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Synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against *Pseudomonas aeruginosa* in an intraperitoneal foreign-body infection mouse model

Louise D. Christensen¹, Maria van Gennip¹, Tim H. Jakobsen¹, Morten Alhede¹, Hans Petter Hougen², Niels Høiby¹,³, Thomas Bjarnsholt¹,³ and Michael Givskov¹,⁴*¹Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark; ²Department of Forensic Medicine, Faculty of Health Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark; ³Department of Clinical Microbiology, Rigshospitalet, DK-2100 Copenhagen, Denmark; ⁴Singapore Centre on Environmental Life Sciences Engineering, Nanyang Technological University, Singapore 639798, Singapore

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Objectives: Quorum sensing (QS)-deficient *Pseudomonas aeruginosa* biofilms formed in vitro are more susceptible to tobramycin than QS-proficient *P. aeruginosa* biofilms, and combination treatment with a QS inhibitor (QSI) and tobramycin shows synergistic effects on the killing of *in vitro* biofilms. We extended these results to an *in vivo* *P. aeruginosa* foreign-body biofilm model. The effect of treatment initiated prophylactically was compared with treatment initiated 11 days post-insertion.

Methods: Silicone tube implants pre-colonized with wild-type *P. aeruginosa* were inserted into the peritoneal cavity of BALB/c mice. Mice were treated with intraperitoneal or subcutaneous injections of the QSIs furanone C-30, ajoene or horseradish juice extract in combination with tobramycin. Mice were euthanized on day 1, 2, 3 or 14 post-infection for the estimation of quantitative bacteriology, histopathology and cytokine measurements.

Results: Combination treatment of *P. aeruginosa* resulted in a significantly lower cfu per implant as compared with the placebo groups for all QSIs tested. For early-initiated treatment, a significant difference in clearing was also observed between the combination group and the single-treatment groups, and between the placebo group and the single-treatment groups. In one case a significant difference in clearing was found between the two single-treatment groups.

Conclusions: Synergistic antimicrobial efficacy could be achieved when treating mice with both a QSI and tobramycin, resulting in an increased clearance of *P. aeruginosa* in a foreign-body infection model. Our study highlights the important prospects in developing an early combinatory treatment strategy for chronic infections.

Keywords: biofilm, *in vivo*, implant model, antibiotics

Introduction

Infections related to the use of foreign bodies are serious complications of medical device insertion.¹,² Each year in the USA alone, about 1 million cases of nosocomial infections are associated with indwelling devices,³ and *Pseudomonas aeruginosa* is frequently linked with infections of indwelling catheters and foreign-body implants.⁴,⁵ It has, on several occasions, been shown that *P. aeruginosa* grow as biofilms on medically implanted foreign bodies, such as prosthetic heart valves, cardiac pacemakers, total joint prostheses and intravenous catheters.⁶–⁸ When *P. aeruginosa* have colonized a foreign body, it is almost impossible to eradicate the biofilm and generally it is necessary to remove the implant to get rid of the infection.⁹,¹⁰ Unfortunately, replacement surgeries have a high failure rate and the replacements are prone to recolonization.⁹ The biofilm mode of growth protects the bacteria from antibiotics such as tobramycin, ciprofloxacin and colistin, and also shields the bacteria from the host immune responses.¹²–¹⁶

When *P. aeruginosa* grow as biofilms, the bacteria use quorum sensing (QS) to coordinately control expression of tissue-...
damaging virulence factors, including proteases and rhamnolipids. The ability to produce rhamnolipids has been shown to be important for \textit{P. aeruginosa}, and we have proposed that rhamnolipids function as a protective shield against the innate immune system where contact causes necrosis of polymorphonuclear leukocytes (PMNs).\textsuperscript{12} In contrast, a \textit{P. aeruginosa} mutant with an inactive \textit{rhlA} gene (which disables the production of rhamnolipids) has lost the ability to cause necrosis. Furthermore, the persistence of the \textit{rhlA} mutant was significantly reduced in two different animal models of infection compared with the parent wild-type.\textsuperscript{15}

