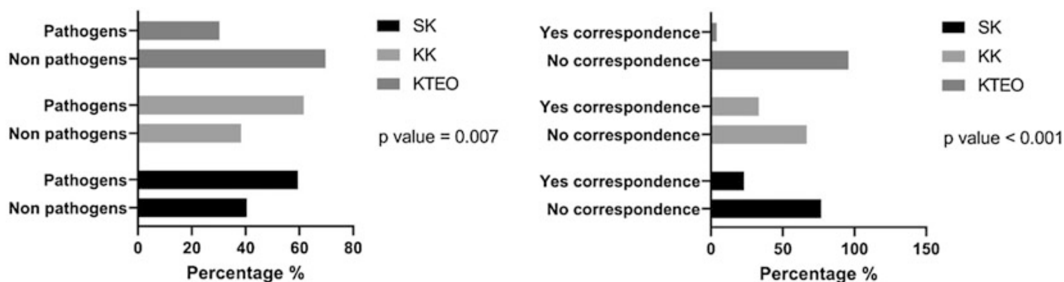

Fig. 3 Presence of pathogens on device surfaces and correspondence to infections found in ICU. The KTEO presented the lowest value in pathogens contamination

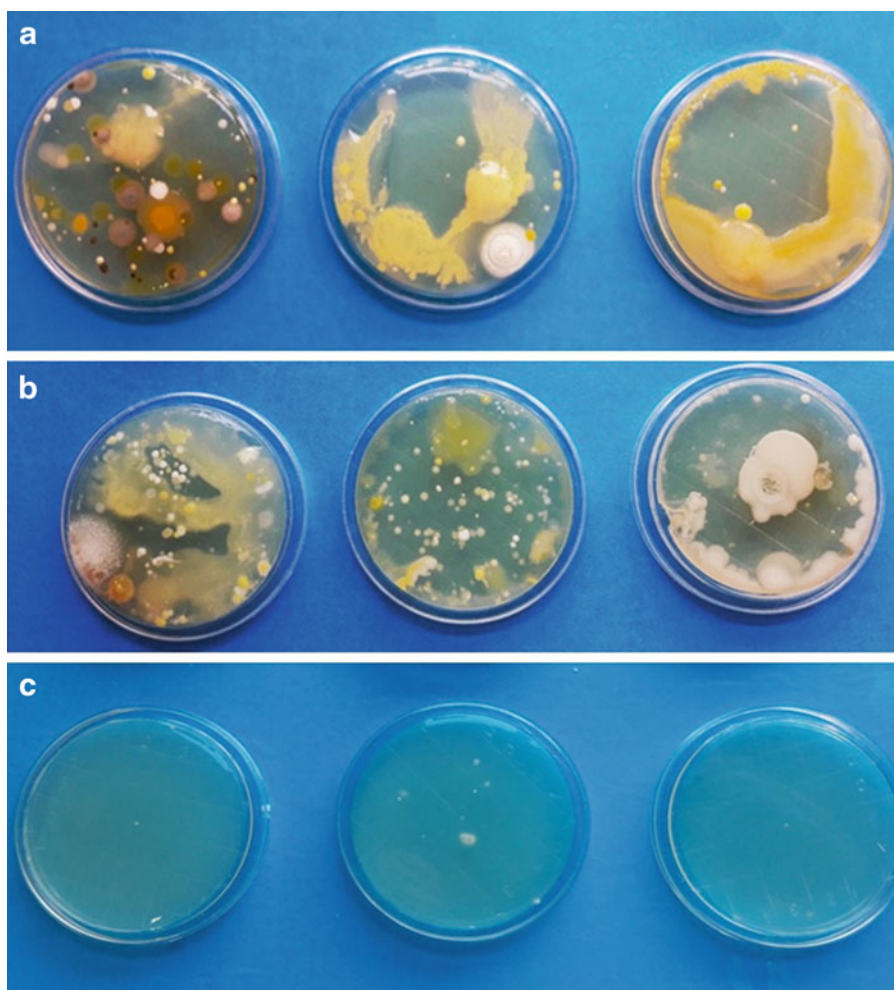

Fig. 4 **a**-**c** Examples of bacterial growth on PCA plates used for surface microbial sampling on SK (**a**), KK (**b**) and KTEO (**c**) devices, respectively. The sampling was taken in three different spots of the keyboards at two times of the day (at 8:00 am and 8:00 pm). The KTEO device was again the least contaminated one

because it showed the lowest microbial load. As shown in Tables 2a and 2b, the results proved high rates of decrease for all the six employed pathogens, despite the forcing bacterial contamination done every 24–48 h, with statistical significance both at 24 h ($p = 0.003^*$) and 48 h ($p = 0.040^*$).

Firstly, the antibacterial activity of KTEO was determined over time on the contaminated surface, and the viable counts of the tested strains were assessed up to 48 h. As shown in Table 2a, a decrease in the viable counts compared to the control, ranging from 3 log to 4.5 log, was observed for all the strains at the last determination (48 h). In particular, *E. coli* ATCC 25922 showed the greatest decrease, with 4 log reduction already after 24 h, followed by *E. coli* ESBL and, in the subsequent times, by the *S. aureus* strains, while both *E. faecalis* ATCC 29212 and VRE showed to be the least sensitive strains. Table 2b shows the results of the study carried out with daily contaminations for 3 days of the KTEO, with the samples collected in three different spots of the keyboards every 5 and 13 h. At the last determination of the first day, we observed a reduction ranging from 1 log to 2.5 log for *E. coli* ATCC 25922, ESBL-producing *E. coli* and *S. aureus* ATCC 6538, and the decrease was less evident for the remaining strains. Furthermore, the reduction in bacterial load has continued over time with trends similar to the previous ones.

4 Discussion

The ideal keyboard should be easy to sanitize, simple to use and not provide a suitable surface for microbial development. It has been shown that a flat profile, the presence of an alarm to indicate the need for cleaning and a surface coating are important features for maintaining a low microbial count (Wilson et al. 2008). Martin et al. (2011) have established that the use of a UV lamp ensures adequate effectiveness in reducing bacterial contamination; on 67% of the irradiated keyboards no pathogens were detected in significant quantities, much better than the devices used as a control (Martin et al. 2011). However, the cost/benefit *ratio* deriving from the massive use of this model should be investigated (Boyce et al. 2011; Gostine et al. 2016).

The present investigation confirms the important role of contaminated environmental surfaces

Table 2a Bacterial decrease with forced contamination at 48 h

Bacteria load UFC/cm^2/h	Time 0	12 h	24 h	36 h	48 h	p-value
***E. coli* ATCC 25922**	100,000	26	9	9	2	**0.040***
***E. coli* ESBL**	100,000	10,000	1000	44	40	**0.040***
***S. aureus* ATCC 6538**	1,000,000	100,000	10,000	168	151	**0.040***
***S. aureus* MRSA**	1,000,000	100,000	10,000	355	293	**0.040***
***E. faecalis* ATCC 29212**	1,000,000	100,000	10,000	1000	1000	**0.040***
***E. faecalis* VRE**	1,000,000	100,000	10,000	1000	1000	**0.040***

Table 2b Bacterial decrease with forced contamination every 24 h

Bacteria load/time UFC/cm^2/h	Time 0	5 h	13 h	Time 0 24 h	29 h	36 h	Time 0 48 h	53 h	60h	p-value
***E. coli* ATCC 25922**	161	33	8	100,000	6	0	100,000	42	2	**0.003***
***E. coli* ESBL**	326	107	0	100,000	36	20	100,000	92	7	**0.003***
***S. aureus* ATCC 6538**	738	542	85	100,000	168	92	100,000	207	121	**0.003***
***S. aureus* MRSA**	100,000	10,000	328	100,000	213	109	100,000	1782	223	**0.003***
***E. faecalis* ATCC 29212**	100,000	10,000	1000	100,000	10,000	1000	100,000	10,000	1000	**0.003***
***E. faecalis* VRE**	100,000	10,000	1000	100,000	10,000	1000	100,000	10,000	1000	**0.003***

in the transmission of healthcare-associated pathogens, also endowed with antibiotic-resistance feature, as widely reported. *S.aureus* in particular was constantly present in all surfaces sampled, with frequency of contamination similar to other published reports (Martin et al. 2011; Gostine et al. 2016). The research has excluded any possible role of airborne contamination, measuring the air microbial load we only found non-pathogenic environmental microorganisms. In fact, as demonstrated by our study, bacteria on keyboards are mostly related to strains of human source transmitted with contact, therefore related to cross-contamination throughout the keyboard surface, frequently touched by many and different healthcare workers.

Several studies have demonstrated the capability of some pathogens, like *S.aureus*, to remain viable on a variety of dry surfaces up to 14 days. We have investigated the possibility of using new methods to reduce the transfer of germs from the environment and from the staff to the computer keyboard and vice versa and, consequently, to hospitalized patients. Due to the high frequency of contamination caused by the repeated contact with the hands of hospital staff, keyboards act as a reservoir of nosocomial pathogens, often resistant to antibiotics, and could be an effective vector for cross-transmission (Schultz et al. 2003; Boyce 2007; Boyce et al. 2011; Costa et al. 2019). The removal of any sources of pathogens becomes even more important in all those situations in which, due to working dynamics, there are weaknesses in the contamination prevention protocols (Das et al. 2018; Hayden et al. 2018). In the present study the KTEO device, composed by a standard keyboard covered with Tegadaderm® and TTEO on the top, showed a bacterial colony count reduction of 26% and 45% compared to SK and KK, respectively. A much lower contamination of the KTEO by pathogenic strains, compared to the other two devices was also observed: the presence of microorganisms was only 31% with an absolute improving effect of 30% and relative of 50% (Kang et al. 2012; Russotto et al. 2015; Hayden et al. 2018). Furthermore, it is important to underline how the use of the keyboard added with the natural antibacterial compound (KTEO) has led to a six-fold reduction in the rate of infections caused by the same nosocomial pathogens isolated from the device. The correspondence between the microorganisms found in the ICU and those isolated from the samples, indicates that the transfer rate from KTEO was 5% only, with a relative decreasing of about 5 and more than 11 times compared to the SK and KK, respectively. The KK showed the worst result, probably because the washing method does not guarantee adequate disinfection, facilitating the spread of microorganisms on the surface, the proliferation of which would be favored by the residual humidity. Hand washing and drying has been shown to reduce the release of bacteria by more than ten times; according to this principle, a keyboard surface that remained wet after cleaning could be the worst reservoir of pathogenic microorganisms (Suetens et al. 2018). With regard to the disinfection practices, the continuous exposure to antimicrobial agents, found in sublethal dose in soaps and hand disinfectants, promotes the development of new resistance in the main pathogens responsible for healthcare acquired infections, with a substantial increase in the microbial load of pathogens after only an overnight exposure. For these reasons, the diffusion and optimization of antiseptic rotation programs play a fundamental role in order to derive a concrete benefit in terms of health (Duckworth and Jordens 1990; Ataee et al. 2017; Nasr et al. 2018), hence the need to explore innovative sanitizing procedures and antibacterial materials to tackle this problem more effectively (Kampf 2016). To this direction, a safe and environmentally friendly alternative may be the use of natural compound as EOs, that have already been successfully evaluated as antibacterial agents. The synergism between antibiotics and phytochemicals and the capability in modulating bacterial drug resistance was also recently reported, thus corroborating the antimicrobial potentiality of these natural compounds. TTO in particular, extracted from *Melaleuca alternifolia* has attracted the attention of the scientific community due to its unique chemical properties and broad-spectrum of activity against

many human pathogens (Russell 2002). Furthermore, Garozzo et al., demonstrated a potential role of TTO in reducing viral load against influenza virus, this aspect should be investigated in the future, to limit a viral outbreak, as happened during the pandemic event of 2020 (Garozzo et al. 2011; Yap et al. 2014). The employment of EOs could be a valuable resource in the SARS-CoV-2 pandemic also. A recent study suggests the use of essential oil from *Melaleuca cajuputi*, a plant of the same genus of *Melaleuca alternifolia* (TTO), in preventing SARS-CoV-2 invasion into human body infections and limiting the spread of the virus (My et al. 2020).

5 Conclusion

In the era of multidrug resistant bacteria, it became mandatory to preserve life-saving antibiotics, especially in wards with critical and vulnerable patients, in which even less aggressive bacteria can be a danger to health. The workplace is just one of many foci that can be a vehicle for microbial contamination. The correct use of the phonendoscope, often subjected to an inadequate disinfection, is an example of how many simple precautions should be put in place to limit the spread of pathogenic strains, sometimes even multiresistant. Our results suggest that the KTEO device, composed by a standard keyboard covered with Tegadaderm® and TTEO on the top may be a feasible strategy for reducing the transmission of pathogens in health care settings. The regular use of this antibacterial cover may be complementary to surface cleaning protocols, thus improving the protection against bacterial transmission when compliance with surface cleaning protocols is not sufficient for the problem.

Further studies will be necessary to better define the role of keyboard and other surfaces handled by more workers in the diffusion of pathogens, with the objective to improve the level of protection against bacterial responsible for HAIs, in particular in the ICU, where immunocompromised and high susceptible patients are commonly present.

6 Strength and Limitations of the Research

The study was not blind and to exclude any type of error such as a possible different use of the device, a laboratory check was planned at the end of the research, with forced contamination. It is important to note that at the beginning of the research the device with a lower rate of bacterial load was not known. The staff who collected the plates were different from those who analyzed the bacterial load.

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Conflict of Interest Statement The author reports no conflicts of interest in this work.

Ethics The research was conducted after Ethics approval by Ethics Committee.

Consort Guidelines The research followed Consort Guidelines

Dataset Dataset is available upon request.

Authors Contributions Enrico Giuliani, PhD, the conception and design of the study, final approval

Alberto Barbieri, Prof, the conception and design of the study, final approval

Martina Mariani,MD, acquisition of data

Valeria Caciagli, MD, acquisition of data

Ramona Iseppi, PhD, analysis and interpretation of data, drafting the article

Gabriele Melegari, MD, analysis and interpretation of data, drafting the article

Patrizia Messi, Prof, analysis and interpretation of data, drafting the article

Elisabetta Bertellini, MD, revising it critically for important intellectual content

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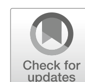

Procalcitonin in the Assessment of Ventilator Associated Pneumonia: A Systematic Review

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Abstract

Background Ventilator-associated pneumonia (VAP) is one of the most common nosocomial infection, associated with considerable mortality and morbidity in critically ill patients; however, its diagnosis and management remain challenging since clinical assessment is often poorly reliable. The aim of this systematic review was to evaluate the role of PCT in the diagnosis and management of critical ill patients affected by VAP.

Methods We performed a systematic review of the evidence published over the last 10 years and currently available in medical literature search databases (Pubmed, Embase, Web of Knowledge, Cochrane Libraries) and searching clinical trial registries. We regarded as predefined outcomes the role of PCT in diagnosis, therapeutic monitoring, antibiotic discontinuation and prognosis. The Open Science Framework Registration number was doi.org/10.17605/OSF.IO/ZGFKQ

Results 761 articles were retrieved and a total of 18 studies (*n° of patients* = 1774) were selected and analyzed according to inclusion criteria. In this 2020 update, the systematic review showed that currently, conflicting and inconclusive data are available about the role of PCT in the diagnosis of VAP and in the prediction (i) of the efficacy of antibiotic therapy, and (ii) of the clinical outcome. These studies, instead, seem to agree on the utility of PCT in the management of antibiotic therapy discontinuation.

Conclusions Currently there is insufficient evidence to support the role of PCT in the routine assessment of patients with VAP. The value of the results published appears to be limited by the deep methodological differences that characterize the various studies available at the present being.

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Keywords

Procalcitonin · PCT · Ventilator associated pneumonia · VAP · Pneumonia · Systematic review · ICU · Intensive care unit · Critical care · Driven discontinuation · Antibiotic therapy · Antibiotic stewardship

1 Introduction

Ventilator associated pneumonia (VAP) is one of the most common hospital-acquired infection occurring in the intensive care unit (ICU) and is associated with a high morbidity and mortality in critically ill patients (Melsen et al. 2013; Torres et al. 2017a). Nevertheless, in the absence of a gold standard for the diagnosis of VAP, the clinician often relies on Johanson clinical criteria (new or progressive infiltrate on chest radiograph along with at least two of the following criteria: fever, leukocytosis or purulent tracheobronchial secretions) that resulted in only 72% specificity and 69% sensitivity when compared with autopsy data (Rea-Neto et al. 2008). For these reasons, early identification and risk stratification (but also therapeutic management and prognosis) remain challenges for clinicians (Woodhead et al. 2011; Tablan et al. 2004; Fagon et al. 2000).

Over the last years, different blood biomarkers have been identified and analyzed to improve the management of patients with severe pneumonia (Palazzo et al. 2011; Hohenthal et al. 2009; Póvoa et al. 2006). In bacterial infections and sepsis, procalcitonin (PCT), the calcitonin prohormone, has emerged as the most investigated and promising blood biomarker (van Vugt et al. 2013; Schroeder et al. 2009), but its role in the management of pneumonia remains under evaluation (Luyt et al. 2005). Furthermore, there is a growing interest towards the role of PCT in fungal infections (Cortegiani et al. 2019). Despite PCT has been approved by the US Food and Drug Administration (FDA) as a useful tool to guide antibiotic therapy in the suspicion of lower respiratory tract infections (Assicot et al. 1993; Muller et al. 2007; FDA News Release 2017), the performance of this biomarker is mainly affected by the etiologies, pathogenic mechanisms and epidemiological characteristics of pneumonia. In particular, the role of PCT in the management of patients with VAP is currently unclear and many questions have been felt unanswered.

The aim of this systematic review is to evaluate the actual role of PCT in the diagnosis and management of critical ill patients affected by VAP. We reported as predefined outcomes the role of PCT in diagnosis, therapeutic monitoring, antibiotic discontinuation and prognosis in patients with VAP.

2 Methods

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement recommendations (Moher et al. 2009; Liberati et al. 2009). Search terms were formulated using the PICO structure. Participants included adults (>18 years old) with VAP or suspected VAP. We included studies that focused on serum PCT levels in adult patients with diagnosis of VAP. Comparisons broadly addressed PCT dosage in VAP versus other indicators of pneumonia. Outcomes included the role of PCT in diagnosis of VAP, in predicting the efficacy of antibiotic therapy, in discontinuation of antibiotic; PCT as predictor of clinical outcome. Study designed selected were: Randomized control trials and observational studies.

