Multiple-dose ophthalmic preparations that do not contain preservatives carry high risks of microbial contamination. However, there are various types of hospital preparations, with different physicochemical properties. In the present study, we evaluated the association between physiochemical properties and microbial contamination in ophthalmic preparations. The investigated hospital preparations included ophthalmic preparations of physiological saline, 0.2% fluconazole, 0.5% vancomycin hydrochloride, and 2% cyclosporine. We investigated the microbial dynamics of each ophthalmic preparation and microbial contamination in ophthalmic preparations used by patients. Remarkable growth of *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Serratia marcescens* was observed in ophthalmic preparations of physiological saline and 0.2% fluconazole. All tested microorganisms displayed decreased counts after inoculation in 0.5% vancomycin hydrochloride. In 2% cyclosporine, all investigated microorganisms were below the limit of detection after inoculation for 6 h. The microbial contamination rates of ophthalmic preparations used by patients were 16.7% (3/18 samples) for 0.5% vancomycin hydrochloride and 0% (0/30 samples) for 2% cyclosporine. All detected contaminants in 0.5% vancomycin hydrochloride were *Candida* spp., one of which was present at a level of $1 \times 10^5$ colony-forming units/mL. The storage method for in-use ophthalmic preparations should be considered on the basis of their physicochemical properties.

**Key words** ophthalmic preparation; hospital preparation; multiple-dose; preservative; microbial contamination

Multiple-dose ophthalmic preparations that are used in hospitals include commercial preparations manufactured by pharmaceutical companies as well as formulations prepared in the hospital. Most commercial preparations contain preservatives to prevent microbial contamination. However, preservative-containing ophthalmic preparations cannot be used for patients with hypersensitivity or allergy to preservatives. Moreover, some ophthalmic preparations are not commercially available due to low stability of the agent or low frequencies of use. In such cases, the use of hospital preparations is considered. Hospital preparations are defined as ophthalmic preparations that are not commercially available and are aseptically prepared using drugs for injection or reagents in the hospital. Hospital preparations have the advantages of being available regardless of the frequency of use and being prepared without preservatives. On the contrary, hospital preparations lacking preservatives have been reported to be associated with a high risk of microbial contamination. However, there are various types of preparations produced in hospitals, and their physicochemical properties differ.

In the present study, to evaluate the association between physicochemical properties and microbial contamination of hospital preparations, we investigated the dynamics of various microorganisms in a variety of hospital preparations and microbial contamination in hospital preparations used by patients.

**MATERIALS AND METHODS**

**Investigated Hospital Preparations** We investigated ophthalmic preparations of physiological saline, 0.2% fluconazole, 0.5% vancomycin hydrochloride, and 2% cyclosporine, each of which was prepared by Yamaguchi University Hospital (736 beds) Pharmaceutical Service. These ophthalmic preparations are prepared in the clean bench using commercially available products as described below. These preparations are dispensed into ophthalmic preparation containers. The ophthalmic preparation of physiological saline is “OTSUKA NORMAL SALINE,” that of 0.2% fluconazole is “Diflucan® Intravenous Solution,” that of 0.5% vancomycin hydrochloride is “ VANCOMYCIN for I.V. Infusion 0.5MEEK” and “OTSUKA NORMAL SALINE,” and that of 2% cyclosporine is “Sandimmun®” and “sesame oil” (Table 1).

**Inoculated Microorganisms in Ophthalmic Preparations** Nine strains of seven species, *Pseudomonas aeruginosa* (ATCC27853, clinical strain), *Burkholderia cepacia* (clinical strain), *Escherichia coli* (NIIHJC-2), *Serratia marcescens* (IFO3936, clinical strain), *Staphylococcus aureus* (209P), *Candida albicans* (IFO1594), and *Aspergillus niger* (IFO4407), were inoculated into the aforementioned hospital preparations. When the ophthalmic preparation is contaminated due to contact between the tip of the container containing the preparation and the finger or eyelid, the contamination level in the ophthalmic preparation is considered to be approximately $1 \times 10^2$ colony-forming units/mL. Therefore, the inoculated doses of microorganisms ranged from $1 \times 10^2$ to $1 \times 10^5$ CFUs/mL.