QS in \textit{P. aeruginosa} involves two acyl homoserine lactone (AHL)-based QS systems: the las and rhl systems. These two systems are organized with the dual-functioning receptors/transcriptional activators LasR and RhlR, and the signal synthetases encoded by \textit{lasI} and \textit{rhlI}. The synthetases synthesize a specific signal molecule named N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), respectively.\textsuperscript{16, 17} In addition to the AHL-based QS systems, \textit{P. aeruginosa} also employs the \textit{Pseudomonas} quinolone signal (PQS) system. The QS systems of \textit{P. aeruginosa} have been shown to be hierarchically arranged with the las system controlling the rhl system,\textsuperscript{18, 22} and the PQS system positioned between the las and rhl systems.\textsuperscript{22} However, the rhl system can operate independently of the las system,\textsuperscript{23} and it has been suggested that the PQS system controls this activation.\textsuperscript{24}

Recently, we have identified 1-isothiocyanato-3-(methylsulfinyl)propane, also known as ajoene, as a QS inhibitor (QSI) compound in horseradish. Furthermore, ajoene (4,5,9-trithiadodec-1,6,11-triene-9-oxide) was found as a QS present in garlic extract. By real-time PCR and DNA microarray analysis, ajoene was shown to specifically and effectively target two of the major QS networks in \textit{P. aeruginosa}, the LasIR and RhlIR systems, and was found to down-regulate QS-controlled rhamnolipid production in wild-type \textit{P. aeruginosa} batch cultures. However, no effect of ajoene could be obtained in vivo using a foreign-body infection model.\textsuperscript{25} Ajoene was shown in vitro to significantly inhibit a subclass of QS-regulated \textit{P. aeruginosa} genes. Additionally, ajoene was found to have an antimicrobial effect on \textit{P. aeruginosa} in vivo using a pulmonary infection mouse model (T. H. Jakobsen, M. van Gennip, R. K. Phipps, M. Shanmugham, L. D. Christensen, M. Alhede, M. Skindersoe, T. B. Rasmussen, K. Friedrich, F. Utte, P. Ø. Jensen, C. Moser, K. F. Nielsen, L. Eberl, T. O. Larsen, D. Tanner, N. Høiby, T. Bjarnsholt and M. Givskov, unpublished results) and horseradish juice extract, which we found to contain QS activity.\textsuperscript{25} We demonstrated the importance of initiating the treatment of the mice prophylactically or early in the infection, as early combination treatment improved the ability to clear the infection and resulted in a better outcome for the mice.

**Materials and methods**

**Bacterial strains**

All experiments were performed with wild-type \textit{P. aeruginosa} strain PA01, obtained from Professor Barbara Iglewski (University of Rochester Medical Center, NY, USA). The strain was tagged with a plasmid-based mini-Tn7 transposon system (pBK-miniTn7::gfp3) constitutively expressing a stable green fluorescent protein, according to Koch \textit{et al.}\textsuperscript{29}

**Animals**

Female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark) at 8–9 weeks of age, and were maintained on standard mouse chow and water ad libitum for 2 weeks before the challenge. The animal studies were carried out in accordance with the European Convention and Directive for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the Danish law on animal experimentation. All experiments were authorized and approved by the National Animal Ethics Committee, Denmark (the Animal Experiments Inspectorate, dyreforsøgsstilsynet.dk), and given the permit number 2010/561-1817.

**Foreign-body infection model**

Silicone implants were prepared as described previously by Christensen \textit{et al.}\textsuperscript{30} with modifications. Silicone tube implants with a size of 4 mm (inner diameter 4 mm/outer diameter 6 mm, Ole Dich) were used instead of square implants and inserted into the intraperitoneal cavity. A bacterial pellet from a centrifuged overnight culture was resuspended in 0.9% NaCl to an optical density at 600 nm of 0.1 and implants were left in this solution for 20 h at 37°C with shaking at 110 rpm. Animals were challenged according to the method of Christensen \textit{et al.}\textsuperscript{30} Mice were anaesthetized by subcutaneous (sc) injections in the groin area with hypnorm/midazolam (Roche) [one part hypnorm (0.315 mg/mL fenbutyl citrate and 10 mg/mL fluanisone), one part midazolam (5 mg/mL) and two parts sterile water]. At the end of the experiments, mice were euthanized by intraperitoneal (ip) injection of 10 mL/kg body weight (BW) pentobarbital (200 mg/mL) with lidocaine hydrochloride (20 mg/mL) (DAK).

