Evaluation of induced and spontaneous contamination of ocular medications after first opening

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SUMMARY
In order to evaluate whether ophthalmic preparations can be safely used within 12 hours after first opening, four different sterile ocular medications, were opened and tested for spontaneous bacterial contamination after exposure to air. Samples (10ml) were collected from 5 containers of each ophthalmic preparation after 0, 2, 4, 8 and 24 hours. No viable microorganisms were found during and at the end of the evaluation time. In order to assess if the ocular bacterial population might contaminate the medications, about 10^5 microbial cells of different species (Staphylococcus aureus, S. epidermidis, S. haemolyticus, Streptococcus pneumoniae, S. pyogenes, Corynebacterium spp., Haemophilus influenzae, Moraxella catarrhalis, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumanii, and Candida albicans) were added to the containers and incubated at 35°C or at room temperature. Samples were collected and the number of viable microorganisms was estimated. The medicaments showed a bacteriostatic or bactericidal activity (<100 survivors after 24 h of exposure) against the majority of all relevant species, with the exception of E. coli, P. aeruginosa, and A. baumanii, which demonstrated to be able to grow in some of these ocular preparations. These data indicate that the risk of spontaneous contamination of the containers after first opening is low, and that all solutions do not offer a favourable substrate for the growth of the microorganisms showing in the great majority of the cases an antimicrobial activity. These results suggest that a safe usage of these ocular medications can be extended from the recommended 12 hours at least 24 hours after first opening.

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INTRODUCTION
The eye, like any other part of the body in contact with the external environment, is naturally colonized by various bacterial species as staphylococci, streptococci, Haemophilus spp. and Corynebacterium spp. (2, 8, 11, 15, 14). The eyes in any case are protected by palpebral movements, presence of antibacterial enzymes, immunoglobulins (particularly those of secretory type) and by the detergent action of tears (2, 15). In the development of the ocular cavity infection many factors are involved, as far as the general health conditions of the subject. Presence of contact lenses may increase the risk of infection because of the frequent manipulations, the easy contamination of the container and the propensity of bacteria of adhering to the lenses and produce biofilm (3, 18, 21).

Because of the difficulty of many drugs to reach therapeutic levels in this anatomic site by systemic route, most diseases of the ocular cavity are treated with topical preparations that present a risk of microbial contamination. It therefore extremely important to prevent bacterial contamination of these preparations and evaluate the impact of contamination especially for those solutions that do not contain any specific antimicrobial agents. Concerning sterile preparations, the time of their utilisation after first opening proposed by the European Agency for the Evaluation of Medicinal Product should be about three hours. This limit was set taking into account a possible environmental contamination and the subsequent growth in these preparations of potential pathogens. In the case of ophthalmic solutions of 0.5 ml each dose, whose reusable limit is within 12 hours, this risk is higher considering the possible contact of this opened container with the surface of the eye. Microorganisms or opportunistic pathogens might enter into the container (10, 17, 19, 16). Depending on the nature of pharmaceutical preparations, this contamination may follow different directions. The number of bacteria can increase considerably if the fluid is nutrient, remain unchanged if no nutritional substances are present in the media, or reduce if the

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ophthalmic solution has antimicrobial properties. Finally, there is the possibility that a certain amount of bacteria from contamination or contact with eye’s surface can be seized by the liquid due to the occurrence of adhesion on the plastic of the container. On the other hand, the mere adherence to a surface may enhance the expression of bacterial genes that control the synthesis of compounds necessary for sessile life and/or expression of pathogenic and virulence traits. A sessile community of microorganisms could therefore be a constant source of virulent bacteria (5, 9).

To evaluate these hypotheses, an experimental contamination of four ophthalmic solutions, has been carried out. The study also examined the outcome of known quantities of bacteria belonging to different species in the ophthalmic preparations in order to simulate contamination by contact with infected conjunctiva and the ability of the plastic container to provide a surface for the adhesion of certain species of staphylococci.

**MATERIALS AND METHODS**

**Microorganisms**

Strains used in this study, representative of the most frequent microorganisms found in the ocular bacterial population (2, 8, 11, 14, 15), belong to the collection of this Institution and were collected from clinical specimens. They included: S. aureus, S. epidermidis, S. haemolyticus, S. pneumoniae, S. pyogenes, Corynebacterium spp., M. catarrhalis, H. influenzae, E. coli, P. aeruginosa, A. baumanii, and C. albicans.