The PubMed, Embase, Web of Knowledge and the Cochrane Libraries database were used to identify all English language articles using the following key words: "ventilator associated pneumonia/VAP and procalcitonin/PCT", "ventilator acquired pneumonia and procalcitonin/PCT", "ventilator acquired pneumonia and procalcitonin", "nosocomial respiratory infection and procalcitonin/PCT", "respiratory infection in ICU and procalcitonin/PCT" or "pneumonia in intensive care unit/ICU and procalcitonin/PCT". Only papers published from 2009 to 2019 were considered suitable for reviewing (last update in 10 January 2020).

All papers classified as Classical Articles, Clinical Studies, Clinical Trials, Clinical Trial Protocols, Clinical Trial, Phase I, Clinical Trial, Phase II, Clinical Trial, Phase III, Clinical Trial, Phase IV, Controlled Clinical Trial, Randomized Clinical Trials (RCTs) evaluating the topic were considered as eligible for this systematic review. Moreover, manual searches of potentially relevant papers from reference lists were performed to identify additional eligible articles.

The ClinicalTrials.gov website, the European Union Clinical Trials Register, the ICH GCP Clinical Trials Registry, the Chinese Clinical Trial Registry, the Thai Clinical Trials Registry, the Australian New Zealand Clinical Trial Registry, the International Clinical Trials Registry Platform (ICTRP), the Cochrane Central Register of Controlled Trials were online consulted for characteristics of currently unpublished RCTs investigating VAP and PCT (last update in 10 January 2020).

Finally, articles were independently screened by two authors (F.A. and G.C.). Full texts (and clinical trial registry data (ClinicalTrials.gov website) of all potentially relevant studies were analyzed to assess eligibility. Disagreements on the inclusion/exclusion of studies were resolved by discussion and consensus with other coauthors. Duplicates were removed. The study selection process is detailed in a flow diagram (Fig. 1).

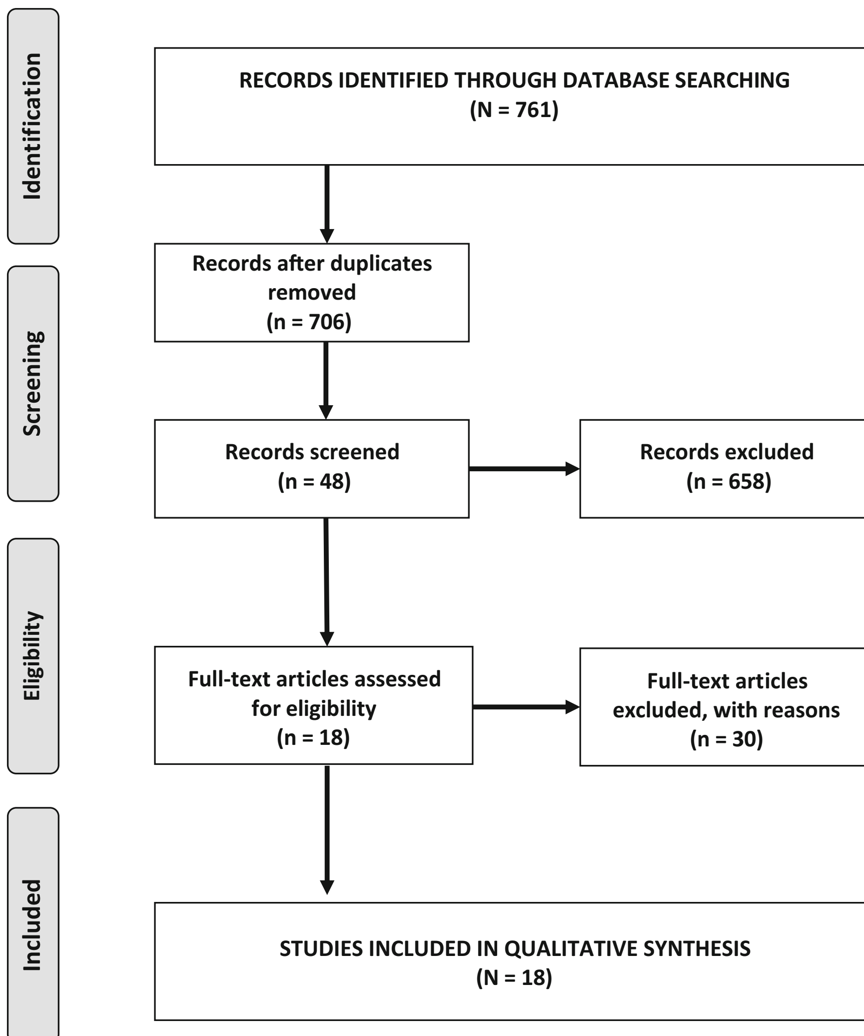

Fig. 1 The study selection process

Registration number on The Open Science Framework (OFS) Register was doi.org/10.17605/OSF.IO/ZGFKQ

3 Results

Literature search led to retrieve a total of 761 studies; after the screening for eligibility, 743 studies were excluded as they did not match the inclusion criteria. A total of 18 studies (involving a total of 1774 patients), were selected and analyzed (Fig. 1).

3.1 The Role of PCT in Diagnosis of VAP

The role of PCT in diagnosis of VAP actually remains an open issue: conflicting data are available especially with regard to the ability of PCT alone to point the diagnosis of VAP. In particular, in a pilot study carried out in 49 adult trauma patients, serum PCT was unable to discriminate between the VAP and non-VAP groups either at day 0 or day 3 (Habib et al. 2016). Similar results were obtained in a prospective, multi-center, observational study involving subjects receiving mechanical ventilation for more than 72 h: in the 138 patients (but only 35 with VAP) enrolled PCT showed a poor predictive performance (Póvoa et al. 2016).

In a more recent pilot study designed to evaluate Histidine-Rich Glycoprotein (HRG), a novel serum biomarker tested for both diagnosis and prognosis of VAP in a group of 186 patients mechanically ventilated with symptoms suggestive of pneumonia, PCT levels measured 72 h after intubation were significantly higher ($p < 0.001$) in VAP group than in no-VAP group (Ding et al. 2018). This data was confirmed also by a small observational study performed to evaluate the diagnostic efficacy of serum PCT in the VAP early diagnosis: in this setting, Clinical Pulmonary Infection Score (CPIS) and/or PCT serum levels were confirmed significantly higher in VAP patients (Chen et al. 2018).

On the other hand, interesting data were obtained in studies evaluating the value of PCT in combination with other markers in the diagnosis of VAP: in a single center prospective observational study recruiting 91 patients, a seven-biomarker panel called Bioscore (BALF/blood ratio mTREM-1 and mCD11b, BALF sTREM-1, IL-8 and IL-1b, and serum CRP and IL-6) was reported to be able to correctly identify 88.9% of VAP cases and 100% of non-VAP cases (Grover et al. 2014). Similarly, Zhou et al., analyzing 124 subjects with suspected VAP in a prospective study, demonstrated that PCT of $\geq$0.25 ng/mL combined with positive lung ultrasound was accurate in the diagnosis of VAP with a sensitivity and specificity of 81.3 and 85.5% respectively (Zhou et al. 2019).

With regard to the chance of simplifying the differential diagnosis between ventilator-associated tracheobronchitis (VAT) and VAP, PCT does not allow adequate discrimination between the two clinical conditions as has been shown by a pre-planned analysis of 404 out of 2,960 patients receiving mechanical ventilation for >48 h enrolled in the prospective multinational TAVeM database collecting data from 114 ICUs (Coelho et al. 2018).

3.2 The Role of PCT in Predicting the Efficacy of Antibiotic Therapy in Patients Suffering from VAP

An analytical and descriptive study conducted in 50 patients with VAP was available on this topic. Serum PCT levels were assessed before and during (every 48 h) antibiotic therapy. Authors reported that PCT serum levels, while over the normal values before starting the therapy, showed a progressive and significant drop after the first 48 h. On the grounds of these results, the authors concluded that PCT could be used as a tool for evaluating and predicting the effect of antibiotic therapy in this setting (Kiaei et al. 2015). On the contrary, no association was found between adequacy of antibiotic treatment and PCT concentration in a prospective, observational study carried

out on 34 non-surgical patients with early onset VAP (Zielinska-Borkowska et al. 2012).

3.3 The Role of PCT in Discontinuation of Antibiotic Therapy

Three studies on PCT and discontinuation of antibiotic therapy were selected. The first one concerned a prospective no-randomized no-controlled trial aiming to evaluate a protocol using a spot PCT assessment combined with CPIS to guide antibiotics discontinuation in patients with VAP caused by non-fermentative gram-negative bacilli. According with the results of the study, this strategy appeared effective in its purpose (Wongsurakiat and Tulatamakit 2018). In a second multi-center, randomized, controlled trial PCT reduced by 27% the time spent on antibiotic therapy in comparison to duration of the antibiotic course of therapy suggested by guidelines in patients with VAP (Stolz et al. 2009). Recently, a 5-year prospective cohort study involving 157 patients with microbiologically-proven VAP, PCT was effective in the reduction of time on antibiotic therapy when used in accordance with a PCT-guided algorithm (antibiotic withdrawal strongly encouraged if PCT < 0.5 ng/mL or < 80% peak value) (Florence et al. 2019).

3.4 PCT Driven Discontinuation of Antibiotic Therapy and Risk of VAP Recurrence

Only one trial, designed to predict and evaluate the risk of VAP recurrence following PCT-guided antibiotic discontinuation, was available: this single arm observational study carried out on 51 patients with VAP showed that even though PCT is effective in the evaluation of antibiotic interruption, the risk of relapse is significative higher when CPIS≥5. Moreover, CPIS and the tracheal secretions characteristics were independent risk factors accounting for infection recurrence (Wang et al. 2019).

3.5 PCT as Predictor of Clinical Outcome

Six studies investigated the role of PCT as predictor of outcome in patients with VAP. The BioVAP study (Biomarkers in the diagnosis and management of Ventilator-Associated Pneumonia) is a prospective, multi-center, observational study, designed to evaluate whether biomarkers may add additional information in the clinical decision-making process of VAP at the bedside. In the sub-group analysis of 37 patients with microbiologically documented VAP, PCT kinetics during the first 6 days of antibiotic therapy did not succeed in differentiating VAP survivors from non-survivors (Póvoa et al. 2017). Likewise, in the study of Hillas G. et al. conducted on 45 adult patients with VAP, neither threshold values or PCT kinetic were able to predict VAP survival or septic shock development (Hillas et al. 2010). Those data are in tune with the results of a prospective observational study conducted to assess the clinical usefulness of monitoring PCT concentrations in 34 non-surgical patients with diagnosis of early onset VAP: in this setting also, PCT was found to be a poor predictor of mortality or development of septic shock (Zielinska-Borkowska et al. 2012).

On the contrary, Seligman R. et al. demonstrated that serum PCT was able to predict mortality in a cohort of 71 patients with VAP enrolled in an observational single center study: PCT decreased as the infection was kept under control and turned out to be an effective biomarker for mortality prediction, showing the highest accuracy on day 4 of treatment (Seligman et al. 2011). Moreover, in a prospective observational study involving 92 patients, PCT levels combined with the pulmonary infection score were reported to be useful in the prognostic assessment and effective to evaluate 28-day survival (Su et al. 2012). Finally, in a small study aiming to evaluate the prognostic value of PCT kinetic on 45 critically ill patients who developed VAP, serum PCT level above 1 ng/mL on day 3 of therapy was found to be a strong predictor of mortality, with an odds ratio of 22.6 (Tanrıverdi et al. 2015).

3.6 Ongoing Researches

The ClinicalTrials.gov website, the European Union Clinical Trials Register, the ICH GCP Clinical Trials Registry, the Australian New Zealand Clinical Trial Registry, the Chinese Clinical Trial Registry, the Thai Clinical Trials Registry, the International Clinical Trials Registry Platform (ICTRP), the Cochrane Central Register of Controlled Trials were consulted online on January 2020 using the following key words: "ventilator associated pneumonia and procalcitonin", "ventilator acquired pneumonia and procalcitonin", "VAP and PCT". Five ongoing clinical trials have been designed to investigate the role of PCT in VAP, in particularly about the PCT-guided antibiotic stewardship (Table 1).

4 Discussion

Both the 2016 IDSA/ATS and the 2017/2018 ERS/ESICM/ESCMID/Asociación Latino Americana de Tórax guidelines for the management of adults with hospital-acquired pneumonia/ventilator-associated pneumonia (HAP/VAP) recommend the use clinical criteria alone for diagnosis, rather than biomarkers such as PCT (Kalil et al. 2016a, b; Martin-Loeches et al. 2018; Torres et al. 2017b, 2018). Anyway, although a regular use of biomarkers is not advised in the VAP management, these guidelines suggest that clinicians may still rely on PCT in specific circumstances (Torres et al. 2018; Dianti and Luna 2018). In particular, in the event of highly antibiotic-resistant pathogens, inadequate initial or second-line antibiotic treatment, treatment failure, severely immunocompromised patients (due to neutropenia or stem cell transplant), PCT may be useful to assess the efficacy and duration of antibiotic treatment (Torres et al. 2018). Moreover, a meta-analysis on the relationship between PCTs and severe pneumonia published in 2016 reported that elevated PCT levels were associated with an increased risk of mortality in critically ill patients with VAP, although the small number of heterogeneous studies available (Liu et al. 2016).

Considering the increasing number of the papers recently published on this topic, in this systematic review we reported a 2020 update on the role of PCT in the assessment of VAP: our analysis showed that currently, conflicting and inconclusive data are available about the role of PCT in the diagnosis of VAP and in the prediction (i) of the efficacy of antibiotic therapy, and (ii) of the clinical outcome. A small number of studies, instead, seem to agree on the utility of PCT in the management of antibiotic therapy discontinuation. These results seem to advise against the extensive use of PCT in the management of VAP, limiting its role to a general indicator not specific for this pathology. The available evidence does not definitively contraindicate the use of PCT in patients with VAP but the judgment on its clinical utility is subject to the availability of new large randomized studies.

The interest in evaluating PCT in the management of patients suffering from VAP is principally related to the need of an effective marker able to minimize the consequences of antibiotic overuse in critical care, including, adverse effects, antibiotic resistance and related costs. Therefore, many studies have been designed and carried out on this topic; however, the value of the results achieved and published appears to be limited by the deep methodological differences that characterize the various studies available at the present being. For this reason, determining in what circumstances the PCT may be useful or not in the management of the VAP is not the focus of the topic. In fact, the validity of this biomarker in severe pneumonia is undermined by a more basic questions, namely if the extremely heterogeneous results currently available depend on methodological study issues. In this prospective, in a recent commentary entitled "Do we need new trials of procalcitonin guided antibiotic therapy?", Lisboa et al. underlined that after more than a decade of studies on the topic, the evaluation of PCT-guided antibiotic stewardship remains burdened by significant limitations in trial designs.

Table 1 Ongoing studies on PCT and VAP (capitation on date 10 January 2020)

Title Sponsor Register	Characteristic of the study	Purpose	Primary outcome measures	Secondary outcome measures
Efficacy and safety of point- of-care Procalcitonin test to reduce antibiotic exposure in ventilator associated pneumonia (VAP) patient in ICU: A randomised controlled trial	***Study Type:*** Interventional	To address efficacy, safety and cost analysis of PCT-guided antibiotic therapy in severe VAP patients	To compare the duration of antibiotic treatment between PCT and standard of care groups	To compare the mortality between PCT and standard of care
Sponsor: University of Science Malaysia	***Estimated Enrollment:*** 100 participants			To compare the rate of recurrence infection between PCT and standard of care
Register: ClinicalTrials.gov Id. NCT03982667	***Allocation:*** Randomized			
	Intervention Model: Parallel assignment			
A randomized clinical trial of 4 vs. 8 days of definitive antibiotic therapy for early ventilator-associated pneumonia in the surgical intensive care unit	***Study Type:*** Interventional	To evaluate if 4 days of antibiotic therapy, as compared to 8 days, is equally effective and results in decreased antibiotic exposure among surgical ICU patients with early VAP.	Clinical response CPIS (at 28 days)	Biomarker response: Procalcitonin (daily for either 4 or 8 days, depen-dent upon treatment arm)
Sponsor: Denver Health and Hospital Authority	***Estimated Enrollment:*** 100 participants			Microbiologic response at 1 day after last day of treatment: BAP culture <10^3 cfu/mL
Register: ClinicalTrials.gov Id. NCT01994980	***Allocation:*** Randomized			Infection with MDR pathogen
	Intervention Model: Parallel assignment			Recurrence
				Ventilator free days
				Mortality
A randomized prospective clinical trial to assess the role of procalcitonin-guided antimicrobial therapy to reduce long-term infections sequelae	***Study Type:*** Phase IV	To demonstrate if using one PCT-guided rule of stop of antimicrobials, the incidence of infections by *C.difficile* and by MDR bacteria during the next 6 months may be significantly decreased.	The change of infection-associated adverse events rate for patients treated by the PCT-guided stopping rule compared to patients treated by standard of care.	Time to first infection-associated adverse events rate until month 6.
Sponsor: Hellenic Institute for the Study of Sepsis	***Estimated Enrollment:*** 280 participants			Rate of CDI until month 6
Register: EudraCT Number: 2017-002011-33	***Allocation:*** Randomized			Rate of infections by MDR until month 6.

