

Original Research Article

Assessment of preservative effectiveness in preserved and preservative-free artificial tears eye drops by microbial challenge test

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Abstract

Purpose: To assess and compare the preservative effectiveness of preserved and preservative-free artificial tears.

Methods: Eight artificial tear products were randomly purchased from registered pharmacies. The products were classified as preserved (n = 4) or preservative-free (n = 4) based on the presence (containing benzalkonium chloride) or absence of a preservative. Physicochemical evaluation, including colour, clarity, particulate matter, and pH, was carried out for all products. In addition, baseline microbiological quality assessment of each product was carried out using a culture-based viable cell count method, targeted at determining the sterility of the test products before the challenge test and after exposure to ambient air to simulate typical usage. Microbial challenge tests were performed using *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger*. Results were periodically (0, 7, 14, and 28 days) monitored and surviving organisms were determined by serial dilution and pour-plate viable cell count.

Results: The study recorded 100 % clear, colourless, and particle-free products, with pH values ranging from 6 to 9. Preserved formulations generally resisted microbial contamination better than preservative-free products. Air exposure led to microbial growth in all products except P-4, which remained stable throughout the 28 days. Preserved drops significantly reduced bacterial and fungal contamination at the end of the microbial challenge test, while preservative-free drops exhibited higher microbial survival over time.

Conclusion: Although the preserved eyedrops offered better protection against contamination, when compared to the unpreserved eyedrops, only one out of 4 tested had a total absence of microbial contamination throughout the 28 days of testing. These results buttress the protective role of preservatives in ophthalmic preparations and the need for careful handling of these products.

Keywords: Artificial tears, Microbial contamination, Ophthalmic preparation, Preservatives

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INTRODUCTION

The eye's surface is protected by several defence mechanisms, including blinking, tear

flow, and antimicrobial substances in the tear film. These mechanisms help prevent microbial colonisation and maintain eye health [1]. Dry eyes, often corrected with artificial tear drops, are

characterised by discomfort, irritation, and inflammation, which can result from disrupted tear film stability or tear production [2,3]. Artificial tears are one of the most commonly used ophthalmic products. As with all ophthalmic preparations, artificial tear eye drops should remain sterile, resisting microbial contamination at production, storage and use [4,5].

Artificial tear formulations are classified as preserved and preservative-free. Preserved eye drops ideally contain preservatives that act as antimicrobial agents, destroying introduced microorganisms, especially those introduced during handling. Common preservatives utilised in the preparation of eye drops include: Benzalkonium chloride, Chlorobutanol and Polyquaternium-1 [6,7]. These help maintain the microbiological stability of multi-dose containers by preventing microbial proliferation caused by handling.

However, studies have documented adverse effects on ocular tissues with repeated exposure to some preservatives, especially benzalkonium chloride. These effects are particularly significant for patients who require frequent or long-term use, such as those with chronic dry eye or glaucoma [7,8].

To minimise preservative-related toxicity, preservative-free formulations are designed and packaged in single-use units or multi-dose systems with advanced filtration or valve mechanisms that prevent microbial entry [9]. These typically rely on sterile manufacturing processes and proper handling to maintain the product's microbiological safety [10,11].

To assess microbiological stability, antimicrobial effectiveness testing (microbial challenge test) is performed. Microbial challenge, also called antimicrobial or preservative effectiveness testing, is used to assess a preservative system's ability to prevent microbial contamination. This test is essential in ophthalmic preparations, and comparing the two preparations' effectiveness in resisting microbial contamination provides valuable insight into their safety profiles, thereby guiding clinicians [7,8,10]. It involves deliberate contamination of test products with selected microbes, such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus* spp., *Proteus mirabilis*, and others, depending on the nature of the product being tested. The effectiveness of the preservative is determined by its ability to prevent, inhibit, or eliminate microbial growth [22,23].