**Growth condition**

*S. aureus*, *S. epidermidis*, *S. haemolyticus*, *Corynebacterium* spp., *P. aeruginosa*, *A. baumanii*, *E. coli* and *M. catarrhalis* were cultured in Mueller-Hinton (MH) Broth (Biogenetics, Padua, Italy) solidified with agar-agar when necessary as suggested by CLSI (2007). To the same medium 5% horse blood was added for testing *S. pneumoniae* e *S. pyogenes*. Haemophilus Test Medium (HTM) was used for *H. influenzae*. Finally Sabouraud agar plates were employed for *C. albicans*.

**Spontaneous contamination of ocular medications**

Considering our previous experience with similar ocular medications made by the same manufacturer, (12), it appears of some interest to evaluate if the ocular preparations recently introduced in the therapy, and here described, are characterised by similar favourable properties. The evaluation of spontaneous contamination of ocular medications has been, then, carried out using five samples of each medication (Naflox lot 780707, Calmostill lot 130707, Xiloial lot 9030907, Ketoftil lot 410207). Samples were opened and maintained unprotected under these conditions for 24 hours at room temperature. At 0, 2, 4, 8 and 24 hours, aliquots (10µl) were collected and seeded onto blood-agar, McConkey–agar and Sabouraud–agar and incubated for 48 hours at 35°C. After this period the plates were examined in order to verify the presence of any microorganisms. Three different culture media were employed to allow the growth of Gram-positive (MH supplemented with 5% horse blood) Gram-negative bacteria (McConkey) and fungi (Sabouraud).

**Viability of microorganisms cultured in ocular medications**

The evolution of a microbial population introduced in the two ophthalmic solutions (simulating a possible contamination by contact with infected eye surfaces) has been studied by adding the compounds to suspensions of about 10^6 CFU/ml of various bacterial species in saline solution. The choice of this concentration derived from considerations relating to the density of pathogens that can colonize the ocular region and produce eye infection. Three samples of each preparations, inoculated with these suspensions, were incubated at both room temperature and 35°C. After 0, 2, 4, 8 and 24 hours, of 10ml of each culture were plated onto the appropriate medium for the growth of each species. After 24 hours of incubation at 35°C the CFU /ml was estimated and the curve of growth was draw.

**Adhesion of the different bacterial species to the plastic container**

To test the adhesion of microorganisms to the plastic material used for the production of containers, two groups of empty plastic containers (size 0.5 ml), provided by Farmigea Spa, were inoculated using a bacterial suspensions in phosphate buffer solution. A portion of cells placed in glass containers was used as control. After 0, 2, 4, 8 and 24 hours of incubation at both room temperature and 35°C, 10ml of each suspension was plated onto the appropriate medium for the growth of each species. After 24 hours of incubation at 35°C the CFU /ml was estimated.

**RESULTS**

**Evaluation of spontaneous contamination of ocular medications**

When five samples of each ophthalmic preparation were opened and exposed unprotected to air at room temperature for 24 hours, no viable
microorganisms (fungal or bacterial) were detected after 0, 2, 4, 8, hours and at the end (24 hours) of the evaluation time (data not shown).

Evaluation of viability of bacteria cultured in ocular medications

When the ophthalmic solutions were infected with a known inoculum of the various representative pathogens, different effects were observed depending on the microorganism analysed. A reduction of CFU/ml was generally noted in all Gram-positive and Gram-negative species, with the exception of fast growing Gram-negative bacilli and S. aureus that were able to grow in Xiloilal. Different reductions of the CFU/ml values were observed depending on the microbial species analysed and experimental conditions adopted. After 24 hours of exposure to the ocular medications, in some experiments, viable microorganisms were not found due to the limit imposed by the technology used (<1x10^2 CFU/ml). However, in other tests, the number of survivors has been reduced by at least 90%.