(continued)

Table 1 (continued)

Title / Sponsor / Register	Characteristic of the study	Purpose	Primary outcome measures	Secondary outcome measures
	Intervention Model: /			28 day -6 month mortality
				Rate stool + for GDH by C. difficile until month 6
				Rate of stool colonization by MDR until month 6
				Microbiome composition on day 28
				Changes of the microbiome between days 1 and 28.
				Consumption of antimicrobials until hospital discharge
				Cost until hosp. discharge.
GRam stain-guided antibiotics ChoicE for ventilator-associated pneumonia (GRACE-VAP) trial	***Study Type:*** Interventional	To reveal whether gram staining can reduce the use of broad-spectrum antibiotics without impairing patient outcomes and thereby provide evidence for an antibiotics selection strategy in patients with VAP. (PCT evaluated as secondary outcome)	Clinical cure of VAP	Select of anti-MRSA or –pseudomonal agents as initial antibiotic therepies
Sponsor: Osaka General Medical Center	***Estimated Enrollment:*** 200 participants			Coverage of initial antibiotic therapies
Register: ClinicalTrials.gov Id. NCT03506113	***Allocation:*** Randomized			28-day mortality
	Intervention Model: Parallel assignment			ICU-free days
				Ventilator-free days
				Duration of antibiotic therapies
				Need of escalation or de-escalation of antibiotics
				Adverse events related to antibiotics
				Laboratory marker of inflammation (CRP, PCT) on 2, 4, 6, 8, and 14 days

(continued)

Table 1 (continued)

Title Sponsor Register	Characteristic of the study	Purpose	Primary outcome measures	Secondary outcome measures
				Organ failure control
				Renal function
Endocan; can be a new biomarker in ventilator-associated pneumonia?	***Study Type:*** Observational [patient registry]	To evaluate relationship between VAP and Endocan and whether correlated with other clinical and laboratory findings [presence of fever, pathological lung X-ray, the number of white blood cells (WBC), procalcitonin (PCT)and C-reactive protein (CRP)] .	Relationship between the level of Endocan and development of VAP	Correlation with the level of Endocan and clinical and laboratory findings
Sponsor: Erzincan University	***Estimated Enrollment:*** 60 participants			The number of white blood cells (WBC)
Register: ClinicalTrials.gov Id. NCT02916277	***Observational Model***: Cohort			Procalcitonin (PCT)
	Time Perspective: Prospective			C-reactive protein (CRP)
	Target Follow-Up Duration: 5 days			

The main problems reported were represented by the design of studies in which fundamental aspects of the PCT biology were not taken into proper consideration, or the control groups were not adequately enrolled or treated (Lisboa et al. 2018). Even in the case of trials exploring the relation between VAP and PCT, the value of the results available was compromised by several bias: the most significant limitation was related to the small sample size of the studies.

Moreover, it is worth it to notice that a number of studies have shown that PCT levels are of limited value in cases of renal failure, hemodialysis, surgical interventions and resuscitated cardiac arrest. As a matter of fact, these pathological events are frequently concomitant in patients with VAP and can affect the meaning of this biomarker (Ceccarelli et al. 2019; Spaziante et al. 2018, 2019; Sargentini et al. 2015, 2017). For these reasons, not only randomized controlled trials, but also additional "real-life" studies are needed in order to clarifying the use of PCT in the VAP. The strengths of this review include the variety of study characteristics and the large number of subjects enrolled. Moreover, focus was placed on a number of outcomes including the role of PCT (1) in diagnosis of VAP, (2) in predicting the efficacy of antibiotic therapy in patients suffering from VAP, (3) in discontinuation of antibiotic therapy and risk of VAP recurrence, (4) in the prediction of clinical outcome in patient with VAP. However, there are several limitations in this study: first of all, we have not defined, in our search strategy, a minimum number of enrolled patients in the source studies. Furthermore, limitations in study design were not considered. This may have led to the inclusion of studies with small sample size and not always methodologically comparable. Nevertheless, we considered relevant to present the entire systematically retrieved spectrum of trials in order to reflect the data currently available.

In conclusion, as suggested by the most updated guidelines, the PCT is currently a diagnostic tool primarily meant for the management of particular circumstances of patients with VAP. However, even in these cases its meaning must be interpreted "cum grano salis", considering the

methodological limitations that afflict the available data. It remains a duty to continue to explore this issue trying to standardize research strategies for future studies and clinical trials.

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Impact of DAA-Based Regimens on HCV-Related Extra-Hepatic Damage: A Narrative Review

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Abstract

Two-third of patients with chronic hepatitis C show extrahepatic manifestations due to HCV infection of B lymphocytes, such as mixed cryoglobulinemia and non-Hodgkin B-cell lymphoma, or develop a chronic inflammatory status that may favor the development of adverse cardiovascular events, kidney diseases or metabolic abnormalities.

DAAs treatments induce HCV eradication in 95% of treated patients, which also improves the clinical course of extrahepatic manifestations, but with some limitations. After HCV eradication a good compensation of T2DM has been observed, but doubts persist about the possibility of obtaining a stable reduction in fasting glucose and HbA1c levels.

Chronic HCV infection is associated with low total and LDL cholesterol serum levels, which however increase significantly after HCV elimination, possibly due to the disruption of HCV/lipid metabolism interaction. Despite this adverse effect, HCV eradication exerts a favorable action on cardiovascular system, possibly by eliminating numerous other harmful effects exerted by HCV on this system.

DAA treatment is also indicated for the treatment of patients with mixed cryoglobulinemia syndrome, since HCV eradication results in symptom reduction and, in particular, is effective in cryoglobulinemic vasculitis. Furthermore, HCV eradication exerts a favorable action on HCV-related lymphoproliferative disorders, with frequent remission or reduction of clinical manifestations.

There is also evidence that HCV clearance may improve impaired renal functions, but same conflicting data persist on the effect of some DAAs on eGFR.

Keyword

HCV extrahepatic manifestations · Hepatitis C virus · Interferon-free DAA regimens

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1 Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection tends to progress towards liver fibrosis and cirrhosis and subsequently to hepatocellular carcinoma (HCC) in the context of bridging fibrosis or liver cirrhosis (Stroffolini et al. 2018; Sagnelli et al. 2013, 2019).

In 2015, 71 million people were living with chronic HCV infection worldwide (Global Hepatitis Report 2017). Exposure to infected blood or blood products (intravenous drug use, iatrogenic exposure, tattooing, piercing) and risky sexual contact (multiple partners, anal sex, presence of genital lesions) were the risk factors most frequently associated with the transmission of this infection (Santantonio et al. 2006; Corey et al. 2006; Daniels et al. 2009; Esteban et al. 2008).

After becoming infected with HCV, almost 35% of the subjects eliminate the virus spontaneously or after asymptomatic acute self-limiting hepatitis, while the remaining 65% progress to chronicity, identifiable by the persistence of HCV RNA in serum for at least 6 months (Alter et al. 1992). The natural history of chronic HCV infection is extremely variable, from a long-term absence of liver lesions to the persistence of minimal liver changes with a slow indolent progression to fibrosis or a rapid progression to liver cirrhosis and its serious complications, such as portal hypertension, liver failure and HCC development (Poynard et al. 1997, 2000). Several factors can accelerate the progression of liver disease, including an older age at the time of infection, concomitant alcohol abuse, presence of diabetes and coinfection with HIV and/or HBV (Alter et al. 1992; Poynard et al. 1997, 2000; Alter and Seeff. 2000; Coppola et al. 2012, 2014, 2015; Webster et al. 2015; Bagaglio et al. 2020; Sagnelli et al. 2020).

Nearly two-thirds of patients with chronic HCV infection (CHC) show extrahepatic manifestations due to HCV infection of B lymphocytes, such as mixed cryoglobulinemia and non-Hodgkin B-cell lymphoma, or to a chronic inflammatory status that may favor the development of adverse cardiovascular events (stroke, coronary artery disease), kidney diseases and metabolic abnormalities (Cacoub et al. 2014; Calogero et al. 2019).

Interferon (INF) alfa was the cornerstone of chronic HCV therapy until 2014, but was later replaced by classes of direct acting antiviral agents (DAA) which, combined with each other, give much better therapeutic results and a marked reduction in treatment times and adverse reactions. Today, thanks to the high and rapid effect of DAA regimens, the sustained virological response rate at the twelfth post-treatment week (SVR12) is about 95%, even in the presence of advanced liver diseases (EASL Recommendations on Treatment of Hepatitis C 2018).

Although the effectiveness of DAA combinations on the eradication of HCV infection is proven by numerous randomized clinical trials and by a day to day worldwide clinical practice, their efficacy on the associated metabolic disorders and related extrahepatic manifestations need further clarification.

2 Chronic Inflammatory Status and Extra-Hepatic Damage

The pathogenesis of extra-hepatic manifestations of HCV infection has not been fully investigated at present (Fletcher and McKeating 2012; Zignego et al. 2007). HCV infection determines the clonal expansion of B lymphocytes (Carbonari et al. 2005; Charles et al. 2008) with the production of rheumatoid factor M immunoglobulin (Ig), which in sensitive subjects causes the deposition of immune complexes in small vessels, with consequent vasculitis. The mechanisms of other manifestations seem multifactorial, including a direct interaction between viral proteins and intracellular signaling pathways, viral replication in extra-hepatic cells and an intensified immune reaction with systemic effects. The activation of the immune system may induce chronic inflammation as occurs in human immunodeficiency virus (HIV) infection (Kuller et al. 2008; Petta et al. 2014; Negro 2014; Negro et al. 2015; Bedimo and Abodunde 2016).

Patients with chronic HCV infection are at risk of developing type 2 diabetes mellitus (T2DM), the most common extra-hepatic manifestation in this infection (Mehta et al. 2000; Wang et al. 2007; Vanni et al. 2016; Mehta et al. 2000). In addition, compared with uninfected controls, HCV infected patients more frequently show high levels of insulin resistance (IR) (Moucari et al. 2008; Younossi et al. 2013). On the other

hand, compared with untreated subjects, HCV-infected patients more frequently show a cardioprotective lipid profile, characterized by significantly lower levels of serum total cholesterol, low-density lipoprotein, and triglycerides (TC) and higher serum levels of high-density lipoprotein (Vassalle et al. 2004; Dai et al. 2008). Nonetheless, data from several studies show an association between HCV infection and atherosclerotic damage (Vassalle et al. 2004; Marzouk et al. 2007; Targher et al. 2007; Dai et al. 2008; Alyan et al. 2008; Petta et al. 2012; Hsu et al. 2015b) with increased mortality by circulatory diseases (Lee et al. 2012). These conflicting data deserve careful and thorough investigation.

It has also been observed that, compared to the general population, HCV-positive subjects more frequently develop chronic kidney disease (CKD) (Latt et al. 2012).

Studies have shown that therapy to eradicate HCV infection improves some extra-hepatic manifestations associated with HCV infection, regardless of the severity of liver disease. The evidence is stronger for mixed cryoglobulinemia, which often resolves entirely with viral clearance (Mazzaro et al. 1996; Cacoub et al. 2005; Dammacco and Sansonno 2013), but it remains unclear for extra-hepatic manifestations due to the chronic inflammatory state which have often been considered contraindications to INF-based treatment for the possibility of their exacerbation, for possible drug interaction with drugs used to treat them or for fear of additional toxicity (Massoumy et al. 2013; Kanwal et al. 2014). The directly acting antiviral (DAA) regimens are more effective and better tolerated than interferon-based therapy and therefore more frequently usable in the presence of extra-hepatic manifestations.

The purpose of this narrative review is to provide an overview of the knowledge available on the action exerted by DAA therapy on the extra-hepatic manifestations of chronic HCV infection. The article is addressed to all young doctors, to all doctors working in infectious diseases, gastroenterology, and internal medicine wards and to general practitioners who aid patients with chronic HCV infection.

3 Methods

We conducted a computerized bibliographic search using MEDLINE and EMBASE involving both medical title terminology (MeSH) and relevant keywords for search strings to locate studies that analyzed until April 2020 the outcome of HCV patients after DAA treatment. The following items were used for research in the studies: “hepatitis c”, “HCV”, “direct active antivirals”, “DAA” and “kidney”, “eGFR”, “kidney function”, “diabetes”, “glycemic control”, “blood sugar”, “lipids”, “triglycerides”, “cardiovascular”, “median intimal thickness”, “hypertension”, “lymphoma”, “B-NHL”, “DLBCL”, “CHL”, “Mixed Cryoglobulinemia Syndrome”, “vasculitis”. Based on the main research objectives, the articles were classified into one of the following research topics: kidney function, glycemic control, lipid control and cardiovascular events.

4 DAAs and Glycemic Homeostasis

The correlation between alterations in the glycid balance and CHC is made evident by the frequent development (30–70%) of insulin resistance in CHC patients, by the 3.8 times higher rate of chronic HCV infection in patients with T2DM than in those without (Vanni et al. 2016), by the more frequent progression of fibrosis in patients with CHC and IR (Hui et al. 2003) and by a more frequent development of HCC (Desbois and Cacoub 2017) in patients with CHC and T2DM than in those with CHC alone. The eradication of HCV infection with IFN-alfa therapy induces a substantial improvement in the markers of glucose metabolism as shown in a meta-analysis (Cacoub et al. 2018a) on 7,000 CHC patients from 40 studies; after IFN treatment, the incidence of IR and T2DM was significantly reduced during a long-term post-treatment follow-up in those who achieved a sustained virological response (SVR). In a retrospective study performed in Japan on 2,842 CHC patients treated with IFN the rate of T2DM was 3.6% at

the 5th year, 8.0% at the 10th year and 17.0% at the 15th year of post-treatment observation, predictive factors for T2DM development being advanced liver disease, failure to achieve SVR after treatment and age of 50 or more years (Arase et al. 2009). In a retrospective study from Spain on 234 IFN treated CHC or liver cirrhosis patients, glucose abnormalities occurred less frequently in those who obtained SVR than in those who did not (14.6% versus 34.1%) (Simó et al. 2006).

Contrasting data comes from an Italian retrospective study (Giordanino et al. 2008) where no association was found between SVR and a lower risk of developing T2DM during an 8-year follow-up.

A substantial improvement in fasting glucose and glycosylated hemoglobin levels were observed in HCV patients who achieved SVR with DAA treatment (Bose and Ray 2014; Meissner et al. 2015; Pavone et al. 2016; Morales et al. 2016; Hum et al. 2017; Ikeda et al. 2017; Fabrizio et al. 2017; Abdel Alem et al. 2017; Dawood et al. 2017; Ciancio et al. 2018; El Sagheer et al. 2018), regardless of the DAA regimen used (Meissner et al. 2015; Pavone et al. 2016; Morales et al. 2016; Abdel Alem et al. 2017; Fabrizio et al. 2017; Ikeda et al. 2017; Dawood et al. 2017; Ciancio et al. 2018; El Sagheer et al. 2018) (Table 1). In a National Veterans Health System study on 2435 HCV patients treated with different DAA regimens, a significantly higher reduction in mean hemoglobin A1c (HbA1c) was observed in the 2180 who achieved SVR compared to the 275 non-SVR-patients (Hum et al. 2017).

In a study on 91 HCV-positive liver transplant patients, 96% achieved SVR and HbA1c dropped from 35.5 $\pm$ 4.3 mmol/L to 33.3 $\pm$ 3.6 mmol/L at the 44th week after treatment; in patients not treated with anti-diabetic agents, a fasting glucose level decreased from 6.8 $\pm$ 1.7 mmol/L before antiviral therapy to 5.7 $\pm$ 1.1 mmol/L at the 24th week after treatment discontinuation (Beig et al. 2018).

The association between SVR induced by DAA regimens and glycaemic control is further supported by the behaviour of other parameters, like IR development, T2DM development and type and doses of anti-diabetic drugs. A prospective study showed that HCV eradication produced a clearance or reduction of IR in 76% of 133 non-diabetic HCV-genotype 1 patients with advanced liver disease who achieved SVR12 (Adinolfi et al. 2018a, b). The DAA-induced SVR also correlates with a reduced risk of developing TDM2 (Adinolfi et al. 2018a) and with an improvement in glycemic control in T2DM patients (Adinolfi et al. 2018a). In addition, HCV patients with T2DM receiving oral anti-diabetic or insulin treatment needed a dose reduction during DAA therapy (Soriano et al. 2016; Hum et al. 2017; Ikeda et al. 2017; Ciancio et al. 2018; Teegen et al. 2019). Compared to the baseline values, a significant improvement in beta-cell function was observed after DAA treatment in a prospective, open-label, multi-center study (107.7 $\pm$ 86.8 vs. 86.7 $\pm$ 44.5, $p = 0.05$), an improvement more evident in patients with high baseline IR (Huang et al. 2017). A post hoc analysis of six studies on CHC genotype-1 patients with advanced fibrosis showed a significant reduction in fasting glucose levels in patients treated with paritaprevir/ritonavir/ombitasvir/dasabuvir, compared to those in the placebo group (Tran et al. 2017): the most significant reduction being observed in T2DM patients (22.1 mg/dL less at the 12th week compared to the baseline), followed by those in a pre-diabetic status (−5.78 mg/dL by week 12) (Tran et al. 2017). As for the HbA1c, a substantial reduction was observed only in responders with a high baseline HbA1c level (Hum et al. 2017). In addition, in an Egyptian study on CHC patients with HCV genotype-4 and T2DM who achieved SVR, the independent prognostic factors for a drop in blood glucose levels >20 mg/dl or a drop in HbA1c levels >0.5% were a T2DM duration less than 7 years, a T2D negative family history and a liver disease severity up to cirrhosis Child-Pugh A (Dawood et al. 2017). Pavone et al. retrospectively evaluated 21 HCV-positive patients with T2DM treated with different interferon-free DAA regimens; fasting glucose serum levels significantly decreased during treatment (mean value −52.86 mg/dL, $p = 0.007$); also glycated

Table 1 Changes in glycemic balance in DAA treated CHC patients

References	Type of study	Number of patients, Setting	HCV-Genotype	Treatment	Results
Meissner et al. (2015)	Cohort study	55 HCV-T2DM patients	HCV-G 1a/b	SOF/RBV	Decrease in HbA1c.
Morales et al. (2016)	Observational retrospective study	60 CHC patients, of whom 38.3% with T2DM	HCV-G-1a/b	SOF-based regimens	Decrease in HbA1c; reduced doses of antidiabetic drugs in 25% of cases.
Pavone et al. (2016)	Observational retrospective	149 CHC patients, of whom 19% with T2DM	12.1% HCV-G 1	SOF-based regimens	Decrease in FGL, decrease in HbA1c; reduced doses of antidiabetic drugs in 23% of cases.
Abdel Alem et al. (2017)	Retrospective observatinal study	65 CHC-T2DM patients	Most cases with HCV-G4	SOF-based regimens	Decrease in FGL, HbA1c decrease.
Fabrizio et al. (2017)	Observational retrospective study	449 CHC patients, of whom 13.1% with T2DM and 2.0% with HIV coinfection	1.3% HCV-G 1a 6.5% HCV-G 1b	SOF/RBV 32.2% SOF/SIM $\pm$ RBV 13.5% SOF/LED$\pm$RBV 25.4% OMV/PTV/r/DSV $\pm$ RBV 27.1% SOF/DCV/RBV 1.6%.	Decrease in FGL.
Hum et al. (2017)	Observational retrospective study	2,435 CHC-T2DM patients	99.3% HCV-G 1	SOF/SMV SOF/LED OMV/PTV/r/DSV	Decrease in HbA1c, reduced doses of antidiabetic drugs in 9% of cases.
Dawood et al. (2017)	Clinical trial open labeled	460 CHC patients, of whom 82.2% with T2DM	HCV-G 4	SOF/DCV	Decrease in FGL, decrease in HbA1c; reduced doses of antidiabetic drugs in 27% of cases.
Ikeda et al. (2017)	Observational prospective study	36 CHC patients, of whom 36.1% with T2DM	HCV-G 1b	SOF/LED	Decrease in HbA1c.
Stine et al. (2017)	Retrospective cohort study	165 CHC patients, of whom 18.5% with T2DM	80.8% HCV-G 1	SOF/LED 38.5% SOF/SIM 26.9% SOF/RBV 19.2% SOF/RBV/peg-INF 7.8% SOF/LED/RBV 3.9% BOC/RBV/peg-INF 3.9%	No decrease in HbA1c; reduced doses of antidiabetic drugs in 13% of cases.