**Measurements of pH and Water Activity in Each Ophthalmic Preparation** The pH and water activity of ophthalmic preparations were measured using a pH meter (Horiba F-52) and water activity meter (Gunze Inc., Tokyo, Japan), respectively. The temperature during the measurement was 25°C.
Investigation of Microbial Dynamics in Each Ophthalmic Preparation

Each microorganism was inoculated into the four hospital preparations. The storage temperature of the ophthalmic preparations during the test was set to 30°C. Following inoculation of the microorganisms for 6, 24, or 48 h or 7 d, the container was manually shaken for 1 min, and the solution obtained using the routine ophthalmic solution dropping procedure was used as the test solution. Each sample was diluted 10-, 100-, 1000-, or 10000-fold in sterile distilled water. Subsequently, 0.2 mL of each dilution and the same amount of an undiluted sample were plated onto Trypto-Soy agar (Nippon Becton Dickinson Co., Tokyo, Japan). Plates were incubated at 30°C for 24–72 h. All the microorganisms were examined twice. After inoculation for 7 d, the bacterial count >10-fold greater than the initial inoculation amount was considered to be “growth (+),” while that <10-fold greater than the initial inoculation amount was considered to be “growth (−).”

Collection Method of Hospital Preparations after Use

We collected hospital preparations used by outpatients and inpatients at the ophthalmological department of Yamaguchi University Hospital (736 beds) between April 1, 2013 and June 30, 2016. The period from the first administration to the day on which the hospital preparations were examined was 1–3 months. A total of 48 samples of hospital preparations (product volume, 5 mL) were examined, including 18 samples of 0.5% vancomycin hydrochloride and 30 samples of 2% cyclosporine.

Identification and Quantification of Contaminants

When ≥1 mL of the ophthalmic solution was considered to remain in the container, the container was manually shaken for 1 min, and the solution obtained via the routine ophthalmic solution dropping procedure was used as the test solution. When the volume of the remaining ophthalmic solution was less than 1 mL, 1 mL of physiological saline was added to the ophthalmic solution on the clean bench using the following procedure. Physiological saline was injected from the nozzle of the container using a syringe for injection. The containers were manually shaken for 1 min, and the solution obtained by dropping was used as the test solution. Each of the samples was diluted 10- and 100-fold in sterile saline. Subsequently, 0.2 mL of each dilution and the same amount of an undiluted sample were plated onto Trypto-Soy agar and Sabouraud Dextrose agar (Eiken Chemical Co., Tokyo, Japan). Plates were incubated at 30°C for 24–72 h (Trypto-Soy agar) or 2–7 d (Sabouraud Dextrose agar). Bacterial species were identified using Gram staining and Oxidation-Fermentation, catalase, and cytochrome oxidase tests performed using the Api20 NE, Api20CAUX, and VITEK® 2 Compact systems (bioMérieux Co., France).

Ethical Consideration

Concerning the collection of ophthalmic preparations, outpatients and inpatients were provided a written explanation that the purpose of the collection of ophthalmic preparations was the “investigation of the state of in-use ophthalmic preparations” and that their participation was voluntary. We consulted the ethics review committee and received a response of “review unnecessary” because of the non-use of the patient’s medical record and biological samples in this study.