In detail, the four different ophthalmic preparations have shown a bacteriostatic effect on S. aureus with the exception, as mentioned above, of Xiloial (table 1). Narflox showed a bactericidal effect after two hours of exposure, while the other compounds demonstrated a bacteriostatic activity. Similarly, Calmostill and Xiloilal evidenced a bacteriostatic activity on S. epidermidis and S. haemolyticus, while Narflox and Ketoftil exhibited a bactericidal effect (table 1).

S. pneumoniae showed a considerable susceptibility to all the ophthalmic preparations, a number of CFU/ml below the technical limit of the assay was found under all the experimental conditions (table 1). The four preparations also showed a lethal activity on S. pyogenes, particularly Narflox and Ketoftil. The fraction of survivors remained almost the same up to 24 hours in samples exposed to Calmostill while no viable microorganism was found in samples treated with Xiloilal and incubated at room temperature (table 1).

The results obtained with Corynebacterium spp. denoted a general bactericidal activity of all the compounds, with the exception of Xiloilal that exhibited a bacteriostatic effect on this species (table 1). The slow-growing Gram-negatives, M. catarrhal-

| Pathogen         | Narflox 0.3% | Calmostill | Xiloilal | Ketoftil |
|------------------|--------------|------------|----------|----------|
| **S. aureus**    |              |            |          |          |
| 0                | 1.4x10^3     | 1.4x10^3   | 1.4x10^4 | 1.4x10^4 |
| 2h               | 0            | 8.4x10^1   | 4.0x10^3 | 1.7x10^5 |
| 4h               | 0            | 1.2x10^5   | 1.8x10^4 | 1.4x10^5 |
| 8h               | 0            | 3.5x10^5   | 3.8x10^5 | 2.0x10^5 |
| 24h              | 0            | 1.5x10^4   | >5x10^7  | 7.9x10^4 |
| **S. epidermidis**|              |            |          |          |
| 0                | 1.3x10^3     | 1.3x10^3   | 1.3x10^3 | 1.3x10^3 |
| 2h               | 6.1x10^4     | 1.6x10^4   | 1.1x10^3 | 6.7x10^3 |
| 4h               | 0            | 1.2x10^3   | 1.5x10^3 | 3.5x10^4 |
| 8h               | 0            | 1.1x10^3   | 8.6x10^4 | 0        |
| 24h              | 0            | 5.4x10^4   | 9.5x10^4 | 0        |
| **S. haemolyticus**|            |           |          |          |
| 0                | 7.2x10^4     | 7.2x10^4   | 7.2x10^4 | 7.2x10^4 |
| 2h               | 2.2x10^4     | 4.4x10^4   | 9.2x10^4 | 1.9x10^4 |
| 4h               | 0            | 3.5x10^4   | 8.8x10^4 | 4.8x10^4 |
| 8h               | 0            | 2.4x10^4   | 6.1x10^4 | 0        |
| 24h              | 0            | 5.5x10^4   | 2.7x10^4 | 0        |
| **S. pneumoniae**|              |            |          |          |
| 0                | 4.3x10^4     | 4.3x10^4   | 4.3x10^4 | 4.3x10^4 |
| 2h               | 0            | 0          | 0        | 0        |
| 4h               | 0            | 0          | 0        | 0        |
| 8h               | 0            | 0          | 0        | 0        |
| 24h              | 0            | 0          | 0        | 0        |
| **S. pyogenes**  |              |            |          |          |
| 0                | 9.0x10^4     | 9.0x10^4   | 9.0x10^4 | 9.0x10^4 |
| 2h               | 1.9x10^4     | 7.5x10^3   | 7.9x10^4 | 9.6x10^4 |
| 4h               | 4.8x10^3     | 2.4x10^4   | 8.4x10^4 | 8.5x10^4 |
| 8h               | 0            | 4.0x10^4   | 4.6x10^4 | 5.0x10^4 |
| 24h              | 0            | 1.6x10^4   | 5.5x10^4 | 0        |
| Corynebacterium spp |         |           |          |          |
| 0                | 5.1x10^4     | 5.1x10^4   | 5.1x10^4 | 5.1x10^4 |
| 2h               | 2.1x10^4     | 7.8x10^3   | 4.3x10^4 | 3.9x10^4 |
| 4h               | 0            | 2.5x10^4   | 3.8x10^4 | 9.5x10^4 |
| 8h               | 0            | 1.4x10^4   | 3.4x10^4 | 0        |
| 24h              | 0            | 0          | 2.7x10^4 | 0        |

RT, room temperature
lis and H. influenzae were susceptible to all the ophthalmic preparations (table 2). The dynamic of lethal effect was different depending on the experimental conditions, the strain, and the compound tested.