(continued)

Table 1 (continued)

References	Type of study	Number of patients, Setting	HCV-Genotype	Treatment	Results
Huang et al. (2017)	Prospective study	65 CHC patients, of whom 21.7% with T2DM	72.3% HCV-G 1 27.7% HCV non-G 1	SOF-based regimens 46.2% PTV-OMV/ DSV/r 35.4% ASV/DCV 18.5%	Significant improvement in beta-cell function, mainly if high basal insulin resistance.
Ciancio et al. (2018)	Observational prospective study	122 CHC patients, of whom 82.8% with T2DM	4% HCV-G 1a 54.5% HCV-G 1b	SOF/ SMV ± RRV 20% SOF/LED ± RBV 41.8% SOF/ DCV ± RBV 10% SOF/RBV 11.8% OMV/PTV r/DSV ± RBV 10.9% OMV/PTV/ r ± RBV 5.5%	Decrease in FGL, decrease in HbA1c; reduced doses of antidiabetic drugs in 21% of cases.
Beig et al. (2018)	Observational retrospective study	91/132 (69%) patients with recurrent HCV infection after liver transplantation, of whom 41.8% with T2DM	51.5% HCV-G 1	SOF/LED ±RBV 60% SOF/ RBV ± PEG 21% G/P 7% SOF/VEL 5% Viekira Pak 3% SOF/ DCV ± RBV 3%	Decrease in FGL and HbA1c; reduced doses of antidiabetic drugs in 40% of cases.
Drazilova et al. (2018)	Longitudinal retrospective observational study	370 CHC patients, of whom 10.2% with T2DM	78.8% HCV-G 1b 11.9% HCV-G 1a	SOF based regimens OMV/PTV/r/ DSV EBR /GZR	Decrease in FGL, reduced doses of antidiabetic drugs in 17.6% of cases.
Chaudhury et al. (2017)	Prospective, longitudinal cohort study	251 CHC patients, of whom 17% with T2DM, and 31% with HIV coinfection	HCV-G 1	DAA/IFN/ RBV 14% DAA/RBV 16% DAA 70%	No decrease in HbA1c, reduced doses of antidiabetic drugs in 3% of cases.
Weidner et al. (2018)	Observational retrospective study	281 CHC patients, of whom 10.0% with T2DM	72% HCV-G 1	DAA ± RBV	Decrease in FGL, HbA1c decrease.
El Sagheer et al. (2018)	Cohort study	80 CHC patients	*HCV-G* 4	SOF + SMV	Decrease in FGL.
Butt et al. (2019)	Retrospective case control study	17,103 CHC patients without a diagnosis of any CVD and a control group	//	PEG-RBV regimen 26% DAA regimens 74%	More significant reduction in T2DM incidence rate in patients treated with DAAs

(continued)

Table 1 (continued)

References	Type of study	Number of patients, Setting	HCV-Genotype	Treatment	Results
		with a number equal to case group			compared to other PEG-RBV regimen or untreated.
Lanini et al. (2019)	Retrospective cohort study	205 CHC patients, of whom 26.3% with T2DM and 15.6% with HIV coinfection	27.37% HCV-G 1a 37.56% HCV-G 1b	SOF/LED± RBV 35.61% 2D/3D ± RBV 3.41% SOF/ DAC ± RBV 19.51% SOF/ SIM ± RBV 28.78% SOF/RBV 12.68%	Decrease in FGL.
El Serag et al. (2019)	Retrospective cohort study	45,260 CHC patients	*57.7% HCV-G1a 26.7% HCV-G1b 9% HCV-G2 5.1% HCV-G3 0.95% HCV-G4 0.07% HCV-G5/ G6*	SOF ± LDP 81.76% OMV/DSV/r 14.85% EBR/GZR 2.44% SIM 6.25% DVC 1.6%	Incidence of DM between the SVR and the non-SVR group did not significantly differ.
Gilad et al. (2019)	Observational retrospective study	122 CHC-T2DM patients		DAA	Decrease in HbA1c in 34% of cases.
Teegen et al. 2019	Observational retrospective study	100 patients with HCV -recurrence after LT	84% HCV-G 1	SOF/LDV RBV 53% SOF/SMV 22% SOF/DCV 21% SOF/RBV 2% OMV/PTV/r 2%	Significant decline in the daily average insulin dose.
Li et al. (2019)	Observational retrospective/ prospective study	192 CHC-T2DM patients	67% HCV-G 1	DAA	Decrease in HbA1c.
Alicia Halim Wong et al. (2020)	Retrospective observational study	996 CHC patients, of whom 22% with T2DM	87.1% HCV-G 1	LDV/SOF 89.6% SOL/VEL 7.8% EBR /GZR 2.6%	Decrease in HbA1c by 0.04%.

CHC Chronic Hepatitis C (some cirrhotic cases are included), *SVR* sustained viral response, *FGL* fasting glucose level, *HbA1c* hemoglobin A1c, *Peg-IFN* pegylated-interferon, *RBV* ribavirin, *HIV* human immunodeficiency virus, *OMV* ombitasvir, *PTV* paritaprevi, *r* ritonavir, *DSV* dasabuvir, *G/P* glecaprevir/pibrentasvir, *SOF* sofosbuvir, *SIM* simeprevir, *LDV* ledipasvir, *DCV* daclatasvir, *ASV* asunaprevir, *EBR* elbasvir, *GZR* grazoprevir, *VEL* velpatasvir, *T2DM* type 2 diabetes mellitus

haemoglobin values (detected in 10 patients at weeks 4, 8 and/or 12) significantly decreased during treatment (−1.95%, $p = 0.021$). The Authors concluded that diabetes could be considered as an element to prioritize treatment in CHC patients (Pavone et al. 2016). Soriano et al. described a significant decrease in fasting serum glucose level during DAA treatment in a CHC patient with T2DM, an event forcing a reduction in insulin dosage (Soriano et al. 2016). Also, in a Teegen's study on liver transplant patients a significant decline in the daily average insulin dose was required to keep stable HbA1c after DAAs therapy (55.3 vs. 38.2 U/d; $p = 0.009$) (Teegen et al. 2019).

To be noticed, however, that a consistent number of studies did not show a long-term persistence in glycemic control, or showed no significant decline in HbA1c, or no effect of DAA treatment in patients with a severe CHC, or no effect at all, or an increase in TC and LDL values. A retrospective study by Weidner and colleagues showed that HCV eradication through DAA treatment was associated to with a significant reduction in fasting plasma glucose level and in the rate of patients with impaired fasting plasma glucose. In some CHC patients, however, the reduction of FPG levels was only transitory and no significant improvement in glycemic values was observed in cirrhotic patients up to 12 months after therapy (Weidner et al. 2018). In a retrospective study on 122 diabetic subjects with HCV infection published in 2019, Gilad et al. reported favorable HbA1c changes after DAA treatment in 42 (34%) of the 122; among these 42, only 20 of the 28 (71%) with available follow-up showed this effect sustained over 1.5 year (Gilad et al. 2019). Chaudury et al. prospectively examined for a median period of 28 months 251 HCV CHC patients, of whom 31% with HIV coinfection, before and after DAA therapy and only minimal changes in HbA1c and glucose were observed, independently of the achievement of SVR, HIV status, diabetes, or stage of liver disease. To be noticed that TC and LDL increased significantly after treatment (Chaudhury et al. 2017). Beig et al. performed a retrospective single-center study on 91 HCV-related liver transplant recipients with recurrent HCV infection who received DAA treatment, of whom 87 (96%) reached HCV eradication. HCV clearance was associated with a reduction in treatment doses for diabetes by 38% and from a decline of HbA1c levels from 35.5 ± 4.3 to 33.3 ± 3.6 mmol/mol 44 weeks post-treatment ($p = 0.03$); however, TC and LDL levels significantly increased posttreatment (Beig et al. 2018).

Some studies evaluated the changes in the incidence of T2DM after effective antiviral therapy. Butt and colleagues analyzed the data from patients of the U.S. Veterans Administration and recorded a more significant reduction in T2DM incidence rate in patients treated with DAAs: the incidence rates per 1000 person-years were 9.89 (95% confidence interval [CI] = 8.7–11.1) in DAA-treated patients, 19.8 (95% CI = 18.3–21.4) in those treated with pegylated interferon (Peg-IFN)/ ribavirin (RBV) treatment and 20.6 (95% CI = 19.6–21.6) in those left untreated ($p < 0.001$) (Butt et al. 2019). A cohort of 5127 nondiabetic patients treated with DAAs was analyzed by Li and coworkers; during an average follow up of 3.7 years they recorded an incidence of T2DM of 6.2% in the SVR group and of 21.7% in the non-SVR group (HR = 0.79; 95% CI = 0.65–0.96) (Li et al. 2019). Similarly, El Serag and colleagues analyzed 45,260 patients treated with DAAs, but the incidence of DM between the SVR and the non-SVR group did not significantly differ (21.04/1000 patients per year versus 23.11/1000 patients per year; hazard ratio (HR) = 0.98, 95% CI = 0.81–1.19, $p = 0.86$) (El Serag et al. 2019).

Concluding on this topic, there is still some disputes and the available data do not allow us to conclude on whether the achievement of SVR induces a persistent reduction in fasting glucose and HbA1c; despite this, the data from most studies strongly indicate good compensation of T2DM in HCV patients treated with DAA. Long-term prospective follow-up studies on the evolution of glycolic metabolism of diabetic and non-diabetic HCV patients who achieved SVR with DAA treatment are still needed to resolve the remaining disputes.

5 DAAs and Lipid Homeostasis

HCV infection has been associated with lipid and lipoprotein metabolism disorders, such as hypobetalipoproteinemia, hypocholesterolemia, and hepatic steatosis (Felmlee et al. 2013). HCV production is dependent on the very-low-density lipoprotein (VLDL) biosynthetic pathway, and circulating virions are associated with VLDL in lipoviral particles containing host apolipoproteins (APOs), including APOB, APOE, and APOC3 (Dai et al. 2008); the interaction between HCV virions, VLDL and low-density lipoprotein (LDL) particles is responsible for increased viral infectivity. In addition, HCV infection activates the sterol-regulatory-element-binding-protein (SREBP) 1c, which is involved in lipogenesis and HCV-related liver steatosis, partially due to ß mitochondrial-oxidation (Nielsen et al. 2006; Waris et al. 2007; Merz et al. 2011). It has also been shown that HCV core protein inhibits the activity of microsomal triglyceride transfer protein (MTP), resulting in liver steatosis and hypolipidemia. Because of the mechanisms mentioned above, spontaneous or treatment-related HCV eradication, down-regulating SREBP 1c and up-regulating both MTP and CPT-1 may reduce liver lipogenesis and increase VLDL secretion. Other data on lipid homeostasis in anti-HCV treatment is shown in Table 2. Eradication of HCV infection with IFN-based treatment has been found associated with normalization of hypolipidemia (Tada et al. 2009) and with an increased risk of cardiovascular events. HCV eradication has also been found associated with a decrease in CAP and LDL-C values, the parameter used as a measure of liver steatosis, which, however, correlates with elevated values of small-dense LDL-C (sdLDL-C), which has been shown to predict atherogenesis and dyslipidemia in patients with SVR24 (Kawagishi et al. 2018).

A rapid increase in serum LDL-C and total cholesterol (CT) values from the baseline to the 28th week of DAA treatment has also been described, the hyper lipid effect being stronger with ledipasvir/sofosbuvir than with daclatasvir/asunaprevir combination (Hashimoto et al. 2016). These Authors also observed a close correlation between the decrease in the HCV core antigen serum titers and the increase in LDL-L values, especially in patients treated with sofosbuvir + ledipasvir, suggesting a direct influence of the HCV clearance on serum cholesterol levels (Hashimoto et al. 2016).

In a prospective multicenter study on HCV infected and HIV-HCV coinfected patients treated with several DAA combinations, a significant increase in LDL cholesterol serum values was observed (Mauss et al. 2017), while HDL cholesterol remained stable. However, contrasting data were reported by Carvalho et al. (2018) who evaluated lipid homeostasis in chronically HCV-infected patients at baseline and 1 year after the achievement of SVR in 105 patients treated with sofosbuvir + ledipasvir ± RBV and 73 with IFN ± RBV: they found a significant increase in TC and LDL levels after treatment with both regimens, while serum triglyceride levels decreased only in the DAA group ($p = 0.015$) (Carvalho et al. 2018).

Another marker of lipid homeostasis is the ApoB/ApoA1 ratio, a better predictor of cardiovascular diseases than LDL alone: ApoB is the best direct marker of low atherogenic density LDL-19 and ApoA1 provides a good estimate of HDL. An Italian real-life study (Gitto et al. 2018) analyzed the metabolic changes in 100 HCV patients during a 24-week follow-up period after DAA treatment discontinuation and observed a significant reduction in ApoA1 blood levels and an increase in the ApoB/ApoA1 ratio and Lp (a) (Gitto et al. 2018). Supporting evidence is given by Younossi et al. who evaluated lipoproteins in genotype-1 patients who achieved SVR after ledipasvir/sofosbuvir ± RBV treatment, with an increase in ApoB and LDL and a decline in ApoA1 and apolipoprotein (Younossi et al. 2015).

Concluding on this point, the effect of DAA regimens, especially the sofosbuvir-based, on lipidic homeostasis is characterized by an increase in TC, LDL and the ApoB/ApoA1

Table 2 Changes in lipid homeostasis in DAA treated CHC patients

References	Study type	Number of patients, setting	Treatment	Results
Younossi et al. (2015)	International multicenter randomized open labelled study	100 CHC patients	50 patients received a 12-week LDV/SOF treatment and 50 a 12-week LDV/SOF + RBV treatment	Increase in ApoB and LDL and decline in ApoA1 and apolipoprotein serum values.
Hashimoto et al. (2016)	Retrospective study	100 CHC patients	DCV/ASV LDV/SOF	Higher increase in serum LDL-C and TC with LDV/SOF than with DCV/ASV; decrease in HCV-core antigenemia correlated with increase in LDL-C, mainly with LDV/SOF.
Mauss et al. (2017)	Prospective multicentre cohort study	520 CHC patients with or without HIV coinfection	SOF/PEG-IFN/RBV SOF/RBV SOF/DCV ± RBV SOF/SMV ± RBV SOF/LDV ± RBV OMV/PTV/r ± RBV OMV/PTV/r/DBV ± RBV	Significant persistent increase in TC and LDL-C with SOF/PEG-IFN regimen; triglycerides increased with SOF/PEG-IFN/RBV.
Younossi et al. (2016)	Retrospective study	127 CHC patients HCV-G2/3	SOF/RBV	Treatment restores distal sterol metabolites and increases TC.
Gitto et al. (2018)	Cohort study	100 CHC patients	SOF ± RBV 20 SOF/LPV ± RBV SOF/DCV ± RBV SOF/SMV ± RBV OMV/PTV/r + DSV ± RBV OMV/PTV/r ± RBV	Patients achieving SVR showed a strong decrease in ApoA1 levels and an increase in ApoB/ApoA1 ratio and in Lp(a)
Kawagishi et al. (2018)	Retrospective study	117 CHC patients	DCV/ASV SOF/LDV SOF/RBV OBV/PTV/r	Decrease in CAP and LDL-C in patients with high baseline values; elevated LDL-C and sdLDL-C in patients with liver steatosis and dyslipidaemia at SVR24.
Carvalho et al. (2018)	Prospective study	178 CHC patients	PEG±RBV SOF/LDV ± RBV	Increase in TC and LDL-C in both regimens; decrease in TG during DAA treatment.
Alessio et al. (2020)	Multicenter real-life study	243 HIV/HCV coinfected patients	DAA	Increase in TC and LDL-C serum level after 12 weeks of treatment.

CHC Chronic Hepatitis (Some cirrhotic cases are included), *SVR* sustained viral response, *LDL* low density lipoprotein, *CAP* controlled attenuation parameter, *sdLDL-C* small-dense low density lipoprotein cholesterol, *TC* total cholesterol, *ApoA1* apolipoprotein A1, *ApoB* apolipoprotein B, *Lp(a)* lipoprotein a, *DCV* daclatasvir, *IFN* interferon, *Peg-IFN* pegylated-interferon, *RBV* ribavirin, *TVR* telaprevir, *BOC* boceprevir, *OMV* ombitasvir, *PTV* paritaprevi, *r* ritonavir, *DSV* dasabuvir, *G/P* glecaprevir/pibrentasvir, *SOF* sofosbuvir, *SIM* simeprevir, *LDV* ledipasvir, *DCV* daclatasvir, *ASV* asunaprevir, *EBR* elbasvir, *GZR* grazoprevir, *VEL* velpatasvir, *cIMT* carotid intima-media thickness, *PWV* pulse-wave velocity, *T2DM* type 2 diabetes mellitus, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins, *sdLDL* small dense lipoproteins

ratio. As a consequence of this, a higher incidence of cardiovascular events could have been expected after HCV eradication, eventuality not occurred probably because the adverse effect possibly induced by this increase could have been balanced or overcame by the favorable effect of the DAA-induced HCV eradication on cardiovascular system.

