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RESEARCH

Microbial contamination of multiple-use bottles of fluorescein ophthalmic solution

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Background: The contamination of ophthalmic solutions in ophthalmic practices remains an important cause of a myriad of secondary eye infections and a source of aggravation of ocular disorders such as corneal ulcers and keratitis. The aim of this study was to investigate the possible microbial contamination of fluorescein sodium dye solutions used in eye clinics in Ghana.
Methods: Fluorescein sodium solutions were collected from various eye clinics in Ghana. Twenty-one samples of multiple-use fluorescein ophthalmic solutions were collected from various regions in Ghana. Eighteen unopened bottles yet to be used were also collected to serve as controls from the same facilities. The solutions were inoculated in different culture plates (blood agar, MacConkey agar, Sabouraud dextrose agar and plate count agar). The resulting microbial growth was identified using standard microbial identification techniques. Susceptibility tests were performed to ascertain the clinical importance of the organisms identified.
Results: Positive cultures were recorded for all 21 multiple-use bottles (in-use) collected, but there were no positive cultures for the unopened bottles (yet to be used). Six different genera of bacteria were identified from fluorescein solutions, including resistant strains of *Staphylococci* spp., *Bacillus* spp., *Klebsiella* spp., *Pseudomonas* spp., *Haemophilus* spp. and *Bordetella* spp. *Pseudomonas* spp. were the most common bacterial contaminants. For fungi contaminations, *Aspergillus* spp., *Penicillium* spp. and *Cladosporium* spp. were isolated. The most common fungal contaminants were *Aspergillus* spp.
Conclusions: Multiple-use bottles of fluorescein solution used in eye clinics in Ghana were contaminated with clinically important strains of bacteria and fungi.

Key words: corneal ulcer, fluorescein, keratitis, *Pseudomonas* spp., resistant strains

Fluorescein is a molecule not found in nature. It was first synthesised by Von Bayor in 1871.[1] It is a yellow acid dye of the xanthene series and was first used on the eye in 1882.[1] It is used in cell and molecular biology, forensic science and geological tracer studies, not just ophthalmic practice. In aqueous media fluorescein exhibits well understood hydrolytic equilibria and can exist in cationic, neutral, anionic and dianionic forms, depending on the pH of the solution. Both negatively charged states emit fluorescence but in the dianionic state it is more luminous. Fluorescein usually absorbs light at 494 nm and re-emits at 517 nm in water.[1]

Fluorescein ophthalmic solutions are extensively used in primary eye care for contact lens fitting, the assessment of the pre-corneal tear film and ocular surface integrity. It is also used in the Seidel test for the detection of aqueous leakage, in the Jones dye test for patency of the nasolacrimal system, and routinely in the tonometric measurement of intraocular pressure.[2]

Notwithstanding the clinical usefulness of fluorescein ophthalmic solutions, potential contamination remains an undisputed cause and/or mode of transmission of several secondary eye infections and the source of aggravation of pre-existing ocular infections such as corneal ulcers and keratitis.[3] These contaminated solutions harbour certain opportunistic and pathological microbes such as *Pseudomonas aeruginosa*, *Pseudomonas pyocyanea* and *Serratia marcescens*, as well as yeast and moulds (fungi).[4] For instance, a contaminated fluorescein administered for the Seidel test can possibly lead to endophthalmitis in the event of a corneal laceration.

The incidence of contamination of various ophthalmic solutions ranges from zero to 37 per cent.[5] In the case of residual contents of ophthalmic solutions, contaminations are highly probable.[6–10]

The susceptibility of fluorescein ophthalmic solution (popular in most eye care facilities in Ghana) to *Pseudomonas aeruginosa* contamination has long been established.[11] Nevertheless, the profile of contamination is undoubtedly influenced by the unique geographical and facility-specific factors. Previously, most studies designed to ascertain the microbial contamination of fluorescein were stimulated or experimental in approach, and may not depict a real clinical situation.[2,12,13]

Previous studies have identified the aetiological causes of suppurative corneal ulcers and fungal keratitis in Ghana and South

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India to be *Fusarium* spp., *Aspergillus* spp., *Pseudomonas* spp. and *Streptococci* spp.[14] These microbes have been associated with contaminated ophthalmic solution meant for diagnostic and therapeutic purposes.[15] In this regard, fluorescein has been identified as one of the most problematic of diagnostic ophthalmic solutions in use. This further highlights the necessity for country-specific studies to be conducted in real clinical settings on the use of this diagnostic solution. Such studies will inform clinical policy aimed at protecting the eye health of the public from the potential risk of resistant strains of ocular infection.