**Bacteriology**

After removal from the peritoneal cavity of the mice, silicone implants were placed in centrifuge tubes containing 2 mL of 0.9% NaCl and kept on ice until the tubes were placed in an ultrasound bath (Branson Inc. model 2510, Branson Ultrasonic Corporation, USA) for 10 min (5 min degas followed by 5 min sonic). After the ultrasound treatment, 100 μL of the NaCl–bacteria solution was serially diluted and plated on blue agar plates (State Serum Institute, Denmark) for bacterial visualization.
Blue agar plates are selective for Gram-negative bacteria. The plates were incubated at room temperature for 2 days before determination of cfu per implant.

Preparation of QSIs and tobramycin for injection

The furanone C-30 solution was prepared as described by Christensen et al.18 The mice were given 1 mg/kg BW furanone C-30 and were treated with the inhibitor every 8 h (ip injections) for 2 days starting 1 h post-insertion. The placebo group was injected with a 2.4% ethanol solution (96% ethanol in 0.9% NaCl), corresponding to the amount of ethanol that the furanone C-30-treated group received. Treatment was continued until 8 h before the mice were euthanized.

Ajoene was dissolved in 96% ethanol to a concentration of 100 mg/mL, followed by a 40x dilution in a 20% (2-hydroxypropyl)-b-cyclodextrin solution (vehicle) to a final stock solution of 2.5 mg/mL. The mice were given 25 mg/kg BW ajoene and were treated with the inhibitor every 24 h (sc injections). For the early treatment experiment, the mice were treated from day 2 pre-insertion to day 2 post-insertion; for the late treatment experiment, the mice received ajoene from day 11 to day 13 post-insertion. Thus, QSI treatment was given for 3 days once the mice were infected for both the early and late treatment experiments. The placebo group was injected with a 2.4% ethanol solution (96% ethanol diluted in vehicle), corresponding to the amount of ethanol that the ajoene-treated group received. Treatment was continued until 24 h before the mice were euthanized.

Horseradish juice extract was prepared by using a juice extractor to obtain the juice from the horseradish root. The horseradish juice was centrifuged at 9000 g for 10 min in 15 mL centrifuge tubes and the supernatant was discarded. The pellet was mixed with 96% ethanol and diluted 40 times in vehicle to a final concentration of 0.5% horseradish juice per mouse before the mice were euthanized.

Tobramycin sulphate (Apodan, Copenhagen, Denmark) was dissolved in 0.9% NaCl to a concentration of 3 g/kg tobramycin. The mice were injected sc with 30 mg/kg BW tobramycin once every 24 h, initiated 24 h post-insertion for the early treatment experiment and 12 days post-insertion for the late treatment experiment. Treatment was continued until 24 h before the mice were euthanized.

MIC and pharmacokinetics of tobramycin

The MIC of tobramycin was determined by Etest (AB Biodisk, Sweden). The pharmacokinetics of tobramycin in non-infected BALB/c mice was determined prior to the combination treatment experiments and performed as described by van Gennip et al.,32 with modifications. In short, mice were divided into groups of four and tobramycin was administered sc. At different timepoints after administration (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3 and 4 h), mice were euthanized and blood was aseptically sampled from the heart with a syringe and transferred to a BD Microtainer SST gold tube (ref. 365956). After centrifugation, serum was stored at −20°C until determination of the concentration using fluorescence polarization immunoassay (Abbott AxSYM, Abbott Diagnostics, USA). Tobramycin sulphate (Apodan, Copenhagen, Denmark) was dissolved in 0.9% NaCl.

Histopathology

The abscesses surrounding silicone implants inserted in the mice were prepared for histopathological examination by fixation in 4% (w/v) formaldehyde solution (Bio & Gene, Denmark), embedding in paraffin wax and cutting into 5 μm thick sections, followed by haematoxylin and eosin staining. The slides were scanned at a low magnification, and, from an average evaluation of a minimum of five representative areas at higher magnification (×400), the type of inflammation was estimated. The inflammatory responses were scored as acute (>90% PMNs), chronic (>90% mononuclear cells [MNs]), both types present, no dominating type (PMNs/MNs) or no inflammation, as described previously for the evaluation of inflammation in lungs.13 The histopathological evaluation was carried out blind.