Against fast-growing Gram-negative pathogens different effects were observed, depending on the species analysed and the temperature used (table 2). In particular, when E. coli was examined, it was found that the number of CFU/ml remained the same during the first phase of the test (8 hours), then it decreased and no viable bacteria were found after 24 hours of exposure using Naflox and Calmostill at 37°C. A bacteriostatic effect was registered in all the other cases with the exception of Xiloial that was utilised by E. coli for its growth (table 2).

P. aeruginosa exhibited a different behaviour depending on the experimental conditions adopted and the compound evaluated (table 2). Naflox was bactericidal on this pathogen. A bacteriostatic effect was registered in the experiments carried out with Calmostill and Ketoftil, while Xiloial did not inhibit the growth of this organism. A similar behaviour was found when A. baumanii was studied (table 2).

The ophthalmic preparations were also infected with a C. albicans strain. The results were comparable to those found with slow-growing Gram-negative and Gram-positive microrganisms (table 2). Naflox and Ketoftil showed bactericidal activity against this microrganism while Calmostill and Xiloial exhibited a bacteriostatic activity even if in some circumstances the number of CFU/ml at the end of the experiment was reduced in comparison with the initial inoculum.

### Evaluation of adhesion to the plastic container by the different bacterial species

Table 3 summarizes the changes in bacterial concentrations depending on time. The study shows that the initial bacterial inoculum decline with the same kinetic irrespectively of the container used. It was noted that S. aureus in the plastic containers

### Tabella 2. Evaluation of viability of Gram-negative bacteria and C. albicans inoculated in ocular medications.

| Pathogen              | Naflox 0.3% | Calmostill | Xiloial | Ketoftil |
|-----------------------|-------------|------------|---------|----------|
|                       | RT 37°C     | RT 37°C    | RT 37°C | RT 37°C  |
| M. catarrhalis        |             |            |         |          |
| 0                     | 5.1 x 10⁴³ | 5.1 x 10⁴³ | 5.1 x 10⁴³ | 5.1 x 10⁴³ |
| 2h                    | 1.9 x 10⁴⁴ | 8.5 x 10⁴³ | 5.1 x 10⁴³ | 5.1 x 10⁴³ |
| 4h                    | 1.3 x 10⁴⁴ | 1.7 x 10⁴³ | 1.2 x 10⁴⁴ | 0          |
| 8h                    | 4.8 x 10³³  | 5.8 x 10³³ | 0        | 5.0 x 10³³ |
| 24h                   | 0           | 0          | 0        | 0         |
| H. influenzae         |             |            |         |          |
| 0                     | 1.9 x 10⁵³ | 9.9 x 10⁴³ | 9.9 x 10⁴³ | 9.9 x 10⁴³ |
| 2h                    | 1.8 x 10⁴⁴ | 3.6 x 10⁴³ | 4.1 x 10⁴³ | 2.0 x 10⁴⁴ |
| 4h                    | 6.1 x 10³³  | 2.1 x 10³³ | 2.9 x 10³³ | 5.0 x 10³³ |
| 8h                    | 1.4 x 10³³  | 9.5 x 10³³ | 1.8 x 10³³ | 1.8 x 10³³ |
| 24h                   | 0           | 0          | 0        | 0         |
| E. coli               |             |            |         |          |
| 0                     | 9.9 x 10⁴³ | 9.9 x 10⁴³ | 9.9 x 10⁴³ | 9.9 x 10⁴³ |
| 2h                    | 7.1 x 10⁴³ | 3.0 x 10³³ | 1.2 x 10³³ | 1.0 x 10³³ |
| 4h                    | 5.0 x 10³³  | 1.0 x 10³³ | 2.0 x 10³³ | 8.3 x 10³³ |
| 8h                    | 1.6 x 10³³  | 2.8 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| 24h                   | 0           | 0          | >5 x 10³³  | >5 x 10³³  |
| P. aeruginosa         |             |            |         |          |
| 0                     | 1.0 x 10³³ | 1.0 x 10³³ | 9.6 x 10³³ | 9.1 x 10³³ |
| 2h                    | 0           | 6.5 x 10³³ | 9.4 x 10³³ | 1.1 x 10³³ |
| 4h                    | 0           | 6.3 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| 8h                    | 0           | 3.6 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| 24h                   | 0           | 7.8 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| A. baumanii           |             |            |         |          |
| 0                     | 2.1 x 10³³ | 2.1 x 10³³ | 2.1 x 10³³ | 2.1 x 10³³ |
| 2h                    | 3.5 x 10³³ | 1.0 x 10³³ | 4.4 x 10³³ | 2.1 x 10³³ |
| 4h                    | 0           | 4.4 x 10³³ | 4.0 x 10³³ | 9.2 x 10³³ |
| 8h                    | 0           | 4.1 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| 24h                   | 0           | 2.5 x 10³³ | >5 x 10³³  | >5 x 10³³  |
| C. albicans           |             |            |         |          |
| 0                     | 4.1 x 10³³ | 4.1 x 10³³ | 4.1 x 10³³ | 4.1 x 10³³ |
| 2h                    | 3.7 x 10³³ | 4.5 x 10³³ | 4.5 x 10³³ | 4.5 x 10³³ |
| 4h                    | 0           | 2.7 x 10³³ | 3.7 x 10³³ | 8.2 x 10³³ |
| 8h                    | 0           | 1.5 x 10³³ | 4.5 x 10³³ | 3.3 x 10³³ |
| 24h                   | 0           | 3.0 x 10³³ | 5.0 x 10³³ | 0         |