6 DAAs and Cardiovascular Diseases

HCV infection has also been described as an independent non-traditional risk factor for cardiovascular (CV) diseases (Domont and Cacoub 2016) since it induces an increased overall CV mortality (Goossens and Negro 2017; Cacoub et al. 2018a, b), a dysmetabolic syndrome (Loria et al. 2014) and a cytokine remodeling towards chronic systemic inflammation, which triggers endothelial dysfunction in response to recombinant HCV envelope protein (Urbaczek et al. 2014; Katsi et al. 2015; Gonzalez-Reimers et al. 2016; Cammarota et al. 2019; Sigon et al. 2019). Other mechanisms responsible for CV diseases in HCV patients have been identified in the procoagulant imbalance and IR/T2DM, which may directly cause vascular and cardiac damage (Domont and Cacoub 2016; Vassalle et al. 2018; Petta et al. 2018). Apart from some indirect mechanisms, HCV has also been proven to be a direct cardiotropic virus and a causative agent in structural cardiomyopathies, such as dilated, hypertrophic, right ventricular arrhythmogenicity (Matsumori et al. 1998; Matsumori 2006) and an inducer of cardiac fibrosis (Pepe et al. 2015) and myocarditis (Okabe et al. 1997; Goossens and Negro 2017) rarely secondarily to mixed cryoglobulinemia (Terrier et al. 2013). HCV-core protein elicits immune-mediated oxidative damage in myocardial tissue (Sanchez and Bergasa 2008; Frustaci et al. 2002), where HCV-RNA is also detectable (Matsumori et al. 1996; Okabe et al. 1997; Matsumori 2006). Also, genetic HLA and non-HLA susceptibility correlated to cardiomyopathy development has been described (Sanchez and Bergasa 2008). In addition, myocardial scintigraphy showed increased perfusion defects in 87% of 217 HCV-positive patients (Maruyama et al. 2013). This evidence has determined a partial shift in the practical interpretation of cardiovascular dysfunction in the management of HCV patients, CV disease increasingly representing a reason for prompt treatment, rather than an exclusion criterion.

Epidemiological studies showed an association between carotid atherosclerosis (Tomiyama et al. 2003; Perticone et al. 2015), carotid intima-media thickness (cIMT) and β-stiffness (Novo et al. 2018; Negro 2014) and the circulating of HCV-core protein (Ishizaka et al. 2003). However, the role of HCV in atherogenesis is still unclear as HCV might only be a "bystander" when detected within atherosclerotic plaques, rather than their cause (Goossens and Negro 2017; Vassalle et al. 2018; Romano et al. 2018), and the HCV-induced protective lipid profile constitutes a confounding factor on this point (Maggi et al. 1996; Oliveira et al. 2013; Novo et al. 2018). (Table 3).

Other studies reported an increased risk of acute coronary syndrome (ACS) (Tsai et al. 2015) and acute myocardial infarction (AMI) (Butt et al. 2017; Vassalle et al. 2018), with an association between the number of affected vessels and HCV viral load (Vassalle et al. 2004, 2018). Left ventricular dysfunction and congestive heart failure (CHF) may also occur, events predictable by N-terminal-pro-natriuretic peptide plasma values (NT-pro-BNP) (Vassalle et al. 2018). The hepatic damage (necroinflammation, steatosis and fibrosis) appears to promote T2DM and CV diseases in HCV patients (Lonardo et al. 2016). The "Heart and Soul" study analyzed 981 patients with CV diseases, of whom 8.6% was HCV-infected. The latter showed increased TNF-α levels and an augmented risk of heart failure and death (Tsui et al. 2009). The NHANES cohort examined 16,668 HCV patients and showed that CHC is an independent risk factor for impaired glucose metabolism (IR/T2DM), hypertension and congestive heart failure (Katsi et al. 2015). (Table 3).

HCV eradication has been associated with an improvement in CV and metabolic syndromes, creating a reduction in all-cause mortality both in patients receiving IFN-based therapy and in those treated with DAA (Goossens and Negro 2017; Adinolfi et al. 2018c; Mohanty et al. 2019; Revuelto Artigas et al. 2019b).

In the prospective CirVir cohort, including 878 HCV cirrhotic patients from 35 clinical centers, the achievement of SVR by IFN-based

Table 3 Impact of DAA therapy on cardiovascular disease

References	Study type	Number of patients, Setting	Treatment	Results, suggestions
Nahon et al. (2017)	Prospective cohort study	1,323 CHC patients	Peg-IFN/ RBV Peg-IFN/ RBV/TVR Peg-IFN/ RBV/BOC	SVR obtained in nearly half of the patients, associated with a lower risk of CV events.
Cacoub et al. (2018a, b)	Prospective cohort study	878 CHC patients	Peg-IFN/ RBV Peg-IFN/ RBV/TVR Peg-IFN/ RBV/BOC	SVR obtained in nearly half of the patients, associated with a reduced rate of CV events; Asian ethnicity, smoking, arterial hypertension, 2TDM and low serum albumin were identified as independent predictors of CV events.
Mehta et al. (2017)	Post-hoc analysis on a phase 3 trial	5,963 CHC HCV-G-1 patients; only those with basal glucose and TC values obtained in a fasting state were included	OMV/PTV/r/ DSV ± RBV	Significant reduction in serum triglycerides and glucose; reduced rate of CV events, particularly in patients with altered baseline biomarkers.
Tran et al. (2018)	Post-hoc analysis on phase 3 trial	1,554 CHC patients	G/P	Significant improvement in biomarkers predictive of extrahepatic diseases, including CV diseases and metabolic syndromes.
Petta et al. (2018)	Prospective cohort study	182 child-Pugh A cirrhotic patients	SOF/RBV SOF/ SIM ± RBV SOF/ LDV ± RBV SOF/ DSV ± RBV OMV/PTV/r/ DSV ± RBV	SVR resulted in amelioration of carotid atherosclerosis, evaluated by US measuring of cIMT; such amelioration was not observed in obese subjects.
Butt et al. (2018)	Cohort study	242,680 veterans with CHC with no history of CV disease	Peg-IFN/ RBV Peg-IFN/ RBV/BOC Peg-IFN/ RBV/TPV SOF/ SIM ± RBV SOF/ DCV ± RBV LDV/ SOF ± RBV PTV/r/OMV/ DSV ± RBV EBR/ GZR ± RBV SOF/VEL	SVR resulted in a lower risk of CV events compared with controls pair matched by age, race, sex, and baseline values.
Novo et al. (2018)	Observational cohort study	39 HCV Child-Pugh A cirrhotic patients	SOF/RBV SIM/SOF/ RBV SOF/ LDV ± RBV SOF/DSV/	SVR resulted in a significant amelioration of subclinical CV alterations (evaluated by cIMT, PWV, β-stiffness, global longitudinal strain); reduction of

(continued)

Table 3 (continued)

References	Study type	Number of patients, Setting	Treatment	Results, suggestions
			RBV OMV/PTV/r/ DSV ± RBV	systemic inflammation, particularly in T2DM patients.
Revuelto Artigas et al. (2019a, b)	Observational prospective study	85 CHC patients without T2DM, kidney disease, nor CV diseases, of whom 38.8% non-responders to previous PEG-IFN/RBV treatment	SOF ± RBV SOF/ LDV ± RBV SOF/ SIM ± RBV SOF/ DCV ± RBV OMV/PTV/r/ DSV ± RBV	HCV clearance wasn't followed by cIMT improvement; worsening in blood lipid composition was observed 1 year after treatment. Mid-term effects should be carefully evaluated.
Ichikawa et al. (2019)	Observational prospective study	48 CHC patients	SOF/RBV SOF/LDV DCV/ASV	An increase in cIMT and unfavourable lipidic profiles were observed 1 year after SVR, suggesting the need for long-term follow up studies for a conclusive statement.

CHC Chronic Hepatitis (Some cirrhotic cases are included), *SVR* sustained viral response, *CV* cardiovascular, *IFN* interferon, *Peg-IFN* pegylated-interferon, *RBV* ribavirin, *TVR* telaprevir, *BOC* boceprevir, *MACE* major adverse cardiovascular events, *HIV* human immunodeficiency virus, *HBV* hepatitis B virus, *OMV* ombitasvir, *PTV* paritaprevi, *r* ritonavir, *DSV* dasabuvir, *G/P* glecaprevir/pibrentasvir, *SOF* sofosbuvir, *SIM* simeprevir, *LDV* ledipasvir, *DCV* daclatasvir, *ASV* asunaprevir, *EBR* elbasvir, *GZR* grazoprevir, *VEL* velpatasvir, *cIMT* carotid intima-media thickness, *PWV* pulse-wave velocity, *T2DM* type 2 diabetes mellitus, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins, *sdLDL* small dense lipoproteins

regimens was associated with a decrease in CV mortality (Cacoub et al. 2018b). In 2018, Tran et al. studied a cohort of 1554 HCV patients with CHC without cirrhosis enrolled in two phase-3 clinical trials to evaluate the tolerability and efficacy of glecaprevir/pibrentasvir combination therapy and found a statistically significant reduction in CV diseases and in metabolic syndromes in those who achieved SVR (Tran et al. 2018); a post-hoc analysis on 5963 HCV patients undergoing ombitasvir/paritaprevir/ritonavir/dasabuvir+RBV combination therapy obtained similar results (Mehta et al. 2017). Thirty-nine Italian HCV-cirrhotic patients showed no major CV adverse events following HCV eradication with different DAAs regimens and a decrease in subclinical cardiovascular alterations as detected by PWV and β-stiffness index (Novo et al. 2018).

Butt et al. investigated the risk of CV events in USA veterans with CHC, 4436 treated with Peg-IFN and 12,667 with DAAs, matched with untreated controls according to potential confounding factors: CV events were observed in 7.2% of treated patients and in 13.8% of controls, indicating a significantly lower risk after SVR achievement (Butt et al. 2018). An Italian study on 182 HCV patients with severe liver fibrosis and SVR to DAA evaluated the dynamics of carotid atherosclerosis by measuring the carotid intima-media thickness (cIMT): there was a significant decrease in cIMT during a long-term post-treatment follow-up (from 0.94 ± 0.29 mm at the baseline to 0.81 ± 0.27 at the end of observation, $p < 0.001$) and a reduction of the number of patients with an increased carotid thickening (from 42.8 to 17%, $p < 0.001$) (Petta et al. 2018). Instead, a Spanish prospective study on 85 HCV-cirrhotic patients did not find cIMT reduction within the 12th month after DAA-mediated HCV eradication (Revuelto Artigas et al. 2019a). In addition, Ichikawa et al. analyzed 48 CHC patients 1 year after they achieved SVR to DAA treatment and observed an increase of cIMT associated with an unfavorable lipidic profile (increase in LDL, HDL, sdLDL) (Ichikawa et al. 2019), suggesting that we cannot conclude on this point and further

studies are required to untangle the remaining controversies (Osibogun et al. 2017). Concluding on this point, despite some undesirable effects eventually due to the increase in Total and LDL cholesterol, an overall evaluation of the available data suggests that HCV eradication with DAA therapy, by eliminating numerous other harmful effects of HCV, exerts a favorable action on cardiovascular system, which results in a reduction of adverse events and reduced mortality.

7 DAAs and Renal Function

HCV infection and chronic kidney diseases (CKD) are joined by two main links, the first being the frequent exposure to HCV of patients with advanced CKD in dialysis units, and the second that HCV infection is able to directly induce kidney disease. Epidemiological investigations have underlined that HCV chronic infection increases the incidence of CKD and accelerates CKD progression to end-stage renal disease (Henson and Sise 2019). A strong correlation between anti-HCV positivity and the incidence of CKD has been demonstrated in a meta-analysis published in 2017 (Fabrizi et al. 2017), where anti-HCV positivity was identified as an independent predictor of death for dialysis patients (Fabrizi et al. 2020). HCV infection may contribute to tissue damage by directly infecting the endothelium, tubular epithelial cells, renal infiltrating leukocytes and other types of renal cells, such as mesangial cells, and is associated with three different kidney lesions: mixed cryoglobulinemic nephropathy, membranous-proliferative glomerulonephritis and membranous nephropathy.

Interferon-based treatment induced HCV eradication in nearly half of the treated patients, an event associated with a decreased rate of patients with renal disease and with a reduced progression to an end-stage renal disease (Arase et al. 2011; Feng et al. 2012; Hsu et al. 2015a; Montero et al. 2018; Fabrizi et al. 2020).

With the introduction of DAA therapies for chronic hepatitis C, the SVR rate also increased significantly in patients with CKD (Perazzo et al. 2020). A meta-analysis evaluating 11 studies on HCV patients with CKD-4/5 and treated with DAA-based therapy showed an SVR12 rate of 93.2%, an incidence of serious adverse events of 12.1% and treatment withdrawal of 2.2%, suggesting that DAA-based therapy is safe and efficient in eradicating HCV infection even in this patient setting (Li et al. 2017). It has also been shown that DAA therapy reduces the risk of kidney disease in CHC patients (Fabrizi et al. 2020).

The favorable effect of DAA treatments on glomerular filtration rate (eGFR) has also been proven, especially in patients with mild or moderate CKD (Calleja et al. 2017; Coppola et al. 2019; Sise et al. 2020; D'Ambrosio et al. 2020) (Table 4). In a cohort of 3,264 patients (9.5% in stage CKD 3 and 0.7% in stage 4/5) eGFR improved more significantly in those in CKD-3a stage ($p < 0.0001$) and CKD-3b ($p = 0.0007$) than in those in stage CKD-4/5 ($p = 0.024$) (D'Ambrosio et al. 2020). Half of 38 Spanish patients with baseline eGFR <60 ml/min/1.73 m^2 showed a remarkable improvement in these values after HCV eradication with DAA treatment (Calleja et al. 2017). Sise et al. in a follow-up of 573 days (SD = 337) after the discontinuation of DAA observed in patients with baseline eGFR <60 ml/min 1.73 m^2 the persistence of a substantial improvement induced by DAA treatment (Sise et al. 2020).

Instead, the results may be different if sofosbuvir-based regimens are used, as observed by Shin et al. in 4 of 28 patients with stage 3 CKD who showed a reduction of eGFR of more than 30% (Shin et al. 2017) (Table 4). In addition, in a cohort of 3264 patients, of whom 89.8% had baseline eGFR >60 ml/min, this index significantly decreased in those with CKD-1 ($p < 0.0001$) and CKD-2 ($p = 0.0002$) under a sofosbuvir- RBV combination treatment (D'Ambrosio et al. 2020). Similar results were found in a Spanish cohort of 1567 patients treated with ombisartan/paritaprenvir/ ritonavir + dasabuvir ± RBV and in 1,758 treated with ledipasvir/sofosbuvir ± RBV, where a mean eGFR reduction of −1.6 (SD = 12.4) ml/min/1.73 m^2 was observed in patients with baseline normal renal function (Calleja et al. 2017). In a

Table 4 Studies on the impact of DAA regimens on renal function

References	Study type	Number of patients, Setting	Treatment	Results, suggestions
Shin et al. (2017)	Observational study	28 CHC-G 1 patients with eGFR 30–60 ml/min	SOF/PEG/RBV SOF/SIM SOF/LDV	Worsening in renal function may occur; careful monitoring is suggested.
Calleja et al. (2017)	Cohort study	3,325 CHC-G 1 patients in various CKD stages and with eGFR >90 ml/min	LDV/SOF ± RBV OMV/PTV/ r + DSV ± RBV	Post-treatment eGFR, available for only 659 patients; for those with normal baseline renal function the mean (SD) change in eGFR was −1.6 (12.4) mL/min/1.73 m^2
Butt et al. (2018)	Cohort study	17,624 CHC patients in various CKD stages and with eGFR >60 ml/min	SOF/LDV ± RBV OMB/PAR/r ± RBV	A decline in eGFR values and development of anaemia were observed in a substantial proportion of patients.
Álvarez-Ossorio et al. (2018)	International, prospective, multicohort study	1,131 CHC patients in various CKD stages and with eGFR >90 ml/min, with or without HIV infection.	SOF/SIM SOF/LDV SOF/DCV OMV/PTV/r ± DSV	eGFR slightly declined during treatment, an effect persisting up to 12 weeks after treatment, regardless of HIV status.
Soeiro et al. (2018)	Observational prospective study	333 CHC patients with HIV coinfection, in various CKD stages and with eGFR >90 ml/min.	SOF/LDV ± RBV	Decrease in eGFR during treatment, reversible after treatment discontinuation.
Taramasso et al. (2018)	Observational prospective study	213 CHC-G 1 patients with HIV coinfection, in CKD stages 1, 2 or 3.	OMV/PTV/r/DSV	eGFR significantly declined during treatment, an effect reversed during a prolonged post-treatment follow-up.
Coppola et al. (2019)	Cohort study	403 CHC patients in various CKD stages and with eGFR >90 ml/min	SOF/RBV SOF/SIM ± RBV OMB/PAR/r ± RBV SOF/LDV ± RBV SOF/DCV ± RBV	Improvement in renal function.
Sise et al. (2020)	Cohort study	2,319 CHC patients in various CKD stages and with eGFR >90 ml/min	INF-containing regimens SOF-based regimens RBV-containing regimens	Treatment slowed CKD progression.
D'Ambrosio et al. (2020)	Observational study	3,264 CHC patients in various CKD stages and with eGFR >90 ml/min	SOF-based and no-SOF-based±RBV	eGFR declined during treatment in patients with preserved renal function and improved in those with CKD; no reversion upon drug discontinuation.