Cytokine levels in peritoneal cavity

To estimate relative cytokine levels in the peritoneal cavity, the cavity was flushed with 3 mL of 0.9% NaCl. The collected saline was preserved at −80°C until assay. The cytokine levels were determined by using a Multi-Analyte ELISArray Kit (SABiosciences, Frederick, MD, USA) according to the manufacturer’s instructions. Using two different arrays (the Mouse Common Chemokines and Mouse Inflammatory Cytokines & Chemokines kits), we were able to profile the levels of the following cytokines (cytokine names are abbreviated): IFN-γ, tumour necrosis factor α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and G-CSF. The plates were read at 450 nm in a Wallac 1420 Victor2 (Perkin Elmer, MA, USA) with wavelength correction at 570 nm.

Staining procedures of ex vivo implants

To visualize DNA, PMNs and biofilm, silicone implants were cut with a scalpel into four pieces and stained directly after removal from the mice. The pieces were placed in a flow cell with a coverslide on top for visualization with a confocal laser scanning microscope (LSM AG) was used to generate pictures of the DNA.

Statistical analysis

To compare the bacterial counts (cfu) between two groups of mice, the Mann–Whitney U-test was used (analysis of non-parametric data) for calculating P values in the statistical program GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

MIC and pharmacokinetics of tobramycin

The MIC of tobramycin was found to be 0.75 mg/L. Before the treatment experiments were performed, the pharmacokinetics of 30 mg/kg BW tobramycin administered sc in non-infected
female BALB/c mice was determined. The pharmacokinetic parameters of the free, unbound fraction \( f \) of tobramycin following the recent pharmacokinetic/pharmacodynamic terminology guidelines\(^{35}\) were estimated to be: \( f_{\text{max}} = 91 \, \text{mg/L} \) (peak concentrations were shown in first sample drawn); \( f_s = 0.37 \, \text{h (half-lives were determined using linear regression analysis on the first five points of the serum concentration–time curve);} \( f_{\text{AUC}} = 57 \, \text{mg-h/L} \) (the areas under the 24 h concentration–time curves were calculated by the trapezoidal/rule integral); \( f_{\text{AUC/MIC}} = 76 \) (the MIC used for the calculations was 0.75 mg/L); and \( f_{\text{Cmax/MIC}} = 121 \), which is well above the recommended ratio for the optimal concentration-dependent killing pharmacodynamics of aminoglycosides of 8–10,\(^{35}\) but similar to what can be obtained, e.g. in the sputum of cystic fibrosis (CF) patients when tobramycin is administered by nebulization.\(^{36}\)

**Combination treatment**

The previous findings that an *in vitro* wild-type *P. aeruginosa* biofilm grown in the presence of a QSI compound is more susceptible to tobramycin encouraged us to conduct the same combinatory treatment experiments in an *in vivo* *P. aeruginosa* foreign-body biofilm model. To do this, mice had silicone implants pre-colonized with a wild-type *P. aeruginosa* biofilm inserted into the peritoneal cavity, after which they were divided into four treatment groups. Two different QSI compounds and a QSI-containing herbal extract were tested in combination with tobramycin: furanone C-30, ajoene and horseradish juice extract.

**Furanone C-30 and tobramycin treatment**

After insertion of the implants, the mice were divided into the following four groups: Group 1 (placebo group) received 0.9% NaCl and 2.4% ethanol solution (n=9); Group 2 (furanone C-30 group) received furanone C-30 and 0.9% NaCl (n=8); Group 3 (tobramycin group) received tobramycin and a 2.4% ethanol solution (n=10); and Group 4 (combination group) received furanone C-30 and tobramycin (n=11).

At 48 h post-infection the mice were euthanized and the foreign-body implants were removed from the mice and the cfu per implant was determined.

By means of this experimental setup, we found a significant difference in the clearing of the bacteria from the implants when treating wild-type *P. aeruginosa* with a combination of furanone C-30 and tobramycin (Group 4) compared with both the placebo group and the single treatments with furanone C-30 (Group 2) or tobramycin (Group 3) (\( P < 0.0002, P < 0.00003 \) and \( P < 0.001 \), respectively) (Figure 1). A significant difference in clearing was also observed between the placebo group and the single-treatment groups (Group 2, \( P < 0.006 \) and Group 3, \( P < 0.0003 \)). Additionally, we found a significant difference in clearing between the two single-treatment groups (Group 2 versus Group 3, \( P < 0.01 \)) (Figure 1).