RT, room temperature
reduced its number from $2.2 \times 10^4$ to $<10^2$ CFU/ml within 24 hours. Similar values ($<5 \times 10^3$) were reported in the experiments carried out in the glass containers at 35°C. Introducing a known concentration of S. epidermidis ($1.6 \times 10^4$ CFU/ml) at 35°C, $1 \times 10^3$ CFU/ml microorganisms were counted after 24 hours in the plastic containers and $1.8 \times 10^3$ CFU/ml in the glass control containers. Similarly, for S. haemolyticus, in the different conditions, the number of CFU/ml was reduced from $4.3 \times 10^4$ to about $10^4$ viable microorganisms within 24 hours. Results obtained at room temperature against the three microorganisms are overlapping with those obtained at 35°C. The reduction in the number of survivors becomes valuable (from 90 to 99%) only after 24 hours at a temperature of 35°C in both the plastic and glass containers.

**Table 3. Evaluation of adhesion (CFU/mL) to the plastic container by the different staphylococcal species.**

| Microorganism | Time (hours) | 0 | 2 | 4 | 8 | 24 |
|---------------|--------------|---|---|---|---|----|
| S. aureus     |              | $2.4 \times 10^4$ | $2.4 \times 10^4$ | $2.4 \times 10^4$ | $2.4 \times 10^4$ | $2.4 \times 10^4$ |
|               | RT           | $1 \times 10^4$   | $7 \times 10^3$   | $1.5 \times 10^4$ | $1 \times 10^4$  | $1 \times 10^4$  |
|               | 35°C         | $8 \times 10^3$   | $7.6 \times 10^3$ | $1.6 \times 10^4$ | $1 \times 10^4$  | $1 \times 10^4$  |
|               |              | $1.3 \times 10^4$ | $2 \times 10^3$   | $<10^2$          | $4 \times 10^2$  | $<10^2$          |
| S. epidermidis|              | $1.4 \times 10^4$ | $1.4 \times 10^4$ | $1.5 \times 10^4$ | $1.5 \times 10^4$ | $1.5 \times 10^4$ |
|               | RT           | $1.2 \times 10^4$ | $1 \times 10^4$   | $1.2 \times 10^4$ | $1 \times 10^4$  | $1 \times 10^4$  |
|               | 35°C         | $8 \times 10^3$   | $5 \times 10^3$   | $1.1 \times 10^4$ | $1 \times 10^4$  | $1 \times 10^4$  |
|               |              | $2.9 \times 10^3$ | $5 \times 10^3$   | $1 \times 10^4$  | $4.5 \times 10^3$ | $4.5 \times 10^3$ |
|               |              | $<1 \times 10^4$  | $1 \times 10^4$   | $2 \times 10^4$  | $1 \times 10^4$  | $1 \times 10^4$  |
| S. haemolyticus|            | $1.1 \times 10^4$ | $1.2 \times 10^4$ | $1.2 \times 10^4$ | $1 \times 10^4$  | $1 \times 10^4$  |
|               | RT           | $9 \times 10^3$   | $1 \times 10^4$   | $9.6 \times 10^3$ | $1.3 \times 10^4$ | $1.3 \times 10^4$ |
|               | 35°C         | $2.5 \times 10^3$ | $2 \times 10^3$   | $9.5 \times 10^3$ | $4.6 \times 10^3$ | $4.6 \times 10^3$ |
|               |              | $1.4 \times 10^3$ | $1 \times 10^4$   | $8 \times 10^3$  | $4.5 \times 10^3$ | $4.5 \times 10^3$ |
|               |              | $<1 \times 10^2$  | $1 \times 10^4$   | $8 \times 10^3$  | $1 \times 10^4$  | $1 \times 10^4$  |