Despite their widespread use, both preparations require evaluation and re-evaluation to assess their safety profile. This study was thus designed to evaluate the antimicrobial effectiveness of the preservative in the test products, addressing knowledge gaps and providing evidence to guide safer use and formulation of ophthalmic products. The study further evaluated the baseline microbiological quality and sterility of the preserved and preservative-free artificial tears eye drops prior to air-exposure and preservative effectiveness testing.

METHODS

Study design

The study was designed to assess and compare the contamination rates of some preserved versus preservative-free artificial tears eye drops available in pharmaceutical outlets across Awka metropolis, Anambra State. The samples were subjected to standardised microbial challenge tests and exposed to ambient air at 28 °C to realistically mimic typical usage conditions.

Sampling and inclusion criteria

The products obtained were divided into two groups, representing the presence or absence of preservatives. Group A comprised eye drops containing a preservative; for all products that were purchased, the preservative was benzalkonium chloride. Group B comprised eye drops free of any preservative. For Group A, only formulations containing at least one preservative were included, while formulations lacking preservatives were excluded. Conversely, for Group B, only preservative-free eye drops were included, and formulations containing preservatives were excluded, ensuring a clear distinction between the two groups. The ophthalmic preparations were obtained in duplicate, maintaining the same batch number for each product to ensure uniformity and manufacturing conditions. One unit from each pair was used for the air-exposure contamination study to obtain baseline control data, while the other was used for the microbial challenge test. This guaranteed that both procedures were carried out on products of identical composition and manufacturing conditions, minimising batch-to-batch variability.

Microbial strains, media preparation and inoculum standardisation

Four organisms reportedly implicated in the contamination of ophthalmic preparations, *Pseudomonas aeruginosa*, *Escherichia coli*,

Staphylococcus aureus, and *Aspergillus niger*, were utilised in the study. Laboratory strains of these organisms were obtained from the Pharmaceutical Microbiology laboratory of Nnamdi Azikiwe University and reconfirmed using macroscopic, microscopic, and biochemical tests as described by Cheesbrough [24]. Each inoculum suspension, upon confirmation, was serially diluted with sterile saline to achieve a standard microbial concentration of 1×10^8 CFU/mL. The actual colony-forming units (CFU) were determined using the dilution pour plate method [11].

Preparation of Microbiological media

For the microbiological media, nutrient agar was prepared by dissolving 28 g of nutrient agar powder in 1 litre of distilled water, with continuous heating and stirring to ensure complete dissolution. The solution was then sterilised using an autoclave at 121 °C for 15 minutes and distributed into Petri dishes. Additionally, nutrient broth was prepared by dissolving 13 g of nutrient broth powder in 1 litre of distilled water, then sterilised using an autoclave at 121 °C for 15 minutes and distributed into sterile test tubes for further use.

Physico-chemical evaluation of eye drops

Physicochemical evaluation was done as it helps determine if the eye drops met the required quality standards, such as colour, clarity, presence or absence of particles, and pH, as these will help determine if the product possesses appropriate characteristics, such as safety, stability, quality, patient comfort, as well as therapeutic effectiveness. Under bright light, parameters such as colour, clarity and presence of particulate matter were assessed. The pH of each sample was assessed equally by placing a drop of the eye drop solution on universal pH paper and comparing the resulting colour with the provided chart to obtain a semi-quantitative measure of acidity or alkalinity [12].

Microbiological quality assessment

To assess microbial contamination in the sample before the experimental manipulations, microbiological quality assessment was performed to determine the presence or absence of bacterial and fungal contaminants. To do this, 1.0 mL of each freshly opened eyedrop was diluted with 9.0 mL of normal saline to obtain a 10-fold dilution and subcultured onto nutrient agar (NA) in duplicate for bacterial enumeration and onto Sabouraud dextrose agar (SDA) for fungal assessment using the pour plate

technique [24]. The cultures were examined for the presence or absence of microbial contaminants, and where growth was present, colony-forming units were enumerated to determine the level of contamination.

