In Vitro Evaluation of a Hypobromous Acid Hygiene Stabilized Solution in the Reduction of Bacterial Load Associated to Blepharitis Conditions

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Research Article

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Abstract

Blepharitis is a chronic inflammation of the periocular skin area and it is characterized by eye itching, burning, dryness and irritation, with progression to chronic dry eye syndrome, where the eyelids margins of blepharitis patients are frequently colonized by bacteria.

The aim of the present study was to investigate the *in vitro* bactericidal activity (BA) of a stabilized active bromine solution (MDI-102) at neutral pH for the potential use in the treatment and prevention of blepharitis.

The time kill assays have been conducted both in *clean* and in *dirty* conditions (by using bovine albumin solution as the interfering substance) at different ranges of concentration.

The results show the topical solution to be capable of inactivating, in less than 0.5 minutes, more than 99.9% of several bacterial species involved in the clinical manifestations of blepharitis: *Enterococcus hirae, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Serratia marcescens.* Dirty condition tests confirm the results shown without albumin (clean conditions).

This study demonstrates that MDI-102 active bromine solution can markedly reduce (*in vitro*) the bacterial activity, responsible of clinical manifestation of blepharitis. Thus, MDI-102 can be considered a promising tool for the periocular area and eyelids cleaning for blepharitis patients.

The use of this formulation may contribute in the long-term prevention and hygienic treatment of blepharitis condition. Furthermore, MDI-102 can be considered as an alternative to reduce the use and the abuse of topical antibiotics in the daily practice, which may contribute to the increase of resistance to the antibiotics in the clinical setting.

Key Points

- Br-based solution (MDI-102) is tested as bactericidal for blepharitis treatment.
- MDI-102 can eliminate more than the 99.9% of the tested bacteria.
- MDI-102 can kill the bacteria in less than 30 seconds.

1. Introduction

Blepharitis is a chronic inflammation of the periocular skin area, which includes eyelids and sometimes eyelashes and may involve the Meibomian glands[1]. This condition is a very common ocular disorder[2] and it is characterized by eye itching, burning, dryness and irritation, with progression to chronic dry eye syndrome[2-4].

Meibomian gland dysfunction is the most common aspect of posterior blepharitis[5] and the local microbiome plays an important role in the clinical manifestation of this condition[6]. This is the reason

why topical antibiotics can be used to reduce the bacterial load and provide symptomatic relief[7].

The eyelids margins of blepharitis patients are frequently colonized by bacteria, predominantly by Gram-positive bacteria, including species of *Staphylococcus, Propionibacterium* (*Cutibacterium*) *and Corynebacterium spp.*[8-10].

Current standard treatment of initial blepharitis includes the use of antibiotics[7], however, the mainstay of the treatment is an eyelid hygiene regimen, which needs to be continued long term to prevent recurrence of the disease[11]. An effective solution for the eyelid and eyelash hygiene thus represents a valid tool to reduce the use, and the abuse, of topical antibiotics in the daily practice, which may contribute to the increase of resistance to the antibiotics in the clinical setting[12].

Hypobromous acid (HBrO) is a weak, inorganic acid mainly produced and handled in aqueous solution. HBrO is used as bleach, oxidizer, deodorant, and disinfectant, due to its ability to kill the cells of many pathogens[13].

The opportunity to test the use of HBrO in the hygiene treatment of blepharitis has the following rationale:

1) HBrO was proven to induce lysis of blood cells at approximately 10-fold lower concentration than HClO[14]. Furthermore, in general, bromine compounds show higher bactericidal efficacy compared to chlorine solutions[15,16];

2) bromine is less toxic than chlorine[17];

3) bromine and HBrO are more stable at neutral pH and more compatible with the physiological pH of the periocular skin area, compared to HClO solutions, used in the same setting[18];

4) Br-species are more effective bactericidal on acneic skin compared to Cl-species[15,19,20].

All these background data, suggest that active bromine compounds are very potent oxidants and that they clearly excel their chlorine analogues.