CHC Chronic Hepatitis (Some cirrhotic cases are included), *SVR* sustained viral response, *CKD* chronic kidney disease, *IFN* interferon, *Peg-IFN* pegylated-interferon, *RBV* ribavirin, *TVR* telaprevir, *BOC* boceprevir, *HIV* human immunodeficiency virus, *HBV* hepatitis B virus, *OMV* ombitasvir, *PTV* paritaprevi, *r* ritonavir, *DSV* dasabuvir, *G/P* glecaprevir/pibrentasvir, *SOF* sofosbuvir, *SIM* simeprevir, *LDV* ledipasvir, *DCV* daclatasvir, *ASV* asunaprevir, *EBR* elbasvir, *GZR* grazoprevir, *VEL* velpatasvir, *T2DM* type 2 diabetes mellitus

cohort of 17,624 patients treated with sofosbuvir + ledipasvir ± RBV or with paritaprevir/ritonavir/ombitasvir ± RBV, Butt et al. observed that 30% and 38% of patients in the two different therapeutic regimens, respectively, showed a reduction in eGFR by at least 10 ml/min/1.73 m^2 compared to the baseline normal value. However, it is useful to underline that these possible reductions in eGFR generally disappear within 12 weeks from the suspension of therapy (Butt et al. 2018).

In a Spanish/Portuguese cohort of 1131 patients, including 658 (58%) HIV/HCV-coinfected patients, the eGFR slightly declined during DAA treatments in patients with normal to moderately impaired renal function (Álvarez-Ossorio et al. 2018). A similar decrease in eGFR was observed in 273 HIV/HCV coinfected patients, more pronounced in those receiving tenofovir, in those treated with DAA for 24 weeks ($p = 0.009$) and in cirrhotic patients ($p = 0.036$) (Álvarez-Ossorio et al. 2018). Similar results were observed in a study on 144 HIV/HCV coinfected patients; a strong eGFR decline was observed in those concomitantly treated with tenofovir ($p = 0.0001$), ribavirin ($p = 0.0001$) or integrase inhibitors ($p < 0.0001$), in those with a longer duration of HIV ($p = 0.0002$) or HCV infection ($p = 0.035$), in those with a lower baseline HCV RNA ($p < 0.0001$), or with a previous HCV treatment ($p < 0.0001$), and in the elderly ($p < 0.0001$) (Taramasso et al. 2018).

In conclusion on this point, the HCV eradication obtained with DAA therapy in CHC patients exerts a beneficial effect even in those with impaired renal function and only some conflicting data persist on the effect of some DAA regimens on eGFR. Similar beneficial effects of DAA therapy are also observed in patients with HCV/HIV co-infection and even here doubts persist only on the use of drugs which may lead to a transient reduction in eGFR.

8 DAA Treatment of Cryoglobulinemia

Cryoglobulinemia is a condition characterized by the presence of cryoglobulins in the blood, which reversibly precipitate and form a gel at less than 37 °C and dissolve over 37 °C (Roccatello et al. 2018). Brouet's classification defines three types of cryoglobulinemia: Type I with single monoclonal immunoglobulins; type II, a mixed cryoglobulinemia with monoclonal and polyclonal immunoglobulins; Type III, a mixed cryoglobulinemia with IgM and IgG, both polyclonal. (Brouet 1983). Cryoglobulins could be detected in 25–30% of HCV-positive patients (Dammacco and Sansonno 2013) and 80–90% of cases with type II and type III mixed cryoglobulinemia carry HCV infection (Minopetrou et al. 2013; Roccatello et al. 2018) associated with a high incidence of severe liver fibrosis and cirrhosis (Roccatello et al. 2018). Arthralgia, asthenia and palpable purpura are the most common clinical manifestations of HCV related mixed cryoglobulinemic syndrome (MCS) and skin the most frequently organ involved; however, hematological disease and severe organ disfunction or failure (kidney, hearth, central nervous system, etc.) may occur (Dammacco and Sansonno 2013; Minopetrou et al. 2013). Treatment with Standard IFN provides HCV eradication in only a quarter of treated cases, with a substantial improvement in both liver function and CMS; in cases of temporary viral response, however, CMS usually relapses (Dammacco and Sansonno 2013). The introduction of treatment with Peg-IFN α-2a or 2b and RBV induced SVR in about half of treated patients. In a cohort study published by Gragnani et al. in 2015, the persistence of cryoglobulinemia was linked to a higher probability of Peg-IFN/RBV treatment failure (HR 2.03, 95% CI = 1.12–3.68, $p = 0.0204$), while the 63 HCV patients with MCS who reached SVR showed a clear improvement in clinical and laboratory MCS manifestation throughout a mean follow up period of 92.5 months (Gragnani et al. 2015).

The combination of a first generation DAA (telaprevir or boceprevir) with Peg-INF and RBV achieved HCV eradication in 65–75% of treated patients with remission or reduction of sign and symptoms of MCS (Humphries et al. 2014; Gragnani et al. 2014; Saadoun et al. 2014, 2015; Cornella et al. 2015).

IFN-based regimes have become obsolete once the second generation DAAs have been introduced, because of their good safety profile and higher effectiveness (SVR in 95% of treated patients). Same studies published in 2015 and in 2016 have shown a frequent remission of symptoms and signs of MCS in CHC patients after a DAA treatment (Makara et al. 2015;

Chak et al. 2015; Koga et al. 2017; Obata et al. 2017). Several cohort studies reported high percentages of patients who achieved a remarkable improvement of cryoglobulinemic vasculitis after HCV eradication with DAA therapy (Sise et al. 2016; Bonacci et al. 2017; Saadoun et al. 2017; Lauletta et al. 2017; Emery et al. 2017; Comarmond et al. 2017; Gragnani et al. 2018; Hassan et al. 2018; Miailhes et al. 2018; Pozzato et al. 2020) (Table 5); our meta-prop analysis on these percentages, shows an overall improvement in 78% of patients (95% CI: 0.69–0.86 p = < 0.001) (Fig. 1). A cohort study by Mahale et al. detected the incidence rate (*IR*) per 1,000 persons-year (Py) of CMS in HCV positive patients, either never treated (*IR* 1000 Py = 0.72; 95% CI = 0.66–0.78), or DAA-treated without SVR (*IR* 1000 Py =0.52; 95%CI = 0.41–0.67), or DAA-treated with SVR (*IR* 1000 Py =0.33; 95%CI: 0.21–0.5), showing that HCV RNA clearance is a protective factor in this setting; the adjusted hazard ratios (aHR) indicate no significant difference between treated patients without SVR vs. untreated, aHR 1.11, (95% CI = 0.85–1.45), whereas the differences between patients who reached SVR versus untreated and versus those treated without SVR ware both statistically significant, respectively 0.61 (95% CI = 0.39–0.94) and 0.55 (95% CI = 0.33–0.90) (Mahale et al. 2018). Cacoub et al. analyzed the effect of DAA-induced SVR obtained on HCV extrahepatic manifestations in a meta-analysis including 16 studies; the achievement of SVR was associated with a reduction in extrahepatic mortality (OR 0.44; 95% CI = 0.28–0.67), a higher complete remissions of clinical signs and symptoms of cryoglobulinemic vasculitis (OR 20.76; CI = 6.73–64.05) and with a greater efficacy in malignant B-cell lymphoproliferative diseases (OR 6.49; CI = 2.02–20.85) (Cacoub et al. 2018a).

Out of 12,985 HCV genotype 4 CHC patients successfully treated with second generation DAAs, Fayed et al. identified 50 patients with de novo detectable serum cryoglobulins and vascular renal affection 4.3 ± 1.3 months after treatment, the most common type of kidney affection observed in renal biopsies being membranoproliferative glomerulonephritis (52%); chronic kidney disease (CKD) developed in 46% of cases. The Authors concluded that de novo cryoglobulinemic glomerulonephritis and progression to CKD rarely complicate a successful DAA treatment (Fayed et al. 2018).

Concluding on this point, DAA treatment finds full application in CHC patients with MCS, since it has a good safety profile, induces HCV eradication in nearly 95% of patients and is associated with remissions of cryoglobulinemic vasculitis and with a reduction extrahepatic mortality. In addition, MCS infrequently occurs in CHC patients after HCV eradication.

9 DAAs and Lymphoma

Strongly characterized as a hepatotropic virus, HCV also infect and replicate within B and T cells (Sarhan et al. 2018) and is capable of driving clonal expansion of B lymphocytes (Kasama et al. 2011) within the complex HCV syndrome; therefore, HCV infection is associated and causally related to lymphomagenesis (Zignego et al. 1997). Worthy of notice, patients with HCV-driven type II mixed cryoglobulinemia are at increased risk for NHL (with a 35-fold higher risk than the general population) (Defrancesco et al. 2020). A vast study by the International Lymphoma Epidemiology Consortium and other epidemiologic studies have identified an association between chronic HCV infection and B-NHL subtypes, particularly with the diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma (MZL), and lymphoplasmacytic lymphoma) (Suarez et al. 2006; De Sanjose et al. 2008; Rattotti et al. 2019; Defrancesco et al. 2020). The double tropism and the double oncogenic potential of HCV is also underlined by some reports on HCV infected patients with both HCC and lymphoma (Shapira et al. 2001; Utsunomiya et al. 2009; Becker et al. 2010).

The first evidences for a link between HCV infection and lymphoma date back to the 1990s (Ferri et al. 1994; Pioltelli et al. 1996; Hanley et al. 1996; Galli et al. 1996; Brind et al. 1996;

Table 5 Impact of DAAs treatment on clinical manifestation of cryoglobulinemic vasculitis

References	Study type	Number of patients	Treatment	Improvement of symptoms related to cryoglobulinemia (complete or partial improvement) (n° pts)
Sise et al. (2016)	Cohort	12	SOF/SIM, SOF/RBV	8 among 12 symptomatic.
Bonacci et al. (2017)	Cohort	64	3D, SOF/LDV, SOF/SIM, SIM/DCV, SOF/DCV, Peg-IFN/RBV/DAAs, GRZ/ELB, FAL/DEL	30 patients among 32 symptomatic.
Saadoun et al. (2017)	Cohort	41	SOF/DCV	37 among 41 symptomatic.
Lauletta et al. (2017)	Cohort	22	SOF/RBV, 3D ± RBV, SOF/LDV, SOF/DCV	16 among 22 symptomatic.
Emery et al. (2017)	Cohort	83	PegIFN/RBV/ TEL or BOC, SOF/SIM, SOF/RBV, SOF/LDV, 3D ± RBV	11 among 18 symptomatic.
Comarmond et al. (2017)	Cohort	27	SOF ± RBV, SOF/DCV, SOF/SIM	24 among 27 symptomatic.
Gragnani et al. (2018)	Cohort	139	3D ± RBV, SOF/DCV ± RBV, SOF/LDV ± RBV, SOF/SIM ± RBV, SOF/RBV	65 among 77 patients symptomatic with SVR.
Hassan et al. (2018)	Cohort	120 (63 with cryoglobulinemia)	SOF/DCV	55 among 63 patients with Meltzer's triad.
Miailhes et al. (2018)	Cohort	47	SOF/RBV, Peg-IFN/SOF/RBV, SOF/NS3/4 PI±RBV, SOF/NS5A inhibitors±RBV, 3D.	14 among 28 symptomatic patients.
Pozzato et al. (2020)	Cohort	67	3D, SOF/SIM, ASUNEPRAVIR/DCV, SOF/LDV ± RBV	40 among 67 symptomatic patients.

SOF sofosbuvir, *SIM* simeprevir, *3D* ombitasvir/paritaprevir/ritonavir/dasabuvir, *LDV* ledipasvir, *DCV* daclatasvir, *GRZ* grazoprevir, *PIB* pibrentasvir, *FAL* faldaprevir, *DEL* deleobuvir, *TEL* telaprevir, *BOC* boceprevir

Zignego et al. 1997), either in association or, more rarely, in the absence of mixed cryoglobulinemia (De Vita et al. 1997, Luppi et al. 1996). Subsequently, the aetiological hypothesis was enriched by the detection of HCV RNA in in NHL lesions lymphoma samples (Ohsawa et al. 1998; Karavattathayyil et al. 2000) and by the demonstration of a positive correlation between viral replication and the risk to develop lymphoma (Amiel et al. 2000). This link was confirmed by a Meta-analysis published in 2006 (Dal Maso et al. 2006) and by the data from the Swiss Cohort Study on HIV/HCV coinfected patients (Franceschi et al. 2006). In 2003, an Italian multicentre case-control study confirmed that B-NHL may originate in CHC patients, suggesting a significant potential benefit of an antiviral treatment in limiting the burden of HCV-related haematological disease (Mele et al. 2003). The association of HCV infection with NHL was further confirmed in a case control study which, however, failed to find a significant correlation with Hodgkin Disease (Montella et al. 2001). Zhou et al., in 2016, proposed the HCV load as a prognostic factor in patients with HCV-positive diffuse large B cells lymphoma. (Zhou et al. 2016); in 2017, Shimono proposed HCV infection as an independent factor in the prognosis of follicular lymphoma (Shimono et al. 2017), In 2019, a meta-analysis by Zhu et al. reaffirmed the prominent role of HCV as a risk factor for NHL. (Zhu et al. 2019) and more recently, in 2020, a Turkish multicentre cohort study proposed HCV as a causative and prognostic factor for splenic marginal zone lymphoma (Okay et al. 2020).

It is worth noticing that some studies have highlighting geographic variations for the HCV-NHL association, suggesting a deeper evaluation of HCV genotypes and cofactors

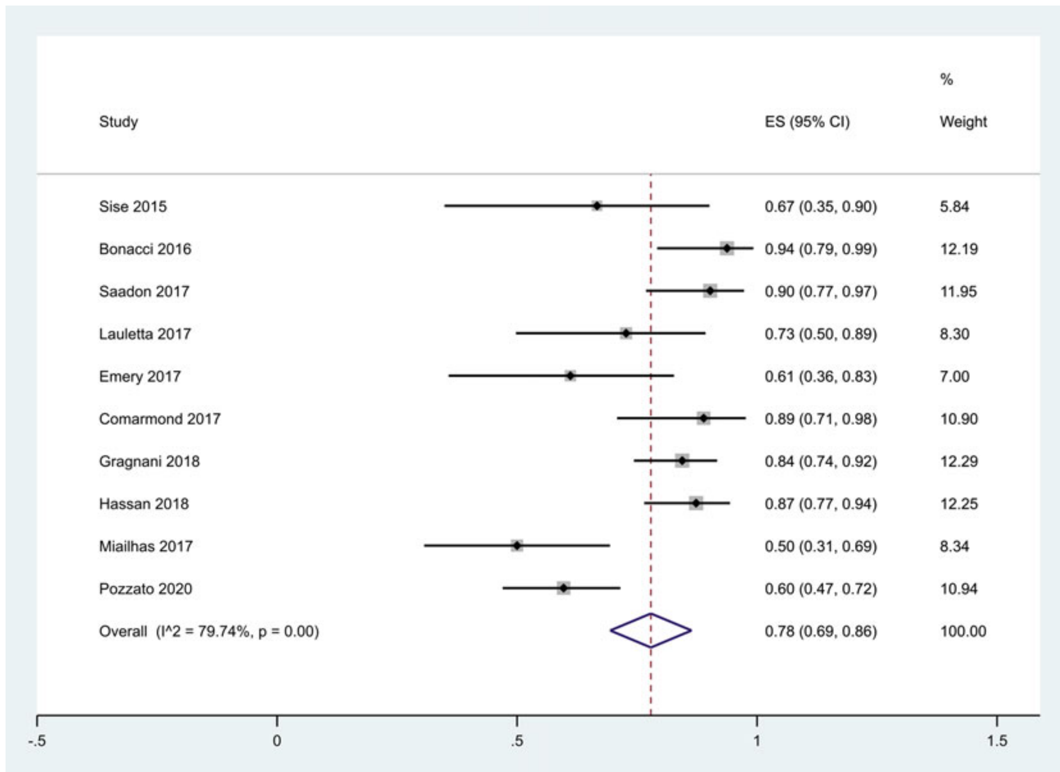

Fig. 1 The proportion of improvement considering clinical manifestation of cryoglobulinemic vasculitis after DAAs treatment and SVR

responsible of discrepancies. In detail, a meta-analysis found a strikingly positive association between HCV seropositivity and NHL only for Italian and Japanese patients (Matsuo et al. 2004); in the 2001 a prospective study on 1576 patients., concluded that HCV positivity was scarcely prevalent (1.83%) in patients with B-NHL in France (Hausfater et al. 2001).

It is worth reporting that a few other some studies denied the association between CHC and B-cell lymphoma (Collier et al. 1999; Avilés et al. 2003).

Considering the complex role of HCV in the related haematological disease the "Fondazione Italiana Linfomi" designed a specific "HCV prognostic score" to its management (Defrancesco et al. 2020).

Underlying mechanisms for HCV lymphomagenesis are far from being fully understood, but seem to revolve around chronic antigen-driven proliferation of B-cells, majorly mediated by viral proteins such as HCV core protein (Suarez et al. 2006; Alisi et al. 2007), and E2 envelope protein (Quinn et al. 2001; Douam et al. 2015), also observed in T cell lines (Zhao et al. 2006), with mechanisms promote a mutator phenotype of immunoglobulin and proto-oncogenes (e.g. Ig heavy chain, BCL-6, p53, and beta-catenin) (Machida et al. 2006a), increasing NF-κB expression and contrasting antiapoptotic functions (e.g. Bcl-2) (Defrancesco et al. 2020). Furthermore, HCV can upregulate B-cell receptor signalling (Dai et al. 2016) and trigger the enhancement of TLR4 expression along with IFN-beta and interleukin-6 production (Feldmann et al. 2006; Machida et al. 2006b). Additional mechanisms involve mitochondrial dysregulation and oxidative damage, with DNA damage, STAT3 activation (Machida et al. 2006a) and epigenetic alterations in microRNA (Peveling-

Oberhag et al. 2012), also in DLBCL (Augello et al. 2014). These complex mechanisms are extensively discussed in some dedicated reviews (Landau et al. 2007; Visco and Finotto 2014). A role in HCV lymphomagenesis has been proposed also for genetic risk factors, like the fibronectin gene polymorphisms (Fabris et al. 2008) and MHC II (e.g HLA-DQ) (De Re et al. 2004, 2009).