Air exposure contamination study and microbial challenge test

To mimic real-world use and assess the formulations' ability to withstand environmental conditions after opening, eye drops were opened and left on open shelves to expose them to ambient laboratory air. This was done to mimic repeated handling and the possible effects of storage conditions associated with multidose ophthalmic preparations during patient use. 0.5 mL aliquots of each sample were collected at 0, 12, 24, 72 hours, 7, 14, and 28 days, inoculated into enrichment thioglycolate broth, cultured, and observed for viable microbes. The preservative effectiveness of the samples was evaluated by challenging them with a standardised inoculum of test organisms. Inoculation was performed such that the final concentration of the microbe ranged between 1×10^5 and 1×10^6 CFU/mL. Inoculated samples were stored under conditions as specified by the United States Pharmacopoeia [23] and examined for viable cells using the pour-plate method on days 0, 7, 14, and 28. For the negative control, the initial microbial load was determined, whereas for the positive control, the microbes were cultured in the absence of the medication to assess organism viability. All inoculated plates were incubated at 37 °C for 24 hours.

RESULTS

Physicochemical evaluation

The physical properties of the test eye drops are as shown in Table 1. The pH of the non-preserved eye drops was largely uniform, with three products (NP-1, NP-3 and NP-4) exhibiting a neutral pH of 7, while NP-2 had a slightly acidic pH of 6. In contrast, the preserved eye drops showed pH variability, with P-1 and P-2 at a neutral pH of 6 and P-3 and P-4 at alkaline pH values of 8 and 9, respectively. All eight ophthalmic preparations under study were colourless, clear and free from visible particulate matter. This suggests that all products met the basic physical requirements for ophthalmic preparations.

Microbial quality assessment

Bacterial and fungal evaluations were conducted on the eight ophthalmic preparations using 10-

fold and 100-fold serial dilutions of cultures on nutrient agar and Sabouraud Dextrose agar, respectively. Following bacterial evaluation, three of the four preservative-free eye drops, NP-1, NP-2, and NP-3, showed no visible microbial growth at either the 10-fold (10^{-1}) or the 100-fold (10^{-2}) dilution levels, while NP-4 at 10-fold dilution exhibited detectable bacterial growth with no visible growth at 100-fold dilution. In the preserved eye drop group, P-2 and P-4 showed heavy bacterial contamination, with colonies too numerous to count at both the 10^{-1} and 10^{-2} dilutions. Fungal assessment across both groups showed no visible fungal growth at both dilutions, indicating compliance with fungal sterility requirements.

Contamination following air exposure

Following air exposure, microbial growth was detected in all NP samples and in P-3 at multiple

sampling times, including 0, 12, 24, and 72 hours, as well as on days 7, 14, and 28. While the above can be considered a failed assessment, P-4 showed no visible microbial growth throughout the 28-day exposure period, indicating superior resistance to airborne contamination. For P-1, absence of growth was recorded up to the 24-hour sampling point, after which varied levels of microbial contamination were noted.

Microbial challenge test

At the end of the microbial challenge test against four organisms: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Aspergillus niger*. The surviving microbes were enumerated on days 0, 7, 14, and 28, and the results are presented in Table 1.

Table 1: Microbial challenge test results of artificial tear products against challenge microorganisms during the 28-day study period

Product	Day 0 (CFU / Plate)	Day 7 (CFU / Plate)	Day 14 (CFU / Plate)	Day 28 (CFU / Plate)
A. <i>Staphylococcus aureus</i>				
NP-1	300	>300	>300	90
NP-2	>300	48	53	17
NP-3	300	>300	>300	>300
NP-4	300	>300	>300	>300
P-1	300	55	18	2
P-2	300	21	19	0
P-3	300	38	0	1
P-4	300	44	0	1
B. <i>Escherichia coli</i>				
NP-1	300	>300	0	>300
NP-2	300	>300	0	0
NP-3	300	>300	0	>300
NP-4	300	>300	0	>300
P-1	300	>300	0	>300
P-2	300	>300	0	>300
P-3	300	>300	0	>300
P-4	300	>300	0	300
C. <i>Pseudomonas aeruginosa</i>				
NP-1	300	>300	>300	>300
NP-2	300	0	>300	>300
NP-3	300	>300	>300	>300
NP-4	300	>300	>300	>300
P-1	300	0	>300	>300
P-2	300	14	>300	>300
P-3	300	0	>300	>300
P-4	300	0	0	>300
D. <i>Aspergillus niger</i>				
NP-1	300	0	0	0
NP-2	300	>300	0	0
NP-3	300	0	>300	>300
NP-4	300	0	>300	>300
P-1	300	>300	300	300
P-2	300	0	0	0
P-3	300	0	0	0
P-4	300	0	0	0