This study describes the effect of active bromine compounds (Br_2, HBrO/BrO^-) stabilized solution, concentration ranged between 0.005% and 0.05%, on bacterial species involved in the periocular skin flora. The aim of the present work is to test this active bromine compounds solution, named MDI-102, for its potential use for the treatment and prevention of blepharitis.

2. Materials And Methods

2.1. Chemicals and solution preparation

KBr was purchased by *CARLO ERBA Reagents*, H_3PO_4 (85%) was from *Merck-Sigma Aldrich*, while the NaClO (3%) was a commercial hypochlorite used for water intended for human consumption (in

compliance with UNI EN 901:2013).

The albumin, used for the *dirty test*, was purchased by *VWR*.

Tryptone soya agar (TSA) from *Microbiol* was used as culture media.

The present solution, named MDI-102, consists in active free bromine solution (Br_2, HBrO/BrO^-) in a concentration ranged between 50 and 550 ppm. MDI-102 was prepared as follows. KBr (0.1-1%), phosphoric acid solution (H_3PO_4 85%) 0.05-0.5% and sodium hypochlorite (NaClO al 3%) 0.005-0.05% were mixed in ultra-pure water solution (90-99%). The exact concentration of the components depends on the final MDI-102 solution concentration (80 or 500 ppm). The reactions occurring in the solution during the preparation are outlined in Figure 1. Briefly, KBr dissolved in water, forms Br^- and K^+ (r. 1). After the addiction of hypochlorite, and the chlorine formation in water (r. 2-3), the bromide reacts with the chlorine forming Br^-, HBrO, Br_2 and Cl- (r. 4-5), which are the main components of the solution MDI-102. Furthermore, as previously described, due to the excess of KBr compared to the hypochlorite, all the ClO^- can be considered converted to Cl^-; it follows that the only antimicrobial effect is due to Br-based species.

Due to the low NaClO stability, the hypochlorite concentration is always analysed before the solution preparation. The free halogen concentration has been assessed using Hach Lange kit. The kit measures the free halogen concentration as follow. The N,N-diethyl-p-phenylenediamine, in the kit, is oxidized by the free halogen making a reddish compound which absorbs at 510 nm. The concentration is measured using Hach DR6000 spectrophotometer (cuvette with 2.5 cm optical path length). The NaClO concentration in the experiments was ranged from 2.9 and 3%.

2.2. Time kill assays

Time kill assay test (kinetic tests) was performed in order to test the bactericidal activity (BA) of MDI-102, using the bacterial strains reported by:

- Standard *UNI EN ISO 11930:2019* (Evaluation of the antimicrobial protection of cosmetics);
- Standard *UNI EN 1276:2019* (Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas);
- Standard *UNI EN ISO 14729:2010* (Contact lens care products - Microbiological requirements and test methods for products and regimens for hygienic management of contact lenses);
- Stroman et al.[21] (blepharitis treatments products) and Groden et al.[10] (blepharitis flora).

The tested microorganisms were:

- *Pseudomonas aeruginosa* ATCC: 9027 (*UNI EN ISO 11930:2019*),
- *Enterococcus hirae* ATCC: 10541 (*UNI EN 1276:2019*),
- *Serratia marcescens* ATCC: 13880 (*UNI EN ISO 14729:2010*),

- *Staphylococcus aureus* ATCC: 6538 (*UNI EN 1276:2019*),
- *Pseudomonas aeruginosa* ATCC: 15442 (*UNI EN 1276:2019*),
- *Escherichia coli* ATCC: 8739 (*UNI EN ISO 11930:2019*),
- *Staphylococcus epidermidis* ATCC:12228 (Stroman *et al.* (2017) DOI:10.214/OPTH.5132851).