Of notice, HCV-related lymphoproliferative diseases present peculiar molecular signature, with possible therapeutic implications (De Re et al. 2012; Peveling-Oberhag et al. 2013; Visco et al. 2017), including an increase in specific oncogene expression, such as Bcl-2, correlated with t(14;18) translocation which disappears following HCV eradication (Zignego et al. 2000). A long past use of IFN in HCV positive patients with lymphoma was linked to the well-known antiproliferative effect of this drug, although toxicity was not negligible and SVR was far from being satisfactory, since HCV eradication was achieved by a quarter of patients receiving Standard IFN-based therapy by approximately 50% of those treated with Peg-IFN + RBV. The Peg-IFN and first-generation DAA-based therapy has been demonstrated able to obtain the SVR in 65–75% of HCV-1 CHC patients. A meta-analysis published by Peveling-Oberhag et al. in 2016 confirmed the strong association between SVR and B-NHL regression, particularly in MZL, suggesting that antiviral treatment may function as a first-line therapeutic approach when I-CT is not immediately required (Peveling-Oberhag et al. 2016), leading to a better overall survival in case of SVR (Hosry et al. 2016, Masarone and Persico 2019). Of notice, Su et al. observed a reduced risk for lymphoma development in patients who received early successful therapy for HCV infection (Su et al. 2019).

Some case-reports published in In 2015 showed that the second generation DAAs exert a favourable clinical effect on HCV lymphoma after HCV clearance: Rossotti et al. described a case of splenic MZL obtaining a favourable rapid hemato-virologic response after a 16-week treatment with faldaprevir, deleobuvir and RBV (Rossotti et al. 2015); Sultanik et al. reported the case of a HCV-positive woman with disseminated extranodal MZL treated with 4 weeks sofosbuvir + RBV, followed by 12 weeks sofosbuvir + daclatasvir, obtaining HCV clearance and a concomitant regression of lymphoma (Sultanik et al. 2015); Lim et al. reported a case of MZL regression by sofosbuvir + RBV (Lim et al. 2015); Carrier et al. reported a satisfactory viro-hematologic response (Carrier et al. 2015). in 5 NHL patients treated with sofosbuvir plus simprevir or daclatasvir, combined with chemotherapy one patient with DLBCL.

The French "ANRS HC-13 Lympho-C" Study observed two prospective cohorts of HCV-B-NHL patients: the first of 61 patients receiving Peg-IFN + RBV (combined with the first generation DAAs telaprevir or boceprevir only in some of them), and the second of 10 patients treated with sofosbuvir plus ledipasvir or simeprevir or daclatasvir or RBV; SVR led to a reduced risk for lymphoma progression, but IFN based regimen was poorly tolerated in DLBCL patients, already weakened by previous chemotherapy (Alric et al. 2016). A beneficial clinical effect of DAA therapy with ombitasvir/paritaprevir/RBV and dasabuvir was described in a HCV patient with aggressive double-hit B-cell lymphoma. (Galati et al. 2016).

In 2016, Arcaini et al. analysed a cohort of 46 patients with HCV-related lymphoproliferative disorders (indolent B-NHL, majorly MZL, and chronic lymphatic leukaemia, CLL): 39 subjects received sofosbuvir plus simprevir or RBV or daclatasvir or ledipasvir, while 7 subjects received an alternative regimen (paritaprevir/ ritonavir/ ombitasvir ± dasabuvir ± RBV or faldaprevir/ deleobuvir/ RBV); 98% of patients achieved SVR, while a hematologic response was obtained in 67% of cases (complete in 12 patients, 26%), more prominent in MZL, while no CLL/SLL patient obtained hematologic regression (Arcaini et al. 2016). Frigeni et al. observed a cohort of 100 patients with indolent HCV-B-NHL (only one with decompensated cirrhosis who failed to obtain SVR, with presenting lymphoma progression); 66 patients were treated with a DAA regimen and the remaining with an IFN-based regimen; nodal involvement was

apparently more severe and less responsive, cryoglobulinemia wasn't a relevant outcome modifier, the strongest responsiveness was observed in MZL and, noticeably, SVR (either $\pm$ IFN) led to an augmented overall hematologic response (Frigeni et al. 2020).

In 2019, a meta-analysis reaffirmed a powerful association between HCV eradication by DAA and favorable hematologic outcomes for HCV-positive B-NHL (Masarone and Persico 2019). Of notice, other studies observed a reduced risk for lymphoma development in patients who received early successful therapy for HCV infection (Su et al. 2019; Iwane et al. 2019) and prevention of relapse of DLBCL and, more generally, of malignant lymphoma (Pellicelli et al. 2018). As a complementary outcome, DAA eradication in HCV-positive DLBCL may reduce the liver toxicity of immune-chemotherapy (I-CT), and it can be offered after or even during I-CT, with the advantage of a timely management (Occhipinti et al. 2018; Merli et al. 2019, 2020).

Although the favourable effect of HCV eradication on the course of lymphoproliferative diseases is evident, some limitations have been underlined. For example, Schiavinato et al. analysed the peripheral blood lymphocytes populations and Ig light chain κ/λ ratio variations as indicators for monoclonal B-cell response in 9 patients with CHC and lymphoproliferative disorders treated with Ombitasvir/Paritaprevir/Ritornavir/Dasabuvir plus RBV; although all patients reached SVR12 and a global reduction of B cells, they still presented monoclonal components (Schiavinato et al. 2017). Rodríguez de Santiago et al. warned the scientific community against an excessive optimism as 6 patients out of 9 HCV patients with a lymphoproliferative disease presented a persistence of monoclonal B lymphocytes in the bone marrow 1 year after SVR; in addition, two NHL patients required additional therapy (chemotherapy and/or immunotherapy) after SVR was achieved (Rodríguez de Santiago et al. 2018). Furthermore, in aggressive B-cells lymphoma, such as DLBCL, there is a limited evidence for the therapeutic aid by DAA (Visco amd Finotto 2014).

In conclusion, available evidence increasingly recognizes the beneficial role of HCV eradication in the treatment of HCV-related lymphoproliferative disorders, particularly obtained with the highly tolerable and effective IFN-free DAA-based regimens, evidence reinforcing the importance of HCV in lymphomagenesis. Antiviral therapy appears majorly important in patients with indolent NHL, but some recent information support the use of HCV eradication also in patients affected by the more aggressive HVC-positive DLBCL, even if further investigation is needed in this topic.

10 Conclusion

In the last decade, emphasis has been placed on the extrahepatic involvement of chronic HCV infection, now fully recognized as a systemic disease, having reversed the previous "liver-focused" holistic paradigm towards an HCV pleiotropic action. In addition to being hepatotropic, HCV is also a lymphotropic virus responsible for polyclonal B-lymphocyte expansion that leads to the development of extrahepatic manifestations, such as type II cryoglobulinemia and some types of B-cell non-Hodgkin lymphomas, such as lymphoplasmacytic lymphoma/immunocytoma and marginal-zone lymphomas. In addition, chronic HCV infection is considered a trigger for immune-mediated disorders through a crossover immune response to self-antigens due to sequence similarities between viral proteins and self-proteins (molecular mimicry theory) or through the activation of autoreactive T-cells due to viral-induced local inflammation (bystander activation theory). In fact, chronic HCV infection has been associated with autoimmune diseases such as psoriasis, lichen planus, Sjogren syndrome and autoimmune thyroiditis, with the presence of organ-specific circulating anti-thyroperoxidase and anti-thyroglobulin autoantibodies and with high titers of non-organ-specific antinuclear, anti-smooth muscle and anti-liver/kidney microsome autoantibodies.

Patients with HCV infection show an increased overall mortality compared to the normal population, probably related to a dysmetabolic syndrome and cytokine remodeling towards chronic systemic inflammation that triggers endothelial dysfunction in response to the HCV envelope protein.

Luckily, recent advances in anti-HCV therapy have led to more efficient well tolerated interferon-free DAA regimens, so most patients can achieve HCV eradication. Ninety-five per cent of CHC patients without cirrhosis treated with DAAs recover completely, but substantial clinical and sometimes even histological improvement is also observed in cirrhotic patients. The beneficial action of the eradication of HCV infection with DAAs is also exerted on the extra-hepatic manifestations of this infection, but some results are contradictory or difficult to explain.

In fact, the available data do not allow a conclusion on whether the eradication of HCV infection induces a persistent reduction in fasting glucose and HbA1c; nevertheless, most studies strongly indicate a good T2DM compensation in patients treated with DAAs. Further long-term prospective studies on the evolution of glucose metabolism in HCV patients who achieved SVR with DAA treatment, diabetic and non-diabetic, are needed to resolve the remaining disputes.

CHC patients frequently show low serum levels of Total and LDL cholesterol, which increase significantly after HCV eradication, in some cases beyond the pre-treatment levels, most likely because the interaction between interaction HCV / lipid metabolism ceases. Despite this negative effect, HCV eradication exerts an overall favorable action on the cardiovascular system, possibly eliminating numerous other harmful effects exerted by HCV on this system.

Mechanisms responsible for direct vascular and cardiac damage in HCV patients have been identified in the procoagulant imbalance and in the IR/T2DM ratio. Furthermore, HCV-core protein can induce an immune-mediated oxidative damage in myocardial tissue and is considered a direct cardiotropic virus, responsible for dilated, hypertrophic right ventricular arrhythmogenicity, cardiac fibrosis and myocarditis. An association has been observed between carotid atherosclerosis, carotid intima-media thickness, β stiffness and HCV core protein. Other studies have reported an increased risk of acute coronary syndrome (ACS) and acute myocardial infarction (AMI), with an association between the number of affected vessels and HCV viral load. The abolition of many negative effects due to DAA induced HCV eradication explains how the increase in IR, Total and LDL cholesterol induced by the same drugs are not very influential. In this regard, it should be also considered that the increase in IR is transitory and therefore has only a temporary negative influence.

Infecting kidney endothelium, tubular epithelial cells, renal infiltrating leukocytes and mesangial cells, HCV is responsible of several kidney lesions, like mixed cryoglobulinemic nephropathy, membranous-proliferative glomerulonephritis, and membranous nephropathy. This infection speeds CKD to an end-stage and has been identified as an independent predictor of death for dialysis patients. The DAAs-induced HCV eradication exerts a beneficial effect in CHC patients with CKD, even in those HIV coinfected, but some conflicting data persist on the effect of some DAA regimens on eGFR. Indeed, the favorable effect of DAA on eGFR is more evident in patients with mild or moderate CKD (stages CKD-3a/CKD-3b) than in those with a more severe illness (stages CKD-4/5).

HCV infection is associated with both mixed cryoglobulinemia and non-Hodgkin's lymphoma, particularly B-cell NHL. MCS is currently considered as a B-cell benign lymphoproliferative disorder frequently induced by HCV infection, but it is also associated with autoimmune or lymphoproliferative disorders. HCV-induced MCS frequently shows a silent, indolent course, but in some cases, it may present a rapidly unfavorable, sometimes life-threatening outcome. Nearly 20% of HCV-related MCS patients show nephropathy at the time of first diagnosis, an index of unfavorable prognosis. DAA treatment finds full application in CHC patients with MCS, since it has a good safety profile, induces HCV eradication in nearly 95% of treated patients and

is associated with remissions of cryoglobulinemic vasculitis and with a reduction extrahepatic mortality. In addition, MCS infrequently occurs in CHC patients after HCV eradication.

The role of HCV virus in the pathogenesis of lymphoproliferative diseases have been shown by several epidemiological studies and is now worldwide accepted. Available studies increasingly recognize the beneficial role of DAAs-induced HCV eradication in treating of HCV-related lymphoproliferative disorders. Antiviral therapy appears majorly important in patients with low-grade B-NHL, but some recent information support using DAAs to obtain HCV eradication also in patients affected by the more aggressive form NHL and even in HVC-positive DLBCL, in this case in combination with chemotherapy.

In conclusion, DAA-induced HCV eradication influences favorably all the extrahepatic manifestations of this infection, with the exception of lipid homeostasis, where the increase in TC and LDL cholesterol could favor, at least theoretically, the occurrence of cardiovascular events. This eventuality, however, is poorly perceived in most cases, possibly because overwhelmed by the effect of HCV eradication in abolishing numerous other harmful effects of HCV infection on cardiovascular system.

Conflict-of-Interest Statement All the authors of the manuscript declare they have no conflict of interest in connection with this paper.

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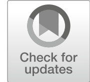

The Ability of a Concentrated Surfactant Gel to Reduce an Aerobic, Anaerobic and Multispecies Bacterial Biofilm *In Vitro*

Anne-Marie Salisbury, Marc Mullin, Lauren Foulkes, Rui Chen, and Steven L. Percival

Abstract

Biofilm formation in wounds can lead to increased inflammation, infection and delayed wound healing. Additionally, biofilms show increased recalcitrance to antimicrobials compared to their planktonic counterparts making them difficult to manage and treat. Biofilms are frequently polymicrobial, consisting of aerobic and anaerobic bacteria, as well as fungi and yeasts. The aim of this study was to evaluate the effects of a concentrated surfactant gel with antibacterial preservative agents (CSG) against wound relevant opportunistic pathogens, including an aerobic biofilm, anaerobic biofilm and multispecies biofilm. The CSG was added to a 48 h anaerobic biofilm of *Bacteroides fragilis*, a 24 h multispecies biofilm of *Acinetobacter baumannii, Staphylococcus aureus* and *Staphylococcus epidermidis* and a 24 h biofilm of *Pseudomonas aeruginosa* grown in an *in vitro* wound relevant environment. Following a contact time of 24 h with the CSG, the bacterial cell density of the biofilms was reduced by 2–4 log in comparison to an untreated control. The results demonstrate the ability of the CSG to disrupt wound relevant biofilms and support the use of the CSG in the clinic to treat wounds caused by biofilm related infections.

Keywords

Anaerobic biofilm · Concentrated surfactant · Drip flow · Multispecies biofilm · Wound dressing

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1 Introduction

Biofilms are formed when microbial cells adhere to a surface and each other and secrete extracellular polymeric substances (EPS), encasing themselves in an extracellular matrix (ECM) (Percival et al. 2014). Biofilms can form on medical devices such as catheters leading to infection (Donelli and Vuotto 2014). There is also increasing evidence showing an association of biofilm formation in chronic wounds, such as diabetic foot ulcers and also acute wounds, such as surgical sites (Banu et al. 2015; Malone et al. 2017; Percival et al. 2017a; Suryaletha et al. 2018).

Biofilm formation in wounds leads to increased inflammation, infection and delayed wound healing causing a large burden on healthcare (Attinger and Wolcott 2012; Zhao et al. 2013).

Staphylococcus aureus and *Staphylococcus epidermidis* are Gram-positive bacteria that often exist as commensal organisms on the skin; however, they are a common cause of skin and soft tissue infections and medical device related infections (Rogers et al. 2009; Mork et al. 2020). *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are Gram-negative bacteria that are highly associated with nosocomial infections and are often multi drug resistant (MDR) (Esposito and De Simone 2017). *S. aureus* and *P. aeruginosa* are the most common microorganisms isolated from chronic wounds (Serra et al. 2015). Although aerobic bacteria such as *S. aureus* and *P. aeruginosa* are frequently isolated from wounds, anaerobic bacteria, such as *Bacteroides fragilis* are also present. *B. fragilis* has been found in a number of different wound types including diabetic foot ulcers (DFUs) and surgical site wounds (Percival et al. 2018; Alexiou et al. 2017).

Although biofilms can consist of a single species, often they comprise of multiple species including aerobic bacteria, anaerobic bacteria and fungal/yeast species (Omar et al. 2017). The multispecies nature of biofilms can create a reservoir of resistance genes and an environment for genetic exchange (Savage et al. 2013; Balcazar et al. 2015; Aguila-Arcos et al. 2017). Due to the close proximity of cells and increased cell-to-cell contact, genetic exchange can occur via plasmid conjugation and DNA transformation following secretion during ECM formation (Molin and Tolker-Nielsen 2003; Madsen et al. 2012; Stalder and Top 2016).

Biofilms are also difficult to treat as they have inherent tolerance to antimicrobials at therapeutic levels that their planktonic counterparts are generally susceptible to. This can be attributed to several factors including the ECM, presence of persister cells, changes in gene expression and slow growth rate (Stewart et al. 2015; Hall and Mah 2017; Singh et al. 2017). The ECM consists of proteins, polysaccharides, lipids and extracellular DNA and often constitutes around 80–90% of the biofilm (Flemming 2016). The ECM has been shown to increase the tolerance of biofilms to antimicrobials through several different mechanisms including reducing the diffusion rate of antimicrobials and subsequently reducing the concentration reaching sessile microbial cells, resulting in exposure to sub-therapeutic levels (Van Acker et al. 2014). The heterogeneity of cells in a biofilm, resulting in differences in gene expression and growth rate and also increases the tolerance of biofilms to antimicrobials (Stewart et al. 2015; Pestrak et al. 2018). Persister cells are present in a biofilm and exist in a dormant state; therefore, they show high tolerance to antimicrobials and antibiotics that target replication and metabolic pathways (Lewis 2010; Pang et al. 2018). Persister cells are hypothesised to reside in infected and non-healing chronic wounds, posing a challenge for treatment (Percival et al. 2011).

Previous studies have demonstrated the ability of the concentrated surfactant gel with antibacterial preservative agents (CSG) included in this study to reduce monoculture biofilms of aerobic strains in various biofilm models (Salisbury et al. 2019b; Percival et al. 2017b). The aim of this study was to evaluate the ability of the CSG to reduce the biofilm cell density of relevant wound pathogens including an anaerobic biofilm of *B. fragilis*, a multispecies biofilm of *A. baumannii, S. aureus* and *S. epidermidis* and an aerobic biofilm of *P. aeruginosa* grown in an *in vitro* wound dressing model.

2 Materials and Methods

2.1 Test Articles

PluroGel® Burn and Wound Dressing, a concentrated surfactant gel with antibacterial preservative agents (CSG) including phenoxyethanol and potassium sorbate, was provided by Medline Industries Inc. (Chicago, IL).

2.2 Anaerobic Direct Contact Method

The effects of the CSG against a 48 h biofilm of *Bacteroides fragilis* ATCC 25285 was evaluated by growing the biofilm in 12 well plates and adding the CSG directly to it.