Statistical Analysis

The association between the types of hospital ophthalmic preparations and microbial contamination was analyzed using Fisher’s exact test. \( p<0.05 \) was considered to be significant in the results of microbial contamination of ophthalmic preparations of 0.5% vancomycin hydrochloride and 2% cyclosporine after use. In this case, however, analysis of the results of microbial dynamics in four types of ophthalmic preparations was performed by applying Fisher’s exact test with the Bonferroni correction; \( p<0.0083 \) was considered to be significant.
Fig. 1. The Microbial Dynamics in Ophthalmic Preparations of Hospital Preparation
(Physiological saline=Fig. 1A, 0.2% fluconazole=Fig. 1B, 0.5% vancomycin hydrochloride=Fig. 1C, 2% cyclosporine=Fig. 1D). Results are expressed as the mean (n=2).
the ophthalmic preparation of physiological saline, E. coli (N1HJJC-2) was below the limit of detection after inoculation for 48 h, S. marcescens (IFO3936) and S. aureus (209P) were below the limit of detection after inoculation for 7 d, and P. aeruginosa (ATCC27853), C. albicans (IFO1594) and A. niger (IFO4407) did not exhibit growth during the period of examination. However, P. aeruginosa (clinical strain), B. cecpacia (clinical strain), and S. marcescens (clinical strain) exhibited remarkable growth (Fig. 1A). In the ophthalmic preparation of 0.2% fluconazole, C. albicans (IFO1594) was below the limit of detection after inoculation for 6 h, S. aureus (209P) was below the limit of detection after inoculation for 48 h, E. coli (NIHJJC-2) was below the limit of detection after inoculation for 7 d, and A. niger (IFO4407) did not display growth during the period of examination. Conversely, P. aeruginosa (ATCC27853, clinical strain), B. cecpacia (clinical strain), S. marcescens (IFO3936, clinical strain), exhibited remarkable growth (Fig. 1B). In the ophthalmic preparation of 0.5% vancomycin hydrochloride, none of the investigated microorganisms displayed growth during the period of examination. P. aeruginosa (ATCC27853, clinical strain), B. cecpacia (clinical strain), E. coli (NIHJJC-2), S. marcescens (IFO3936, clinical strain), and S. aureus (209P) were below the limit of detection after inoculation for 7 d (Fig. 1C). In the ophthalmic preparation of 2% cyclosporine, all of the investigated microorganisms were below the limit of detection after inoculation for 6 h (Fig. 1D).

The microbial contamination rate in the collected ophthalmic preparations was 16.7% (3/18 samples) for ophthalmic preparations of 0.5% vancomycin hydrochloride, versus 0% (0/30 samples) for ophthalmic preparations of 2% cyclosporine. The microbial contamination rate in ophthalmic preparations of 0.5% vancomycin hydrochloride was significantly higher than that in ophthalmic preparations of 2% cyclosporine (p=0.0472). The detected contaminants in the ophthalmic preparations of 0.5% vancomycin hydrochloride were C. famata in two cases and C. guilliermondii in the remaining preparation. A contamination level of $1 \times 10^4$ CFUs/mL was observed in one of the samples (Table 3).

**DISCUSSION**

Ophthalmic preparations that do not contain preservatives are unlikely to cause hypersensitivity and allergic reactions. However, hospital preparations have been reported to be associated with a high risk of microbial contamination.3–5 In the present study, we evaluated the association of physicochemical properties such as pH and water activity with microbial contamination in ophthalmic preparations.

From the results of the dynamics of Gram-negative bacilli in each ophthalmic preparation, it can be suggested that the microbial contamination risk in ophthalmic preparations of physiological saline and 0.2% fluconazole is higher than that in ophthalmic preparations of 0.5% vancomycin hydrochloride or 2% cyclosporine. Of the nine microorganisms tested in preparations of physiological saline and 0.2% fluconazole, three and five strains displayed growth, respectively. In ophthalmic preparations of physiological saline and 0.2% fluconazole, P. aeruginosa growth was observed. P. aeruginosa is an important contaminant in ophthalmic preparations, inducing corneal ulcers.7–10 B. cecpacia and S. marcescens displayed growth at a level of $1 \times 10^4$ CFUs/mL after inoculation for 48 h in both preparations. Therefore, ophthalmic preparations of physiological saline and 0.2% fluconazole may permit the growth of microorganisms due to higher water activity and neutral pH. In addition, Gram-positive cocci do not grow well in the presence of the small amount of nutrients present in intravenous fluids, but the growth of Gram-negative bacilli has been observed.11,12 This may explain the growth of Gram-negative bacilli in ophthalmic solutions. Furthermore, it was reported that in-use ophthalmic preparations of physiological saline and 0.2% fluconazole exhibited microbial contamination at levels of $1 \times 10^4$–$1 \times 10^6$ CFUs/mL.13 Therefore, ophthalmic preparations of physiological saline and 0.2% fluconazole are associated with a high risk of microbial contamination, and strict cold storage and daily replacement are recommended for these preparations.