MDI-102 bactericidal activity was tested at 500 and 80 ppm (active bromine concentration). The tests were carried out following the Standard *UNI EN 1276:2019* with slight modification. Briefly, the solution was composed by 8 mL of testing solution (MDI-102), 1 mL of inoculum (microorganisms solution) and 1 mL of water. Microrganisms were inoculated at concentration ranged between $2x10^5$ and $3x10^7$ colony-forming unit (CFU) mL^{-1} [21]. Microbial concentration was monitored at different times: 0.5, 1, 2, 5, 20, 30 minutes after the addition of the bactericidal solution (MDI-102). After the selected time a neutraliser (Dey/Engley Neutralizing broth) was added as inactivator. A blank test (control) without the bactericidal solution was carried out before each test in order to assess the initial microbial concentration, thus to monitor the microbial concentration over time. Tests were performed at 25 ± 1 °C, room temperature. After the fixed time, a solution aliquot was sampled, inoculated on a Petri dish and incubated at 36 ± 1 °C for 48 h. These tests are following referred as “clean tests”. All the tests were carried out in triplicates; the standard error for all the tests was ranged between zero and 1.5%.

Time kill assay tests were also carried out in “dirty condition” in order to assess the solution’s BA under real conditions. Dirty tests were performed adding albumin (3 g L^{-1}) as interfering substance (*UNI EN 1276:2019*).

The tests were carried out as previously described, thus with same microorganisms’ strains and concentration, times, temperatures, etc. The only difference between the clean and the dirty condition was the presence of albumin in the dirty condition tests, 1 mL of albumin solution (3 g L^{-1}) was used instead of 1 mL of water. The dirty condition tests were carried out using the only MDI-102 at 80 ppm (MDI-102_{80ppm}) solution because it has been considered sufficient to prove the bactericidal activity of the solution. In fact, if MDI-102_{80ppm} is effective in dirty condition, the solution at higher active bromine concentration (MDI-102_{500ppm}) must be effective as well.

3. Results

3.1. Time kill assays

Time kill assays were carried out in order to:

1. assess the BA of MDI-102 towards periocular microorganism;
2. determine the time required to kill the microorganisms connected to the blepharitis.

Time kill assays carried out with MDI-102_{500ppm} are reported in Figure 2 (a-g).

Figure 2 (a-g) shows that MDI-102 (500 ppm) can effectively kill the inoculated microrganisms, concentration ranged between 10^5 and 10^7 CFU mL^{-1}, after only 30 seconds of contact (100% of removal) between the microorganisms and the bactericidal solution. Indeed, after 30 seconds, 0 CFU are determined in the Petri dish.

The same results are shown with the tests with MDI-$102_{80\ ppm}$, as reported in Figure 3 (a-g).

Figure 3 (a-g) shows after 30 seconds a concentration equal to zero for all the tested microorganisms (100% of removal after 30 seconds) with the exception of the *Pseudomonas aeruginosa* (ATCC:9027): its concentration after 30 seconds is indeed 5 CFU mL^{-1}. On the other hand, considering a *Pseudomonas aeruginosa* (ATCC:9027) starting concentration equal to $2*10^5$ CFU mL^{-1} the removal % is 99.998% after just 30 seconds. MDI-102_{80ppm} thus can be considered as effective as the MDI-102_{500ppm} solution.

Bactericidal time kill assays were carried out also in dirty conditions (see paragraph 2.2.) The selected microorganisms were put in contact with MDI-102_{80ppm} and 3 g L^{-1} of albumin, as protein-disruptor; the bactericidal activity of halogenated biocidal product is indeed often tested with peptone[16]. Figure 4 (a-g) shows the tests in dirty conditions.

Dirty condition tests have confirmed the results shown without disruptor (clean condition tests): MDI-102_{80ppm} can effectively kill more than 99,98% of *Enterococcus hirae* ATCC: 10541, *Escherichia coli* ATCC: 8739, *Pseudomonas aeruginosa* ATCC: 9027, *Pseudomonas aeruginosa* ATCC: 15442, *Staphylococcus aureus* ATCC: 6538, *Staphylococcus epidermidis* ATCC:12228, and *Serratia marcescens* ATCC: 13880, after only 30 seconds.