Briefly, a single colony of *B. fragilis* was inoculated into Tryptone Soya broth (TSB) (Scientific Laboratory Supplies, UK) + 5% laked horse blood (Scientific Laboratory Supplies, UK) and incubated anaerobically at 37 °C and 125 rpm for 24 h. The overnight culture was added to a 12 well plate, which was then incubated anaerobically at 37 °C for 48 h.

After incubation, the liquid was removed and the CSG was added to the biofilm in triplicate by adding 3 g per well to ensure complete coverage of the biofilms. Phosphate buffered saline (PBS) (Scientific Laboratory Supplies, UK) was also added to the biofilm in triplicate by adding 2 mL per well for an untreated control group. The plates were then incubated anaerobically at 37 °C for 24 h.

After the challenge period, the contents of each well were transferred to falcon tubes containing 10 mL Dey-Engley neutralising broth (Scientific Laboratory Supplies, UK) and sonicated on full power for 30 min. Samples were then vortexed briefly, serial diluted 1:10 in PBS and plated onto Tryptone Soya agar (TSA) (Scientific Laboratory Supplies, UK) + 5% sheep defibrinated blood (Scientific Laboratory Supplies, UK) in duplicate. The plates were incubated anaerobically at 37 °C for 48 h. After incubation, colonies were enumerated to calculate average CFU/mL.

2.3 Multispecies Biofilm Direct Contact Method

The effects of the CSG against a 24 h biofilm of *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 35984 and *Acinetobacter baumannii* ATCC 19606 was evaluated by growing the biofilm on membrane filter discs utilising a hydrogel as a nutrient source and adding the CSG directly to it.

The hydrogel was prepared by dissolving 3-sulfopropyl acrylate potassium salt (polymer) in PBS and then adding PEG dissolved in PBS, foetal bovine serum (FBS) and 1% 1-hydroxy cyclohexyl phenol ketone prepared in 70% ethanol (photo-initiator) to it. The mixture was added to a 12 well plate (2 mL/well) and set by exposing the hydrogel to 366 nm UV light.

An overnight inoculum of *S. aureus* ATCC 29213, *S. epidermidis* ATCC 35984 and *A. baumannii* ATCC 19606 was set up by inoculating 10 mL of TSB with a single colony and incubating at 37 °C and 125 rpm. Overnight cultures were adjusted to 1×10^8 CFU/mL before adding all 3 strains together in TSB at a final concentration of 1×10^6 CFU/mL. Durapore 13 mm (1 μM) membrane filter discs (Merck, UK) were incubated with the adjusted culture for 2 h at 37 °C and 125 rpm. Following this, the filters were transferred to a 12 well plate containing the hydrogel (1 filter/well) and incubated at 37 °C for 24 h.

Following 24 h biofilm growth, the filters were transferred to fresh 12 well plates and treated with the CSG by adding 3 g directly to each well to ensure complete coverage of the biofilm (n = 3). PBS was added to the untreated control by adding 2 mL per well. Biofilms were treated for 24 h at 37 °C.

To determine bacterial cell density, the contents of each well were transferred to 10 mL Dey-Engley neutralising broth and sonicated at full power for 30 min. Samples were vortexed briefly, serial diluted 1:10 in PBS and plated out onto TSA. The plates were incubated overnight at 37 °C and the following day counts were enumerated to calculate average CFU/mL.

2.4 Drip Flow Bioreactor Wound Dressing Model

The effects of the CSG was evaluated against a 24 h biofilm of *Pseudomonas aeruginosa* ATCC 700888 by growing the biofilm in the drip flow bioreactor. The biofilm was grown at an air/liquid

interface, under low fluid shear conditions to represent an exuding wound environment.

The drip flow bioreactor was prepared by adding a clean borosilicate microscope slide, with a 2.5 cm^2 absorbent pad attached, to each channel of the bioreactor. The drip flow bioreactor was then autoclaved at 121 °C.

An overnight inoculum was set up by inoculating 10 mL TSB with a single colony of *P. aeruginosa* ATCC 700888 and incubating at 37 °C and 125 rpm. The following day the absorbent pads were moistened with TSB and 2 cm^2 membrane filter discs were added to each pad. The overnight culture was adjusted to 1×10^8 CFU/mL and used to inoculate the filter membrane discs. The inoculated discs were air dried for 30 min before connecting the drip flow to a nutrient flow of 270 mg/L TSB at 5 mL/h/channel.

After 24 h, sterile gauze was cut into 2 cm^2 sections and 4 g of the CSG was added to each gauze to completely coat it. The coated gauze was added to the biofilm in triplicate before reconnecting the drip flow to the nutrient flow. A biofilm growth control group was included and remained untreated (n = 3). Treatment was applied for 24 h.

To determine bacterial cell density, each membrane filter disc was transferred to 10 mL Dey-Engley neutralising broth and sonicated on full power for 30 min. Samples were then vortexed briefly, serial diluted 1:10 in PBS and plated onto TSA. The plates were incubated overnight at 37 °C and the following day bacterial colonies were enumerated.

2.5 Statistical Analysis

Raw data was entered into Microsoft Excel and average CFU/mL was calculated. To determine if there was a statistical difference between the untreated control and the CSG treated biofilms the unpaired t-test was carried out using Prism 7 software.

3 Results

3.1 Effects on Anaerobic Bacteria

Following growth of a 48 h biofilm of *B. fragilis* ATCC 25285, the untreated control had a bacterial cell density of 1.01×10^9 CFU/mL (Fig. 1). In comparison, biofilms treated with the CSG had a bacterial cell density of 3.47×10^7 CFU/mL, showing nearly a 2 log reduction in cell count. The log reduction in bacterial cell count of *B. fragilis* following 24 h treatment with the

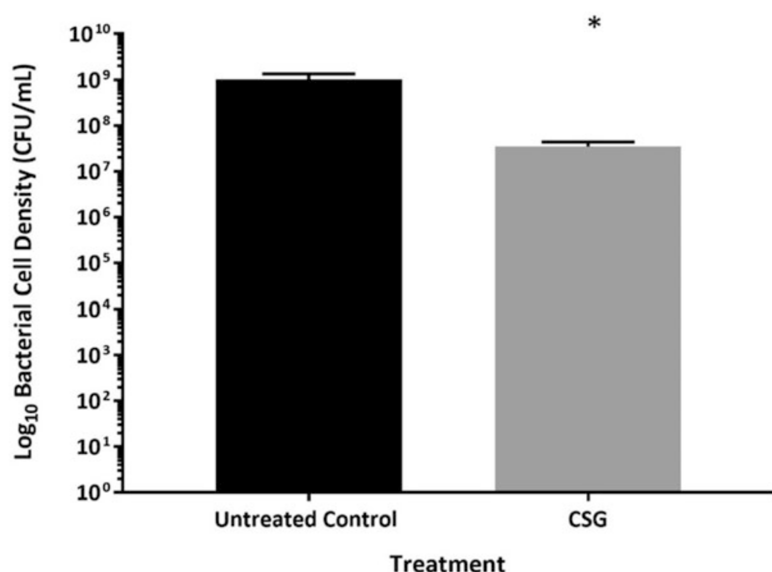

Fig. 1 Log_{10} cell density of *B. fragilis* ATCC 25285 48 h biofilm following 24 h treatment with a CSG. Error bars represent standard error of the mean. * represents a significant log reduction in comparison to the untreated control (p 0.0432)

CSG was significant in comparison to the untreated control (*p* 0.0432).

3.2 Effects on a Multispecies Biofilm

Following growth of a 24 h multispecies biofilm of *S. aureus*, *S. epidermidis* and *A. baumannii* the untreated control had a bacterial cell density of 6.43×10^8 CFU/mL (Fig. 2). Following treatment with the CSG a bacterial cell density of 2.74×10^4 CFU/mL was found, showing a 4 log reduction in cell count. The log reduction in bacterial cell count of the multispecies biofilm following 24 h treatment with the CSG was significant in comparison to the untreated control (*p* 0.0014).

3.3 Effects on a Biofilm Grown in a Wound Dressing Model

Following growth of a 24 h *P. aeruginosa* biofilm in a wound dressing model, the untreated control had a bacterial cell density of 9.72×10^8 CFU/mL (Fig. 3). Biofilms treated with the CSG showed a bacterial cell density of 1.09×10^7 CFU/mL showing ~2 log reduction in cell count. Although a ~ 2 log reduction in the *P. aeruginosa* biofilm cell density was found in this model the difference was not deemed as statistically significant (*p* 0.1762).

4 Discussion

In this study the effects of a CSG on an anaerobic biofilm of *B. fragilis*, a multispecies biofilm of *S. aureus*, *S. epidermidis* and *A. baumannii* and a *P. aeruginosa* biofilm grown in an *in vitro* wound dressing environment was evaluated.

B. fragilis is a common anaerobic strain isolated from wounds, with it being one of the most frequent strains isolated from patients with diabetic foot ulcers (DFUs) in several clinical studies (Ramani et al. 1991; Percival et al. 2018; Al Benwan et al. 2012). *B. fragilis* is also a common anaerobic strain isolated from surgical site infections (SSIs). In a recent study *B. fragilis* was the fourth main pathogen isolated from

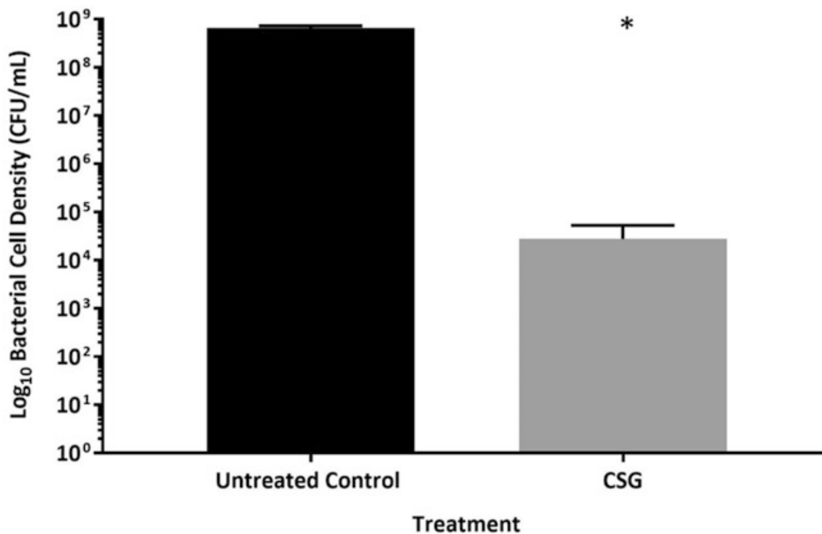

Fig. 2 Log_{10} cell density of a 24 h multispecies biofilm of *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 35984 and *Acinetobacter baumannii* ATCC 19606 following 24 h treatment with the CSG. Error bars represent the standard error of the mean. * = a significant log reduction in comparison to the untreated control (*p* 0.0014)

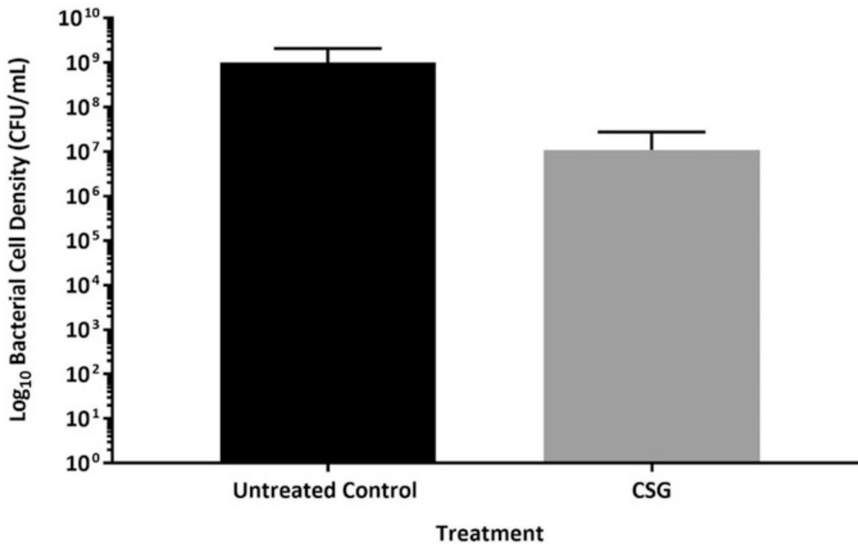

Fig. 3 Log_{10} cell density of *P. aeruginosa* ATCC 700888 24 h biofilm grown in the drip flow bioreactor wound dressing model following 24 h treatment with the CSG. Error bars represent standard error of the mean. No significant difference was found between the CSG treated biofilm and the untreated biofilm (p 0.1762)

patients with SSIs, with the other strains being aerobic strains (Alexiou et al. 2017). In this study, treatment of a *B. fragilis* biofilm with the CSG resulted in nearly a 2 log reduction in bacterial cell density (p 0.0432), showing the ability of the CSG to reduce the bacterial cell count of the anaerobic biofilm and potentially disruption of the biofilm.

Clinical studies have demonstrated that biofilms present in wounds, such as surgical site wounds, are often multispecies (Alexiou et al. 2017). The ability of *A. baumannii, S. epidermidis* and *S. aureus* to form biofilms is well documented (de Oliveira et al. 2016; Pakharukova et al. 2018; Olwal et al. 2018). Additionally, *A. baumannii* and *S. aureus* are both included on the list of ESKAPE pathogens, a list of the most common MDR (multi drug resistant) bacterial species causing nosocomial infections (Esposito and De Simone 2017). The presence of *S. aureus*, including methicillin resistant *S. aureus* (MRSA), is frequently reported as a predominant organism colonising wounds, such as surgical sites, diabetic foot ulcers and chronic wounds (Krishna and Gibb 2010; Banu et al. 2015; Serra et al. 2015; Obermeier et al. 2018). Additionally, increasing incidents of *A. baumannii* infection have been found, particularly in military unit associated wounds has been found (Davis et al. 2005; Schafer and Mangino 2008; Aurora et al. 2018). *S. epidermidis* is a commensal bacterium that is part of the normal skin flora; however, it has been shown to be a common cause of nosocomial infections in the immunocompromised, being associated with medical device related infections (Ziebuhr et al. 2006). It is estimated that up to 80% of infections of medical implant devices are caused by *S. epidermidis* biofilms (Rogers et al. 2009). In this study, the CSG reduced a multispecies biofilm by 4 log in comparison to an untreated control (p 0.0014), showing a reduction in the biofilm bacterial cell count and potentially disruption of the biofilm.

The ability of the CSG to disrupt a *P. aeruginosa* biofilm grown in the drip flow bioreactor was also evaluated. The drip flow bioreactor test method, ASTM E2647–13, is designed to grow a biofilm close to the air/liquid interface in an environment with continuous nutrient flow under low shear conditions (ASTM 2013). In this study, the method was adapted to represent a highly exudative wound environment,

as described previously (Bourdillon et al. 2017; Lipp et al. 2010). The constant flow of proteinaceous media allows the formation of a robust biofilm, while potentially washing away antimicrobials, which could occur in an exuding wound environment (Bourdillon 2016). In this model, the CSG reduced the *P. aeruginosa* biofilm cell density by ~2 log, showing a reduction in bacterial cell count and potentially disruption of a pre-formed biofilm in *an in vitro* model simulating the exuding wound environment.

The authors have previously shown a 3 log reduction in monoculture *A. baumannii* 24 h biofilms of clinical isolates grown in the CDC bioreactor model following treatment with the CSG (Salisbury et al. 2019b). Additionally, a reduction in biofilm cell density of *P. aeruginosa, S. aureus,* Methicillin resistant *S. aureus* (MRSA), *S. epidermidis* and *Enterococcus faecalis* has also been demonstrated in various biofilm models, but no antimicrobial activity was found against the same strains in the zone of inhibition assay, suggesting a potential detachment or dispersion effect in the biofilm models (Percival et al. 2017b). A more recent study demonstrated that treatment of a *P. aeruginosa* biofilm with the CSG reduced components of the biofilm ECM, particularly the extracellular DNA (eDNA) (Salisbury et al. 2019a).

Several publications have demonstrated the importance of eDNA for bacterial adhesion, structure of the biofilm and maturation of the biofilm (Whitchurch et al. 2002; Yu et al. 2019; Blakeman et al. 2019; Cherny and Sauer 2019). Therefore, the ability of the CSG in this study to disrupt the biofilm may be through its ability to reduce eDNA present in the ECM (Salisbury et al. 2019a). Presence of eDNA in the ECM has also been shown to contribute to biofilm mediated antimicrobial resistance of certain antibiotic classes. One study showed the presence of eDNA increased resistance to cationic antimicrobial peptides and aminoglycosides, but not fluoroquinolones or β-lactams, by upregulating PA3552-PA3559 cationic antimicrobial peptide resistance operon (Mulcahy et al. 2008). Therefore, the CSG in this study could potentially be used in combination to increase the susceptibility of biofilms to certain antimicrobials and improve treatment outcome of chronic wounds.

The data presented in this study highlights the potential ability of a CSG to disrupt wound related biofilms, with it demonstrating a reduction in bacterial cell count of an anaerobic biofilm of *B. fragilis* by nearly 2 log (p 0.0432) and a multispecies biofilm of *S. aureus*, *S. epidermidis* and *A. baumannii* by 4 log (p 0.0014). The CSG also exhibited a potential ability to disrupt *P. aeruginosa* reducing a biofilm grown in an *in vitro* model simulating the exuding wound environment by ~2 log, but this outcome was not deemed significant (p 0.1762). Previous studies support the ability of the CSG to cause biofilm disruption, with treatment of biofilm with the CSG resulting in reduction of biofilm ECM components. However, to further confirm biofilm disruption, it would be useful to carry out additional studies such as fluorescent staining of the ECM and bacterial cells and visualisation using confocal scanning laser microscopy. Additionally, to further investigate the activity of the CSG on biofilms, it would be interesting to compare the CSG to control gels, for example one without the antimicrobial preservatives, to evaluate the components having the largest impact on biofilm disruption. The data helps to support the use of the CSG in the clinic to aid in the management of biofilms in chronic wounds.

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