Overall, P-1 to P-4 demonstrated greater antimicrobial effectiveness than the preservative-free products (NP-1 to NP-4). For *S. aureus*, the preserved products recorded lower colony counts over the study period than the preservative-free products, with no detectable growth in P-2 by Day 28. Heavy growth of *E. coli* was observed in all products at Days 0 and 7, while no colonies were recovered on Day 14. By Day 28, growth reappeared in most products. A reduction in *P. aeruginosa* was noted in some products during the early stages of the study, although all products showed heavy growth at the final sampling point. For *A. niger*, no growth was detected in P-2, P-3 and P-4 from Day 7 onward, whereas NP-3 and NP-4 showed growth from Day 14.

formulations. For *E. coli*, no growth was detected in any product on day 14; however, growth reappeared in most by Day 28. Similarly, reductions in *P. aeruginosa* counts were observed in some products at Days 7 and 14, but heavy growth was recorded in all products by Day 28. These findings indicate limited long-term control of both Gram-negative organisms by the tested formulations. With *A. niger*, preserved products showed minimal or no growth in most cases, whereas preservative-free products displayed variable growth patterns. In general, the diagram highlights the protective role of preservatives in reducing microbial contamination in ophthalmic preparations.

DISCUSSION

Figure 1 shows the comparative analysis of the microbial challenges against the test organism. The preserved eye drops generally exhibited a greater reduction in microbial colony counts over time compared to preservative-free products, indicating better antimicrobial protection. For *S. aureus*, preserved products showed a rapid, sustained decline in colony counts throughout the study period. In contrast, preservative-free eye drops NP-3 and NP-4 showed persistently high microbial growth throughout the study, while NP-1 and NP-2 showed varied degrees of reduction.

E. coli and *P. aeruginosa* showed temporary reductions in some products, and complete inhibition was not maintained across all

Ophthalmic preparations, by definition, are required to be sterile, as they are applied directly onto the ocular surface. Although produced with the aim of sterility, contamination may occur at the point of production, during storage, during repeated handling, or during patient use. Such contamination can compromise physicochemical stability, leading to adverse ocular outcomes [13,14]. To mitigate this risk, antimicrobial preservatives are incorporated, or, for non-preserved preparations, an advanced multi-dose delivery system with one-way valves or filters is used to inhibit microbial proliferation and maintain sterility throughout use [15].