4. Discussion

This study showed MDI-102 can effectively kill common eyelid bacteria connected to blepharitis. A single application with 0.008% HBrO led to a complete removal (100% reduction) of bacterial load after 60 seconds (99.998% removal after 30 seconds).

Of particular interest are *S. epidermidis* and *S. aureus* data, which can be considered two strong indicators of blepharitis syndrome[8, 16, 21, 23-25]. The results have been compared with the literature study carried out by Stroman and coworkers[21], which tested a HClO (0.01%) solution for the periocular area hygiene. Stroman et al.[21] tested the solution with several periocular microorganisms at concentration ranged between 10^5 and 10^7 CFU mL^{-1} (same concentration range of the current study) and showed that the hypochlorite solution (100 ppm) can effectively kill more than the 99% of *Staphylococcus* species after 20 minutes and, in detail, a concentration higher than 99.5% of *Staphylococcus epidermidis*. MDI 102_{80ppm} (active halogen 20 ppm lower than the solution tested by Stroman et al. [21]) can kill, after just 30 seconds (thus an interval of time 97.5% lower than the one chosen by Stroman and coworkers[21]), the 99.99% of the tested microorganisms' (calculated on all the average tested microorganisms' concentration).

A single MDI 102_{80ppm} application is thus enough to kill the blepharitis microorganism of concern. MDI 102_{80ppm} has shown a microbial removal, at least, comparable to the commercially available bactericidal solution Chlorine-based and a much faster kinetic removal. This is particularly convenient because it allows to kill the blepharitis related microorganisms in a shorter time after the product application.

Furthermore, the lower bromine toxicity, compared to chlorine, makes MDI 102_{80ppm} potentially more tolerable for the skin than the commercially available competitors chlorine based.

MDI 102_{80ppm} was also tested in dirty condition to check its bactericidal activity in real conditions. Data have shown that albumin does not interfere with MDI 102_{80ppm} BA; the bromine-based solution can effectively kill the 99.98% of the tested bacteria indeed. No comparable kinetic study has been carried out with chlorine-based solutions in “dirty conditions”, thus no further comparison is possible. On the other hand, Gottardi et al.[16] compared the bactericidal activity of N-bromine compounds to the N-chlorine compounds in presence of peptone. The tested compounds were: dichloro- and dibromoisocyanuric acid, chlorantine and bromantine (1,3-dibromo- and 1,3 dichloro-5,5-dimethylhydantoine), chloramine T and bromamine T (*N*-chloro- and *N*-bromo-4-methylbenzenesulphonamide sodium), and *N*-chloro- and *N*-bromotaurine sodium. Their study demonstrates that the N-bromine and N-chlorine BA is strictly related to the selected specific compound. Furthermore, the BA of N-bromine compounds decreases in presence of peptone. The present study shows albumin does not lead to a BA loss; the activity is still higher than 99% after the disruptor addition. This aspect might confirm bactericidal activity is strictly related to the specific compound used as bactericidal.

For the best of our knowledge, it is not possible to carry out a deeper comparison with other literature studies. This aspect confirms the novelty of the present study both in terms of bactericidal solution for blepharitis treatment and in terms of experimental set-up.

Furthermore, as well as chorine based bactericidal solutions, this study demonstrates MDI-102 can be used to kill the susceptible strains equally well as those that are resistant to various antibiotics.

5. Conclusion

A new biocidal solution bromide-based has been developed in this study to treat the blepharitis syndrome. The solution can be effectively used to clean eyelashes, eyelids and the periocular area.

MDI-102 represents an effective alternative to the available chlorine-base biocidal solutions. Bromine based solution is more effective (superior and faster bacterial removal), less toxic and more stable than chlorine base solutions.

Last but not least, based on the present *in vitro* results, as well as chlorine-based bactericidal solutions, MDI 102_{80ppm} can be considered as an alternative to reduce the use and the abuse of topical antibiotics in the daily practice, which may contribute to the increase of resistance to the antibiotics in the clinical setting. Appropriate clinical investigation on the bromine-based solutions in patients with blepharitis

syndrome (*in vivo* study) might be particularly important to be designed, in order to definitively consider the standard use of Br-based biocidal solutions in the clinical practice.