Methods

Selection of facilities

Fifty major eye clinics across the 10 regions of Ghana were approached without prior notice so as to avoid a change in their usual practice. Of these, only 21 facilities granted the team access to collect samples. This was because participation was strictly voluntary with the sole decision by management of the facilities as whether to grant permission or not for sample collection.

Drugs used

The fluorescein-anaesthetic combinations were compounded extemporaneously at the various facilities by mixing one part of two per cent w/v fluorescein with two parts 0.5 per cent w/v anaesthetic. The fluorescein solutions were obtained from a local pharmaceutical company (Archdiocesan Health Pharmacy Ltd, Kumasi, Ghana) and others imported from Aurolab, India. Benzalkonium chloride was the named preservative in all fluorescein solutions. The anaesthetics included Proparacaine HCl (Alcon, Fort Worth, Texas, USA and Ashford Laboratories, Macau, China) and Tetracaine HCl (Adcock Ingram Limited, Johannesburg, South Africa). The preservative was benzalkonium chloride for the Proparacaine HCl and chlorobutanol for the Tetracaine HCl.

Collection of samples

One millilitre each of fluorescein ophthalmic solution (in use) at the various facilities (including fluorescein only and fluorescein + anaesthetic as per their availability), was collected into screw-top containers which had been pre-sterilised by washing in 70 per cent alcohol and sterilised in an ultraviolet chamber (Grant-Bio UVT-B-AR, Cambridge, England) for 30 minutes. These containers were sealed into a sterilised zip bag.

At the site of fluorescein sample collection, the containers were picked out of the sterilised zip bag. A sterile 3 ml syringe fitted with a 30 gauge needle was used to collect the sample. The sample collection was performed while wearing sterilised hand gloves. The samples were placed in their individual containers, sealed firmly and placed back into the sterilized zip bag.

The samples were transported to the laboratory over ice packs to reduce the metabolism of any organisms present. All samples were processed within 12 hours of collection. As a control, samples were collected from unopened containers obtained from the dispensary of the facilities selected. The fluorescein-anaesthetic combinations obtained from these facilities were compounded. The sample collection was undertaken between January and June, 2017.

Preparation of culture media

Culture media used were peptone water, blood agar, MacConkey agar, plate count agar and Sabouraud dextrose agar. These culture media were prepared as directed by the manufacturer, under standard and sterile conditions, in order to prevent external contamination. Other media, such as tryptone soy agar, were used for storing pure isolates for further evaluation. Triple sugar iron agar and citrate agar were used to test the biochemical activity of the various microbes found. Mueller–Hinton agar was used in the culture and sensitivity tests. All these were prepared as directed by the manufacturer.

Incubation and cultivation of samples

One millilitre of the sample was added to 9 ml of peptone water and incubated for six hours. Peptone water serves as a maximum recovery diluent. The samples were serially diluted to prevent bacterial overgrowth during culturing, then plated using the following pour plate method. First, 1,000 μl of the serially diluted samples were inoculated into clean autoclaved Petri dishes. Some 20 ml of the culture media (blood agar, Sabouraud dextrose agar, MacConkey or plate count agar) were poured into the plates, swelled gently and allowed to solidify. The solidified plate samples were incubated in Panasonic cooled incubator MIR-154 at 37°C for 24 hours for bacteria and 25°C for fungi, respectively. Individual visible viable colonies seen after 24 hours were counted and recorded. This was to enable the calculation of the colony forming units (CFUs), of viable bacteria which were expressed as CFU/ml.

The colonies identified were classified based on their morphological characteristics, and isolated and sub-cultured in tryptone soy agar. The pure cultures were then stored under −80°C for later identification studies.

Identification of microbes

Micro-organisms isolates were identified using various criteria which included biochemical, morpho-cultural characteristics, and Gram staining. Appearance (haemolysis pattern) on blood agar and colony appearance made up the morpho-cultural properties. Gram-negative bacteria were further identified using biochemical testing such as catalase, indole, triple sugar iron reduction and citrate tests. However, Gram-positive bacteria were identified using haemolysis pattern on blood agar, catalase, and coagulase tests. Lactophenol blue was used to identify the organisms growing on the Sabouraud dextrose agar.

Microscopy

In observing the prepared slide, a drop of oil was placed on the smear and using ×100 magnification of the light microscope (Olympus CX4, Tokyo, Japan), the morphology of the bacteria was observed. This, together with their growth characteristics on the agar, was used to identify the bacteria.