**Ajoene and tobramycin treatment**

Ajoene and tobramycin combination treatment was investigated both against an initial biofilm infection, as the case with furanone C-30, and against a chronic biofilm infection. For the initial biofilm infection, mice were divided into the following four groups 2 days pre-insertion of the implants: Group 1 (placebo group) received 2.4% ethanol in vehicle and 0.9% NaCl (n=25); Group 2 (ajoene group) received ajoene and 0.9% NaCl (n=22); Group 3 (tobramycin group) received tobramycin and 2.4% ethanol in vehicle (n=24); and Group 4 (combination group) received ajoene and tobramycin (n=23).

Ajoene treatment was initiated 2 days prior to implantation, as done previously with this QSI using a pulmonary infection model (T. H. Jakobsen, M. van Gennip, R. K. Phipps, M. Shamugham, L. D. Christensen, M. Alhede, M. Skindersoe, T. B. Rasmussen, K. Friedrich, F. Utte, P. Ø. Jensen, C. Moser, K. F. Nielsen, L. Eberl, T. O. Larsen, D. Tanner, N. Høiby, T. Bjarnsholt and M. Givskov, unpublished results), and tobramycin treatment was initiated 24 h post-insertion. Both treatments were injected sc every 24 h. At 72 h after insertion, the implants were removed from the mice and the cfu per implant was determined. We found a significant difference in the cfu recovered when comparing the treatment of wild-type *P. aeruginosa* with a combination of ajoene and tobramycin (Group 4) with that of both the placebo group (Group 1) and the single-treatment groups (Group 2 or Group 3) (\( P < 0.0001, P < 0.02 \) and \( P < 0.02 \), respectively) (Figure 2a). A significant difference in clearing was also observed between the placebo group and the single-treatment groups (Group 2,

![Figure 1.](http://jac.oxfordjournals.org/): Clearance of implants pre-colonized with wild-type *P. aeruginosa* inserted in the peritoneal cavity of BALB/c mice treated with either placebo (open circles), C-30 (filled triangles), tobramycin (TOB) (open triangles) or a combination of TOB and C-30 (C-30+TOB) (filled squares). Squares, triangles and circles represent cfu/implant in individual mice and horizontal bars represent the medians. The cfu/implant on control implants, not inserted into mice, is represented by filled circles. Mice were euthanized at day 2 post-insertion and the cfu/implant was determined. \( P \) values for the experiments and the number \( n \) of mice in each group are shown.
For the initial biofilm infection, mice were divided into the following four groups 2 days pre-insertion of the implants, and treatment with horseradish juice extract and vehicle was initiated: Group 1 (placebo group) received 2.4% ethanol in vehicle and 0.9% NaCl (n=5); Group 2 (ajoene group) received ajoene and 0.9% NaCl (n=5); Group 3 (tobramycin group) received tobramycin and 2.4% ethanol in vehicle (n=6); and Group 4 (combination group) received ajoene and tobramycin (n=6).

Treatments with ajoene/vehicle were initiated day 11 post-insertion and tobramycin/NaCl treatments at day 12 post-insertion. Treatments were injected sc every 24 h. At day 11 post-insertion, three mice were euthanized to determine the cfu per implant before starting treatment (see Figure 2b). Here we observed that all implants were embedded in abscesses. Fourteen days after insertion the rest of the mice were euthanized, and the implants were removed from the mice and the cfu per implant was determined. Furthermore, it was observed that all implants, except one from Group 4, were embedded in abscesses that were collected for histopathological evaluation where the type of inflammation was evaluated. No difference in the cellular inflammatory response between any of the groups was observed. By means of cfu we found significantly less wild-type *P. aeruginosa* on the implants when treating the mice with a combination of ajoene and tobramycin (Group 4) compared with the placebo group (Group 1) (P<0.02) (Figure 2b), even though the cfu was only reduced by ~4-fold (the median cfu count± SEM for Group 1 was 3700000±7200000 and for Group 4 was 10600000±8400000). No significant difference was observed between the other groups.

**Horseradish juice extract and tobramycin treatment**

Horseradish juice extract was also tested against both an initial biofilm infection and a chronic biofilm infection. We used horseradish juice extract instead of only the main QSI present in the juice extract, iberin, as iberin has no QSI effect *in vivo*. For the initial biofilm infection, mice were divided into the following four groups 2 days pre-insertion of the implants, and treatment with horseradish juice extract and vehicle was initiated: Group 1 (placebo group) received 2.4% ethanol in vehicle and 0.9% NaCl (n=18); Group 2 (horseradish group) received horseradish juice extract and 0.9% NaCl (n=24); Group 3 (tobramycin group) received tobramycin and 2.4% ethanol in vehicle (n=24); and Group 4 (combination group) received horseradish juice extract and tobramycin (n=24).