Declarations

Acknowledgement

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Conflict of interest

The Authors declare no conflict of interest.

Author contribution

Ludovica Silvani: paper writing, experimental test planning.

Andrea Bedei: scientific director.

Giulia De Grazia: laboratory test.

Castellini Laura: clinical study and feasibility.

Stefano Remiddi: project responsible.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

$$KBr \xrightarrow{H_2O} Br^- + K^+ \qquad \text{r. 1}$$

$$ClO^- + H_3O^+ \overset{H_2O}{\rightleftharpoons} HClO \qquad \text{r. 2}$$

$$HClO + HCl \rightleftharpoons Cl_2 + H_2O \qquad \text{r. 3}$$

$$Cl_2 + 2Br^- \xrightarrow{H_2O} Br_2 + 2Cl^- \qquad \text{r. 4}$$

$$Br_2 + H_2O \rightleftharpoons HBrO + Br^- \qquad \text{r. 5}$$

Figure 1

MDI-102 preparation: reactions outline, from reaction (r.) 1 to 5.

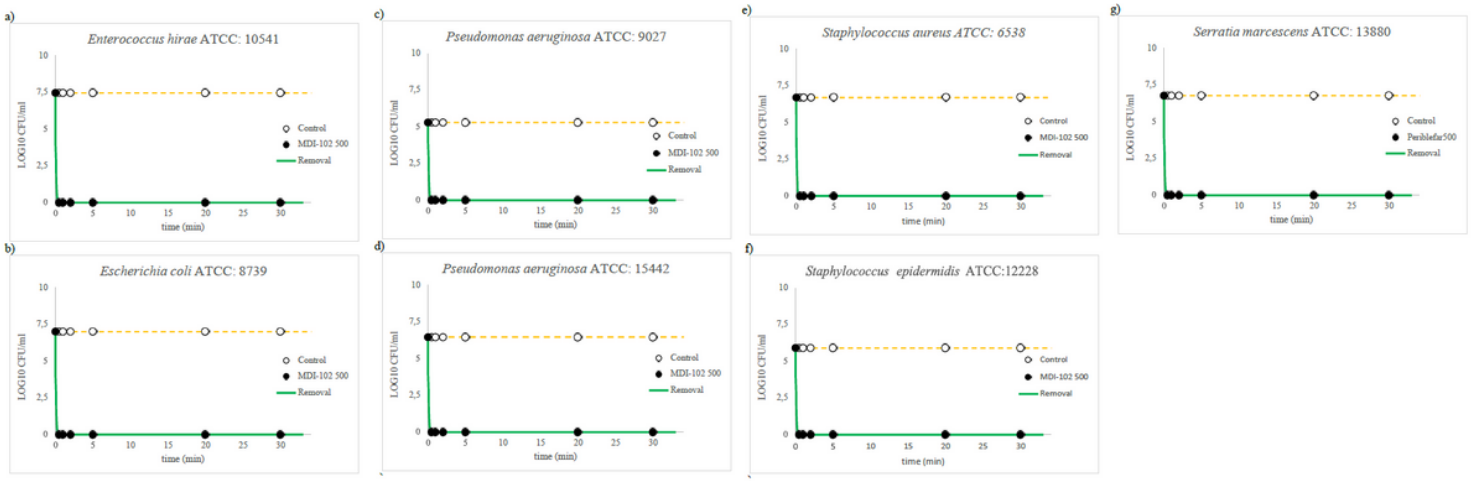

Figure 2

a-g MDI-102 (500 ppm) kinetic tests with the following microorganisms: (a) Enterococcus hirae ATCC: 10541, (b) Escherichia coli ATCC: 8739, (c) Pseudomonas aeruginosa ATCC: 9027, (d) Pseudomonas aeruginosa ATCC: 15442, (e) Staphylococcus aureus ATCC: 6538, (f) Staphylococcus epidermidis ATCC:12228, (g) Serratia marcescens ATCC: 13880. The white dots (yellow dashed line) represent the “control” or blank which are the microorganisms’ trend without MDI-102 (bactericidal solution) along with time. The black dots represent the microbial concentration trend along with time with MDI-102500ppm (concentration of active free Br). The green line shows the kinetic bactericidal trend along with time