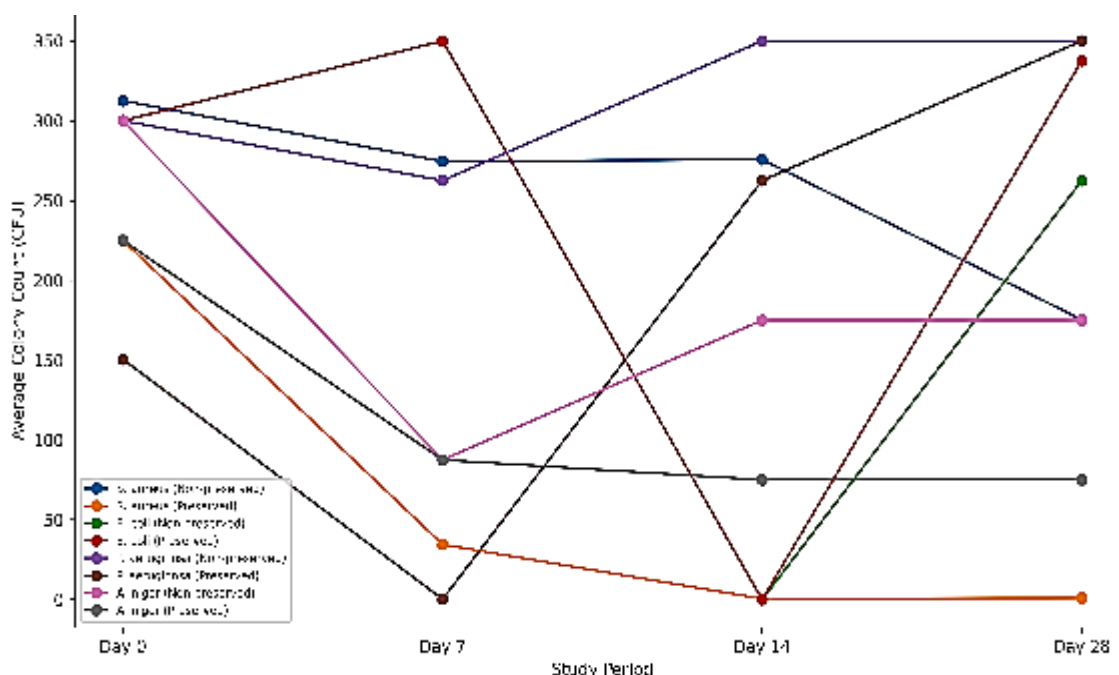


Figure 1: Comparative microbial growth pattern of preserved vs preservative-free artificial tears

Consistent with expected quality for ophthalmic preparations [16], all eight products satisfied preliminary physicochemical parameters, appearing clear, colourless and free from visible particles. Although visual inspection alone cannot confirm sterility, it remains an essential first-line quality control assessment. pH values of the tested preparations ranged from 6.0 to 9.0, consistent with other reports suggesting that the pH of ophthalmic formulations is ideally within the neutral range. Preparations outside the physiological tear pH may cause ocular irritation or stinging upon instillation [17]. The use of indicator strips instead of a digital pH meter is limited by the small amount of drugs in each container and limited measurement precision.

Microbiological evaluation demonstrated that the absence of microbial growth following incubation indicates maintained sterility, whereas detectable growth suggests contamination occurring during manufacture, storage, handling, or laboratory processing. It is acknowledged that, despite strict aseptic techniques, procedural contamination cannot be completely excluded. Following air exposure and enrichment in thioglycolate broth, antimicrobial growth was observed in all products except P-4, indicating superior antimicrobial protection in the preserved formulation. This finding reinforces the protective role of preservatives in limiting microbial contamination during repeated use. Several studies have reported that multidose preservative-free ophthalmic solutions are particularly prone to contamination during use [18].

The preserved eye drops were generally more effective in reducing microbial survival than the preservative-free formulations. This finding is in agreement with observations by Saisyo et al. [18] and Chantra et al. [19], who highlighted the microbiological challenges associated with preservative-free ophthalmic products. Although microbial contamination has also been reported in preserved eye drops [20,21], the extent of microbial control appears to depend on the preservative system and formulation characteristics. In the present study, P-2 showed the best overall performance among the preserved products, producing the greatest reduction in the challenge organisms over the study period.

CONCLUSION

The artificial tear products examined in this study were microbiologically acceptable before use. However, differences were observed in their ability to control microbial growth after challenge. In general, the preserved formulations performed

better than the preservative-free products, although the level of protection varied among products. P-2 showed the best overall antimicrobial performance. These findings emphasize the importance of effective preservative systems in maintaining the microbiological quality of ophthalmic preparations during use.

DECLARATIONS

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Conflict of interest

The authors declare no conflict of interest in the work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

CKE conceptualised the study and supervised the work. BAO and FEO contributed to the development of the methodology and carried out the investigations. INE performed data analysis and contributed to the manuscript draft; CEE drafted the original manuscript. All authors reviewed and approved the final version of the manuscript.

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