**DISCUSSION**

The experiments carried out in this study show that spontaneous contamination of the opened ophthalmic solutions, even after a period of 24 hours, is highly unlikely. All the ophthalmic preparations studied here manifested a modest antibacterial activity, but enough to limit the proliferation of the microorganisms considered pathogens in ophthalmology, with the exception of Naflax that resulted under all circumstances strongly bactericidal (2, 8, 11, 14, 15). The physico-chemical properties of plastic containers do not present pro-adhesive effects on the analyzed pathogens and on the basis of the technique employed. These findings suggest some considerations: with respect to the present experimental experience, the 0.5 ml containers of the ophthalmic preparations Farmigea, for their shape with a rather small opening and for the nature of their content that normally limits the proliferation of microorganisms, are not easily subject to contamination, not only in the short period of time suggested by international regulation but even within a period of 24 hours (20). All the compounds, although preservative-free and not containing specific antimicrobial substances, with the exception of Naflax, demonstrated a bacteriostatic or bactericidal activity not only against S. pneumoniae, S. pyogenes, Corynebacterium spp., H. influenzae and M. Catarrhalis, exigent microorganisms in terms of nutrition and difficult to culture if not grown in their appropriate medium), but also against staphylococci and C. albicans, microorganisms certainly more adaptable and much more resistant in disparate and adverse environments. The compounds exhibited also an initial bacteriostatic activity on E. coli, and P. aeruginosa. The risk that bacteria with variable and considerable biochemical activity, such as fast-growing Gram-negatives, staphylococci and Candida, appears very high especially after 8 hours of exposure. Ophthalmic solutions provide substrates like organic molecules that cannot be easily used by the different microorganisms tested (some of them can only partly metabolize the compounds included in the medicaments). As mentioned above, with the exception of Naflax, these preparations have not antibacterial function and are not intended to be used for the treatment of eye cavity infections. Therefore, it was found as an unexpected characteristic of the compounds to inhibit microbial proliferation thus representing a further favourable feature to be added to their therapeutic property. Containers of 0.5 ml of the preparation Farmigea are inert, like glass and do not seem to provide a specific substrate for enhanced adhesion of bacteria (1, 7, 20). Microorganisms can therefore not be seized in a significant way by plastic. However this seems not to be important because, as shown in this study, the probability of a spontaneous contamination such as environmental exposure is remote (12). The important problem concerning contamination of these compounds by colonized or infected eye surfaces appears irrelevant due to the unexpected antibacterial activity expressed by the medications.

On the basis of the present findings, it seems possible to suggest that the period of time after the first opening for a safe usage of the ocular medications tested here can be extended.

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REFERENCES

1. Anderson JM, Marchant RE. Biomaterials: factors favoring colonization and and infections. In “Infections associated with indwelling medical devices.” 3rd Edition (F.A. Waldvogel and A.L.Bisno Eds), ASM Press, Washington, DC, 2000; 89-109.