Treatments were injected sc every 24 h, and treatment with tobramycin and 0.9% NaCl was initiated 1 day post-insertion. Mice were euthanized at day 3 post-insertion and the cfu per implant was determined. We found that there was a significant difference in the clearance between the placebo group (Group 1) and the combination treatment group (Group 4) (P<0.0001) (Figure 3a). Furthermore, a significant decrease in the number of bacteria recovered from the implants was found when treating with the combination therapy compared with the two single-treatment groups (Groups 2 and 3) (P<0.0001 for both). Additionally, a significant difference was observed between the placebo group and the single-treatment groups (P<0.04 and P<0.02, respectively), but no difference was found between the single-treatment groups (P=0.57) (Figure 3a).
Horseradish juice extract not only had an effect on the bacterial count on the implants, but it was also clear that mice treated with the extract, and especially the combination of horseradish juice extract with tobramycin, had cleaner fur and were less influenced by the infection compared with the mice treated with vehicle. We speculated that the better state of the mice corresponded to a lower inflammation in the peritoneal cavity. To test this, we profiled the cytokine levels in the peritoneal cavity using a MultiAnalyte ELISAArray Kit (SABiosciences). Saline samples collected from three mice were pooled to provide sufficient material to measure the cytokine levels; the experiment was performed twice. We found a clear trend showing that the levels of inflammatory cytokines (KC, MCP-1, MIP-1b, G-CSF and IL-6) were lower in the single-treated horseradish group compared with the placebo group (Figure 4). This difference was not found in non-infected animals.

To address the outcome in a chronic biofilm infection study, mice had silicone implants inserted at day 0 and the mice were divided into four groups: Group 1 (placebo group) received horseradish juice extract and 0.9% NaCl (n = 6); Group 2 (tobramycin group) received tobramycin and 0.9% NaCl (n = 6); Group 3 (horseradish juice extract/vehicle treatment) was initiated at day 3 post-insertion and the cfu per implant was determined. P values for the experiments and the number (n) of mice in each group are shown.

![Figure 3. Clearance of implants pre-colonized with wild-type P. aeruginosa inserted in the peritoneal cavity of BALB/c mice treated with either placebo (open circles), horseradish juice extract (HR) (filled triangles), tobramycin (TOB) (open triangles) or a combination of TOB and HR (HR+TOB) (filled squares). Squares, triangles and circles represent cfu/implant in individual mice and horizontal bars represent the medians. The cfu/implant on control implants removed day 11 post-insertion is represented by open squares and control implants, not inserted into mice (day 0), are represented by filled circles. (a) Early treatment experiment where the implants were removed 3 days post-insertion and the cfu/implant was determined. (b) Late treatment experiment where the implants were removed 14 days post-insertion and the cfu/implant was determined. P values for the experiments and the number (n) of mice in each group are shown.](http://jac.oxfordjournals.org/)

![Figure 4. Cytokine levels (KC, MCP-1, MIP-1b, G-CSF and IL-6), measured using a MultiAnalyte ELISAArray Kit, in fluid obtained from flushing the peritoneal cavity of mice treated with either horseradish juice extract (black bars) or placebo (vehicle, white bars).](http://jac.oxfordjournals.org/)

Figure 3. Clearance of implants pre-colonized with wild-type P. aeruginosa inserted in the peritoneal cavity of BALB/c mice treated with either placebo (open circles), horseradish juice extract (HR) (filled triangles), tobramycin (TOB) (open triangles) or a combination of TOB and HR (HR+TOB) (filled squares). Squares, triangles and circles represent cfu/implant in individual mice and horizontal bars represent the medians. The cfu/implant on control implants removed day 11 post-insertion is represented by open squares and control implants, not inserted into mice (day 0), are represented by filled circles. (a) Early treatment experiment where the implants were removed 3 days post-insertion and the cfu/implant was determined. (b) Late treatment experiment where the implants were removed 14 days post-insertion and the cfu/implant was determined. P values for the experiments and the number (n) of mice in each group are shown.