Antibiotic sensitivity test

Kirby–Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute,[16] was used to determine the *in vitro* susceptibility of the identified bacteria isolates to penicillin 1.5 IU, ampicillin 10 μg, flucloxacillin 5 μg, erythromycin 5 μg, tetracycline 10 μg, co-trimoxazole 25 μg, cefuroxime 30 μg and gentamicin 10 μg. A standardised suspension of the isolated bacteria was prepared by inoculating a colony into 10 ml peptone water and incubated at 37°C for 24 hours. It was then diluted to 0.5 MacFarland turbidity standards. A sterile swab was dipped into the standardised inoculum and used to inoculate evenly the surface of already prepared Mueller–Hinton agar (Oxoid, Basingstoke, UK). The agar was left for 15 minutes for the surface moisture to dry.

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The antibiotics discs were then placed on the surface of the inoculated medium. The plate was then incubated at 37°C for 18 hours. The zones of growth inhibition were recorded. The results were compared with the table provided by the Clinical and Laboratories Standards Institute.[15] The well diffusion method was used in the fungal sensitivity; this test assessed the efficacy of ketoconazole at dose levels of 0.5 to 10 mg/ml. A sterile swab was dipped into the standardised inoculum and used to inoculate evenly the surface of already prepared Sabouraud dextrose agar. The wells were created in the plated media and then incubated at 25°C for five days.

Statistical analysis

Comparison of microbial contamination between fluorescein alone and fluorescein mixed with an anaesthetic was performed using a paired t-test. Two-way analysis of variance followed by Bonferroni post-test was used to compare the mean values of the control against the test sample. Values were expressed as the mean ± standard error of the mean. $p \leq 0.05$ was considered to be statistically significant. Data analysis was performed using GraphPad Prism (version 5.03; GraphPad, La Jolla, California, USA).

Ethics consideration

Ethics clearance (ID NO: UCCIRB/CHAS/2017/03) was obtained from the Institutional Review Board of the University of Cape Coast. Biosafety guidelines for protection of personnel in the laboratory were observed.

Results

Cultivation of samples

There were positive cultures for all 21 samples (four fluorescein solutions only and 17 fluorescein + anaesthetic solutions) on blood, plate count and MacConkey agars. Nineteen samples (four fluorescein only and 15 fluorescein + anaesthetic) resulted in positive culture on Sabouraud dextrose agar (Figure S1). This indicated the presence of microbes in the cultured samples. There was no positive culture for the control.

Microscopy

After the colonies on the blood, MacConkey and plate count agars were sub-cultured on Sabouraud dextrose agar and Gram-stained, microscopy indicated the presence of Gram-positive cocci bacteria and Gram-negative bacilli (Figure S2). The colonies on Sabouraud dextrose agar stained with lactophenol blue indicated the presences of filamentous and yeast-like fungi (Figure S2).

Biochemical test

The isolated bacteria were examined for biochemical activity using various tests. The triple sugar iron and citrate agars were prepared in a test tube as directed by the manufacturer. Bacteria were inoculated in the media and the result observed and recorded. Other biochemical tests performed were indole formation using Kovac reagent, oxidase test using hydrogen peroxide and catalase using fresh serum. Results from these tests were recorded, aiding in the identification of the bacteria.

Fluorescein versus fluorescein-anaesthetic combination

There was no significant difference ($p = 0.679$) in microbial contamination between fluorescein and fluorescein-anaesthetic combination solutions in multiple use bottles (Figure 1).

Profile of microbial contamination

Of the 21 facilities involved in this study, there were at most six samples per region. From the test conducted, various bacterial contaminants were identified, totalling 49 pure isolates. Of these, 13 (26.5 per cent) were *Pseudomonas* spp., 11 (22.4 per cent) were *Bacillus* spp., nine (18.4 per cent) were *Klebsiella* spp., seven (14.3 per cent) were *Staphylococci* spp., five (10.2 per cent) were *Bordetella* spp. and four (8.2 per cent) were *Haemophilus* spp. Upon identification (using Guy St-Germain *Identifying Fungi: A Clinical Laboratory Handbook*), the genera of fungi were identified. These were *Aspergillus* spp. 14 (38.9 per cent), *Penicillium* spp. 13 (36.1 per cent), and *Cladosporium* spp. nine (25 per cent) isolates, of the 36 pure isolates.

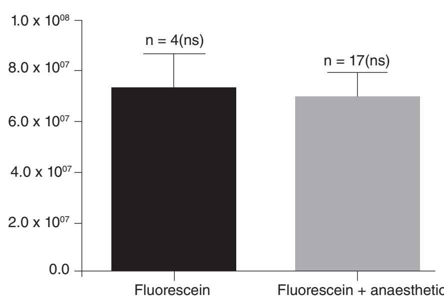

Figure 1. Comparing average microbial loads of fluorescein alone with fluorescein mixed with an anaesthetic. Using paired t-test show a p-value of 0.679. ns: no significance.