2. Barnes SD, Pavan-Langston D, Azar DT, Durand ML. Eye Infections. In Principles and practice of infectious diseases, 6th edn (Mandell, G.L., Bennett, J.E. & Dolin, R., Eds). Churchill Livingstone, New York. 2005; 1387-1425.

3. Cheng KH, Leung SL, Hoekman HW, et al. Incidence of contact-lens-associated microbial keratitis and its related morbidity. Lancet 1999; 354: 181-185.

4. Clinical and Laboratory Standards Institute. Performance Standard for Antimicrobial Susceptibility Tests; Seventeenth Informational Supplement. CLSI document M100-S17, 2007. Wayne, PA.

5. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilm: a common cause of persistent infections. Science 1999; 284: 1318-1322.

6. European Pharmacopoeia, Annex to ICH/CPMP note for guidance on stability testing of new active substance and medical products (CPMP/ICH/380/95): maximum shelf-life for sterile products after first opening of following reconstitution. 1998.

7. Goldmann DA, Pier GB. Pathogenesis of Infections Related to Intravascular Catheterization. Clin Microbiol Rev 1993; 6: 176-192.

8. Gulletta E. Microbiologia clinica dell’apparato oculare. Ed Sci. Documenta Mascia Brunelli- Biolife. 1992.

9. Hall-Stoodley LJ, Costerton W, Stoodley P. Bacterial biofilm: from the natural environment to infectious diseases. Nat Rev Microbiol 2004; 2: 95-108.

10. Høding G, Sjursen H. Bacterial contamination of drops and dropper tips of in use multidose eye drop bottles. Acta Ophthalmologica, 1982; 60: 213-22.

11. Lanciai E. Infezioni dell’occhio. In Principi di Microbiologia Clinica. Terza Ed. Casa Editrice Ambrosiana, Milano, 2007; 428-430.

12. Marchese A, Bozzolasco M, Gualco L, Schito GC, Debbia EA. Evaluation of spontaneous contamination of ocular medication. Chemotherapy 2001; 47: 304-308.

13. Marchese A, Debbia EA, Massaro S, Campora U, Schito GC. Brodimoprim: effects of sub-MIC on virulence traits of respiratory and urinary tract pathogens and on plasmid transfer and stability. J Chemother 1996; 8: 171-177.

14. Mits C, Dockrell H., Goering RV, Roitt I, Wakelin D, Zuckerman M. Infezioni dell’occhio. In Microbiologia Clinica, 3rd Ed., (Bistoni et al. 1°Ed. Italiana) EMSI, Roma, 2006; 347-349.

15. Molina-Romanzi AM. Malattie infettive dell’occhio e della congiuntiva. In Microbiologia Clinica, Gli aspetti microbiologici delle malattie infettive (A.M. Molina-Romanzi Ed.), UTET, Torino, 2002; 269-290.

16. Rahman MQ, Tejwani D, Wilson JA, Butcher I, Ramaesh K. Microbial contamination of preservative free eye drops in multiple application containers. Br J Ophthalmol 2006; 90: 139-141.

17. Schein OD, Hibberd PL, Starch T, Baker AS, Kenyon KR. Microbial contamination of in-use ocular medications. Arch. Ophthalmol. 1992; 110: 82-85.

18. Sousa HM, Lira M, Elisabete M, Oliveira R, Oliveira R, Azeredo J. Adhesion of Pseudomonas aeruginosa and Staphylococcus epidermidis to silicone-hydrogel contact lenses. Optom Vis Sci 2005; 82: 446-50.

19. Su CY, Yang YC, Peng CF, Hsu YC, Lin CP. Risk of microbial contamination of unit-dose eyecups within twenty four hours after first opening. J Formos Med Assoc 2005; 104: 968-971.

20. Van Santvliet L, Sam T, Ludwig A. Packing of ophthalmic solutions-Influence on satbility, sterility, eye drop instillation and patient compliance. Eur. J. Pharm. Biopharm. 1996; 42: 375-384.

21. Yung MS, Boost M, Cho P, Yap M. Microbial contamination of contact lens wearers (university students) in Hong Kong. Ophthalm Physiol Opt 2007; 27: 11-21.