Previously, we have shown that functional QS systems play a key role in the ability of *P. aeruginosa* to persist in a foreign-body infection model and a pulmonary infection model. Treatment with the QS inhibitor horseradish extract reduces the bacterial count in both mouse models of infection. However, the present finding that horseradish extract can reduce the bacterial count per lung is in agreement with previous reports. In this study, we found that the treatment of mucoid *P. aeruginosa* isolates with tobramycin initiated 1 h post-infection resulted in a significant reduction of the bacterial count per lung compared with treatment initiated 24 h post-infection.

In vitro studies have shown that horseradish extract can reduce the bacterial count in both mouse models of infection. However, the present finding that horseradish extract can reduce the bacterial count per lung is in agreement with previous reports. In this study, we found that the treatment of mucoid *P. aeruginosa* isolates with tobramycin initiated 1 h post-infection resulted in a significant reduction of the bacterial count per lung compared with treatment initiated 24 h post-infection.

In conclusion, our study provides evidence that horseradish extract can be used as a potential therapeutic agent against *P. aeruginosa* biofilms. The combination therapy with tobramycin and horseradish extract may have significant synergistic effects, leading to a reduction in bacterial counts and improved clinical outcomes. Further research is needed to evaluate the long-term efficacy and safety of this combination therapy in clinical settings.
and it was found that ajoene down-regulated 11 genes, where 10 were shown to be QS regulated. Furthermore, ajoene (at 25 mg/kg BW) was found to significantly facilitate the ability of mice to clear a P. aeruginosa lung infection (T. H. Jakobsen, M. van Gennip, R. K. Phipps, M. Shanmugham, L. D. Christensen, M. Alhede, M. Skindersoe, T. B. Rasmussen, K. Friedrich, F. Utte, P. Ø. Jensen, C. Moser, K. F. Nielsen, L. Eberl, T. O. Larsen, D. Tanner, N. Haiby, T. Bjarndahl and M. Givskov, unpublished results). The present data clearly demonstrate that combination therapy shows a greater clinical potential due to the synergistic antimicrobial efficacy.

Treatment with horseradish juice extract not only had an effect on the cfu on implants, but also left the mice in a better state, which we found corresponded to lower inflammation in the peri-implation cavity. Treatment with horseradish juice extract resulted in reduced levels of KC, IL-6, G-CSF, MCP-1 and MIP-1b. All cytokines are involved in the inflammatory response in mice when exposed to bacteria, e.g. G-CSF is the major mobilizer of PMNs from the bone marrow and KC is involved in PMN cell activation. Since the difference in cytokine levels was not observed in non-infected mice, it is not likely that horseradish exerts anti-inflammatory effects but rather works through virulence quenching.

The various mechanisms behind biofilm-specific antibiotic resistance are important to understand in order to develop new antibiofilm drugs. Although the mechanisms are not clarified, the development of aminoglycoside tolerance is, in part, regulated by QS.14,28,41 Mulcahy et al.14,41 found that the presence of extracellular DNA (eDNA) can reduce the susceptibility per se to aminoglycosides by neutralizing the activity of the antibiotic by means of its cation-chelation properties. It has been shown that eDNA release is QS regulated and that QS-deficient biofilms are less tolerant of antibiotics, including tobramycin.14,43 This offers an explanation as to why we observe synergistic effects of combination treatments. Furthermore, we have previously shown that wild-type P. aeruginosa are able to lyse PMNs, which subsequently spill their internal content of DNA.12 The molecular basis for this DNA spill is the QS-controlled production of rhamnolipid,12 which we have proposed to function as a PMN shield.44 Furthermore, in vitro experiments of the exposure of freshly isolated PMNs to bacterial biofilms results in the up-regulation of rhamnolipid production, an effect that is likely transmitted through the QS controllers.44 The QSIIs used inhibit rhamnolipid production by disabling the QS-mediated control. Recently, using this mouse model we visualized PMNs on top of a wild-type P. aeruginosa biofilm when investigating implants with scanning electron microscopy. Furthermore, eDNA was also observed on the implants using CLSM (M. van Gennip, L. D. Christensen, K. Qufterup, C. Prats, M. Alhede, H. P. Hougen, R. Phipps, P. Ø. Jensen, N. Haiby, M. Givskov and T. Bjarndahl, unpublished results). It is therefore likely that DNA from lysed mouse PMNs and eDNA from the bacteria are both present on top of the biofilm, and in this way contribute to the neutralization of tobramycin and other aminoglycosides (see Figure 5 for PI staining of DNA on an implant removed from a mouse 24 h post-insertion).

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**Transparency declarations**

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