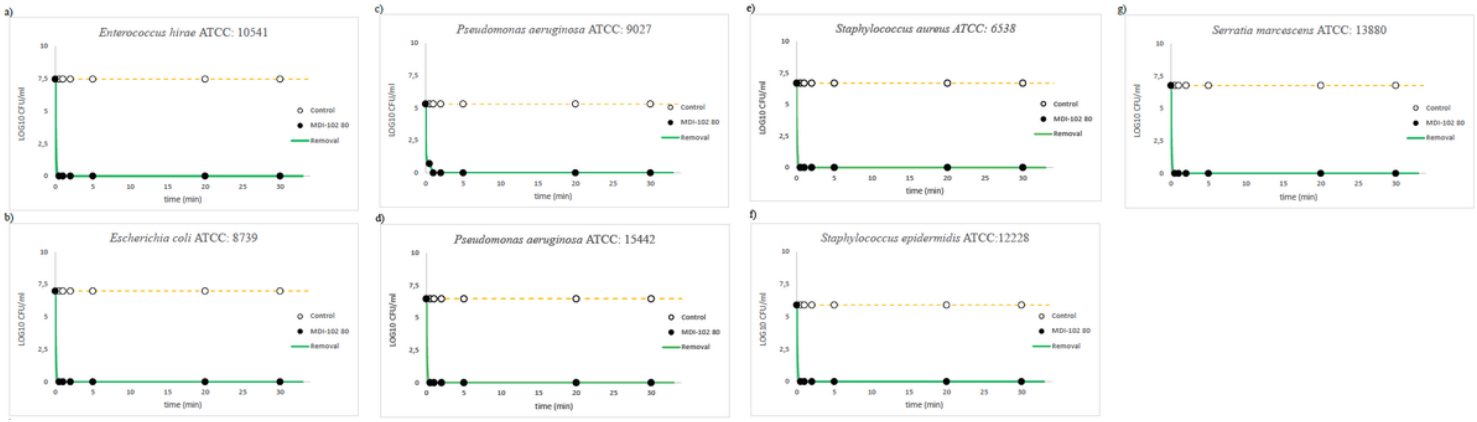

Figure 3

a-g MDI-102 (80 ppm) kinetic tests with the following microorganisms: (a) Enterococcus hirae ATCC: 10541, (b) Escherichia coli ATCC: 8739, (c) Pseudomonas aeruginosa ATCC: 9027, (d) Pseudomonas aeruginosa ATCC: 15442, (e) Staphylococcus aureus ATCC: 6538, (f) Staphylococcus epidermidis ATCC:12228, (g) Serratia marcescens ATCC: 13880. The white dots (yellow dashed line) represent the “control” or blank which are the microorganisms’ trend without MDI-102 (bactericidal solution) along with time. The black dots represent the microbial concentration trend along with time with MDI-10280ppm (concentration of active free Br). The green line shows the kinetic bactericidal trend along with time.