The levels of all tested microorganisms decreased in 0.5% vancomycin hydrochloride after inoculation. However, the presence of C. albicans (IFO1594) and A. niger (IFO4407) was confirmed in 0.5% vancomycin hydrochloride even after inoculation for 7 d. In addition, microbial contamination was observed in 0.5% vancomycin hydrochloride preparations used by patients (3/18 samples). All of the detected contaminants were Candida spp., one of which was detected at a level of $1 \times 10^4$ CFUs/mL. Generally, microbiological growth is possible at neutral pH.14 However, microbial contamination could not be prevented in strongly acidic solutions (pH 3.3) such as ophthalmic preparations of 0.5% vancomycin hydrochloride. Another study illustrated the stability of 25 mg/mL vancomycin deep frozen for 3 months at $-20\pm 2^\circ\text{C}$, and the stability was maintained when the ophthalmic solutions were stored for 48 h in a refrigerator after thawing.15 Furthermore, other research suggested that ophthalmic preparations of 31 mg/mL vancomycin should be kept refrigerated at 4°C and that solutions stored at 24°C should be replaced every week.16 Therefore, strict cold storage is necessary for in-use ophthalmic preparations of 0.5% vancomycin hydrochloride.

Contrarily, all tested microorganisms in ophthalmic preparations of 2% cyclosporine were below the limit of detection after inoculation for 6 h. In addition, none of the 30 samples of 2% cyclosporine that were personally used by patients exhibited microbial contamination at levels of $1 \times 10^4$ CFUs/mL. Generally, microbiological growth is possible at neutral pH.14 However, microbial contamination could not be prevented in strongly acidic solutions (pH 3.3) such as ophthalmic preparations of 0.5% vancomycin hydrochloride. Another study illustrated the stability of 25 mg/mL vancomycin deep frozen for 3 months at $-20\pm 2^\circ\text{C}$, and the stability was maintained when the ophthalmic solutions were stored for 48 h in a refrigerator after thawing.15 Furthermore, other research suggested that ophthalmic preparations of 31 mg/mL vancomycin should be kept refrigerated at 4°C and that solutions stored at 24°C should be replaced every week.16 Therefore, strict cold storage is necessary for in-use ophthalmic preparations of 0.5% vancomycin hydrochloride.
hibited microbial contamination. This may be explained by the apparently lower water activity of 2% cyclosporine. In general, microorganisms have difficulty growing in environments with low water activity. Therefore, oily formulations with lower water activity such as ophthalmic preparations of 2% cyclosporine are considered to be associated with a lower risk of microbial contamination.

The investigated ophthalmic preparations in this study are highly needed in ophthalmic practice. Generally, ophthalmic preparations of physiological saline are often used to relieve dry eye symptoms in patients with chronic Sjögren’s syndrome or Stevens-Johnson syndrome and histories of medication allergies. It is an important ophthalmic preparation for patients difficult to be used variety drugs, such as preservatives. In addition, ophthalmic preparations of 0.2% fluconazole and 0.5% vancomycin hydrochloride have been reported to be effective for treating patients with keratomycosis, *Acanthamoeba* keratitis, and methicillin-resistant *S. aureus* keratitis. Furthermore, ophthalmic preparations of 2% cyclosporine has been used for immune suppression after corneal transplantation.

For much-needed ophthalmic preparations, it is desirable to manage their use in accordance with their physicochemical properties. However, there is less evidence regarding the efficacy and safety of hospital preparations compared to commercially available ophthalmic preparations. Therefore, in the future, the widespread use of commercialized preserved-free ophthalmic preparations equipped with a filter to prevent microbial contamination or single-dose preparations is desired as an alternative to hospital preparations.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