Antimicrobial susceptibility testing

The susceptibility test was performed using a standard antibiotic disc which grouped the isolates into resistant, intermediate and susceptible, to each of the antibiotics found on the disc.[17] Table 1 is a summary of these results for the antimicrobial susceptibility test of isolated bacteria. Figure 2 is also a summary of the minimum inhibition concentration of ketoconazole on identified fungi species. Ketoconazole at dose levels 0.5 to 10 mg/ml inhibited fungi growth significantly ($F_{(4,40)} = 121.5$, $p < 0.001$), with maximum effect at 10 mg/ml. The area under the curve for the plot of concentration against zones of inhibition indicated that the most susceptible was *Aspergillus* spp. and the least susceptible being *Penicillium* spp. (Figure 2).

Discussion

Ophthalmic solutions are liquids, semi-solid and solid preparations that when reconstituted according to the label instructions, result in a solution intended for instillation in the conjunctiva, the conjunctival sac, cornea or the lids of the eye.[18] Sterility is one of the key concerns given the route of administration of ophthalmic solutions.[19] There are risks of serious infection and blindness for users if sterile products become contaminated.

Ocular infections and loss of vision resulting from contaminated extemporaneously prepared ophthalmic products have been reported.[20] Infectious conjunctivitis and keratitis are among the three most common ocular infectious conditions in Sub-Saharan Africa[21–23] with aetiological trace to microbes (*Chlamydia trachomatis*, *Neisseria gonorrhoea*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Staphylococcus albus*, *Haemophilus influenza*, *Pseudomonas aeruginosa*, *Escherichia coli* and filamentous fungi) that bioburden ophthalmic solutions.

Fluorescein has been found to be liable to *Pseudomonas aeruginosa* contamination and *Pseudomonas* spp. were found to be the most common bacterial contaminant in this study, with *Haemophilus* spp. being the least common. *Aspergillus* spp. were the most

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Organism	AMP 10 μg	TET 10 μg	COT 25 μg	GEN 10 μg	CRX 30 μg	CHL 10 μg	CTR 30 μg	CTX 30 μg	FLX 5 μg	ERY 5 μg	PEN 1.5 IU
Haemophilus spp.	R	R	R	S	R	R	R	R	n/a	n/a	n/a
Pseudomonas spp.	R	R	R	S	R	R	R	R	n/a	n/a	n/a
Bordetella spp.	R	R	S	S	R	I	R	R	n/a	n/a	n/a
Klebsiella spp.	R	S	S	S	R	I	R	R	n/a	n/a	n/a
Staphylococcus spp.	n/a	S	S	S	S	n/a	n/a	n/a	R	R	R
Bacillus spp.	n/a	R	S	S	I	n/a	n/a	n/a	R	R	R

AMP: ampicillin, COT: co-trimoxazole, CRX: cefuroxime, ERY: erythromycin, FLX: flucloxacillin, GEN: gentamicin, I: intermediate, PEN: penicillin, R: resistant, S: susceptible, TET: tetracycline.

Table 1. Zone of inhibition by antibiotic standard disc

common fungal contaminants, in contrast to *Fusarium* spp. often being reported as the aetiological cause of fungal keratitis in Ghana and other Sub-Saharan African countries.[14,22–26] Nevertheless, elsewhere, in southern India, *Aspergillus* spp. is the most frequently reported cause of fungal keratitis.[14]

Pseudomonas spp. and *N. gonorrrhoeae* – both of which have been found to penetrate intact cornea epithelium – were two of the infectious agents identified. The use of fluorescein as a diagnostic agent for assessing corneal integrity in suspected cases of abrasion and ulcers provides a probable 'porte d'entree' for most of the other microbes, which are incapable of traversing the intact cornea, increasing the risk of infectious keratitis.[27,28]

Corneal opacity (most of which are caused by recalcitrant infectious keratitis) remains an important contributor to visual disability and unilateral blindness in low-resource settings such as Ghana. Studies from other poor regions of the world have indicated a population incidence of infectious keratitis to be 113–799 per 100,000/year, that is, 10–70 times higher than in Western countries[23] and has therefore been identified as a 'silent epidemic' which requires serious attention.[21–23]

In the present study, fluorescein solutions were found to be contaminated with clinically important microbes of bacterial and fungal species. The bacterial contaminants were resistant to conventional antibiotics, except gentamycin which showed appreciable activity against all the isolated bacterial contaminants including *Pseudomonas* spp. (Table 1). Consequently, critical effort needs to be directed toward controlling the menace of infectious contaminants of fluorescein solution so as to overcome the 'silent epidemic' of corneal disorders.[21–23]