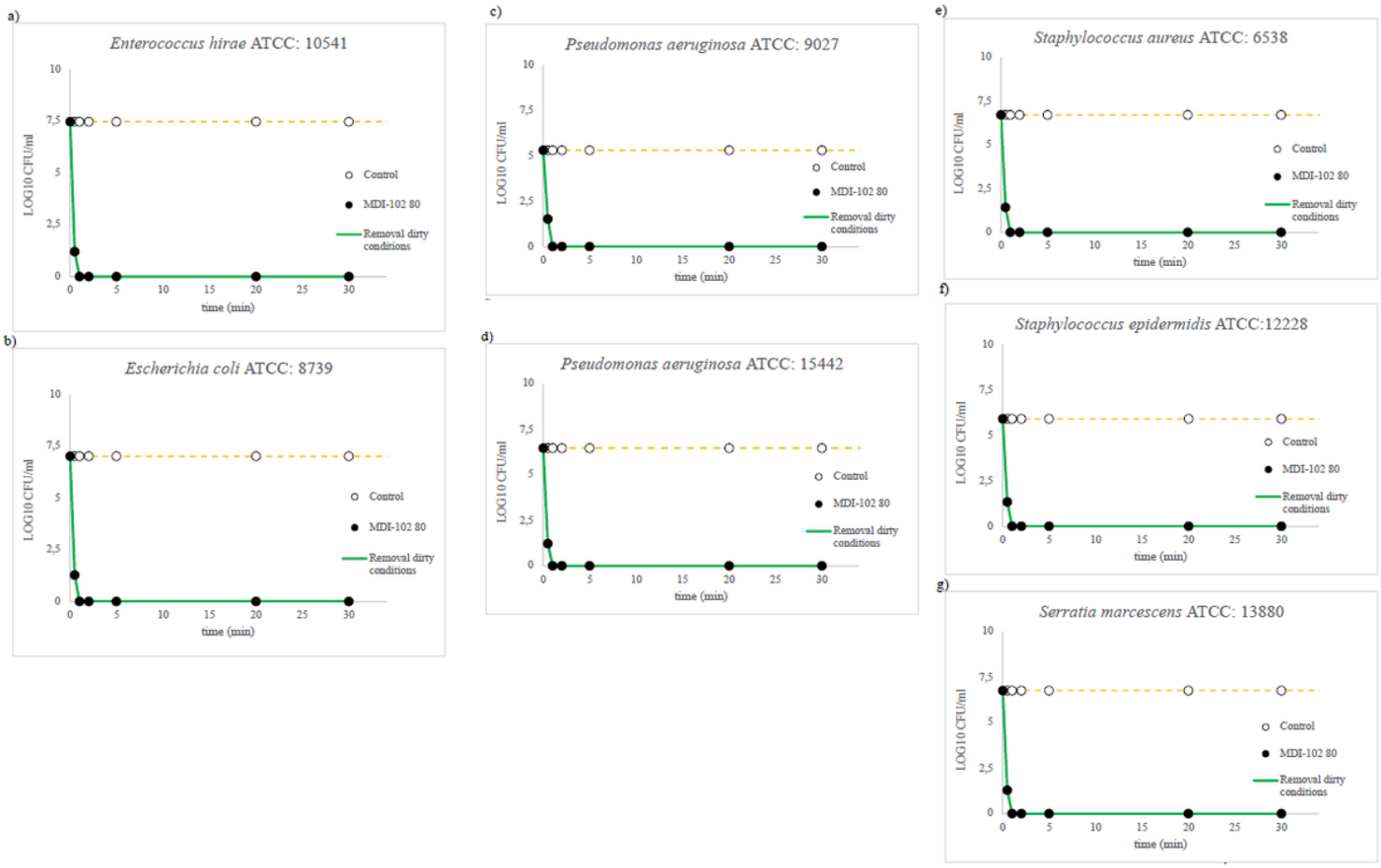

Figure 4

a-g MDI-102 (80 ppm) + albumin 3 g L-1 kinetic tests with the following microorganisms: (a) Enterococcus hirae ATCC: 10541, (b) Escherichia coli ATCC: 8739, (c) Pseudomonas aeruginosa ATCC: 9027, (d) Pseudomonas aeruginosa ATCC: 15442, (e) Staphylococcus aureus ATCC: 6538, (f) Staphylococcus epidermidis ATCC:12228, (g) Serratia marcescens ATCC: 13880.The white dots (yellow dashed line) represent the “control” or blank which are the microorganisms’ trend without MDI-102 (bactericidal solution) + albumin 3 g L-1 along with time. The black dots represent the microbial concentration trend along with time with MDI-10280ppm (concentration of active free Br). The green line shows the kinetic bactericidal trend of along with time in dirty condition.