Although the fungal contaminants were susceptible to ketoconazole, the reported unavailability and inaccessibility of topical antifungal ophthalmic formulations on the Ghanaian market pose a challenge for optimal treatment. This is because clinics resort to the use of extemporaneously prepared ophthalmic products, which could still serve as a potential source of aggravation for a pre-existing condition.[20,29]

Recent studies have indicated that fluorescein-anaesthetic combination solutions exhibit ample fortification against microbial contamination.[2] However, in the present study, no such fortification was exhibited. This could be due to the fortification conferred by such a combination being active in the short term, and may not reflect the long-term usage of these fluorescein solutions in Ghanaian clinical settings.

The limitation of the present study was the low facility participation; less than 50 per cent of the major facilities approached consented and granted access. This has implications for generalising the findings in this study. There was no information on duration of use and the number of people exposed to these solutions. This information was not sought, to minimise the possibility of participating facilities modifying their routine practices with respect to the use of fluorescein ophthalmic solution. There was no attempt to investigate the association between the season of sample collection and the contaminants found.

Nevertheless, the findings of the present study are of public health significance, as any contamination for ophthalmic solutions is unacceptable, regardless of season and profile of use. Although this study did not investigate the association between the use

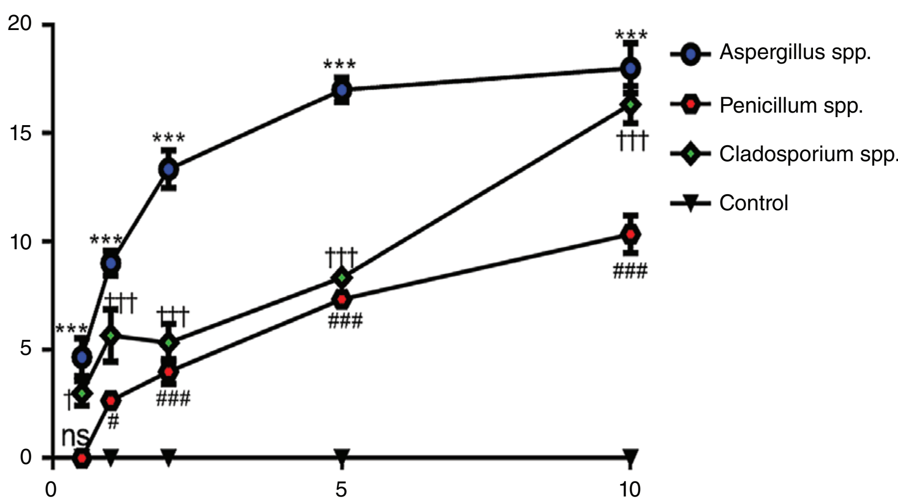

Figure 2. MIC of ketoconazole to three fungi found in the sample, using two-way analysis of variance followed by Bonferroni test. *$p < 0.05$, ***$p < 0.001$ and ns is $p > 0.05$. *Indicates the effect on *Aspergillus* spp., †represents the effect on *Cladosporium* spp. and $^{\#}$effect on *Penicillum* spp.

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of these contaminated ophthalmic solutions and the incidence of ocular infection in the various facilities, there has been a reported increase in the incidence of infectious keratitis in poorer regions of the world.[24]

Conclusion

Samples of multiple-use bottles of fluorescein ophthalmic solution obtained from some eye care facilities in Ghana were heavily contaminated with resistant strains of bacteria of clinical importance. Multiple-use bottles of fluorescein-anaesthetic combination were no better in microbial contamination profile than fluorescein only.

Recommendations

Based on the findings of the present study, it is recommended that:

1. multiple uses bottles of fluorescein ophthalmic solutions (in use) in eye clinics in Ghana be replaced as frequently as possible;
2. alternatives such as the use of fluorescein strips could be encouraged; and
3. there should be a policy guideline on the usage of fluorescein ophthalmic solutions.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1. Plates of cultures of multiple-use bottles of A: fluorescein solution on blood agar; B: fluorescein solution on plate count agar (PCA); C,D: fluorescein solution on Sabouraud; and D: control (unopened fluorescein).

Figure S2. A: Gram-positive cocci bacteria viewed under ×100 lens (oil emersion); B: Gram-negative bacillus (rod) visualised under the oil emersion lens of a light microscope; C: filamentous fungi; and D: a yeast-like structure. Both fungi were stained with lactophenol blue and viewed under microscope with a magnification of ×40.

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