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Microbiological and physicochemical assessment of some brands of gentamicin eye drops marketed in registered retail pharmacies in Port Harcourt, Nigeria

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Abstract

Gentamicin eye drop is the most commonly imported, often abused, cheap anti-infective for most superficial eye infections in Nigeria. This study therefore aims at determining the physicochemical properties and the antimicrobial efficiency of nine brands of the multi-dose Gentamicin eye drops purchased from registered retail pharmacies in Port Harcourt, Rivers state, Nigeria. Nine brands of gentamicin sulphate eye drops were purchased from different pharmacies in Port Harcourt metropolis. Physical appearance of the different brands were examined for integrity of the packaging and closure system. In addition to physicochemical analysis (colour and clarity evaluation, pH) sterility testing was done by inoculating each differentiating nutrient media (liquid thioglycollate, soya casein, and Sabouraud dextrose media) with different eye drop sample. Microbial challenge test on the effectiveness of the preservative (using *E. coli, P. aeruginosa, S. aureus* and *C. albicans*) and pyrogen testing were conducted on the samples. All the samples in vials were packaged properly with no particulate matter in any of them. The pH of the brands ranged from 5.40 - 7.26. All the nine samples of gentamicin eye drops passed the "on the spot" sterility testing. One of the 9 samples (11.11%) failed the preservative challenge test while 4 samples (44.44%) failed the pyrogen test for bacterial endotoxin. An eye product may be sterile but not pyrogen-free. The efficiency of the preservative system in a sterile gentamicin eye drop solution is to confer the eye drop with the ability to withstand contamination by opportunistic microorganisms during usage.

Keywords: Gentamicin; Eye drop; Preservative; Pyrogen testing; Microbial challenge test, Nigeria.

INTRODUCTION

Eye drops are sterile multi- dose pharmaceutical preparation produced with emphasis on sterility both during usage and also over the shelf life of the product [1,2]. They are required to be produced in an aseptic condition, sterile on storage and possess a preservative to prevent microbial growth in the eye solution. They are packaged for multi-use and therefore have a potential risk of microbial contamination [3,4] during usage. Users of multi-dose eye drops are usually advised to discard after 28 days of opening. Thus, they are usually formulated to contain a preservative, which is commonly benzalkonium chloride, phenylmercuric nitrate or organic alcohols (chlorobutanol) etc. This is to inhibit microbial growth that could alter the pH of the solution thereby causing eye irritation, degradation of active

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constituent and eventual loss of activity and secondary infection from opportunistic organisms [5]. Previous research studies have shown a high incidence of fungal and bacterial contamination of in-use eye drops product both for out- and for in-patients during their use [6,7]. There is therefore the need to test the efficiency of the preservative by microbial challenge test to determine its ability to handle and reduce the inadvertent in-usage contamination after opening the seal of the eye drop [8].

Pyrogen testing (official method in United States Pharmacopoeia) is a quality control test based on the use of limulus amebocyte lysate test (LAL). It is a simple, sensitive and convenient *in vitro* assay designed to limit to an acceptable level the bacterial endotoxin present in sterile products especially parenteral preparations and surgical devices. The animal model of pyrogen testing (rabbit pyrogen test) officially recommended in the British Pharmacopoeia has some limitations such as difficulty in quantification of result and variability of biological systems. Generally quality assessment based on the limit test for bacterial endotoxin in sterile products is advantageous as it reveals the safety and the level of contamination by the cell- wall components of gram-negative bacteria which was not filtered off and therefore can induce life threatening fever [9]. Pyrogen testing therefore gives information on the efficiency of the membrane filtration used in the production and the presence / absence of airborne microbial and non-microbial pyrogen in the production environment.

Gentamicin is a broad spectrum aminoglycoside antibiotic with activity against both pathogenic Gram positive and -negative organisms though increase in resistance to its activity has been recorded in literature [10,11]. The chemical structure of gentamicin (Figure 1) shows the presence of three primary amines and other functional groups that enhances its solubility in water. The eye drops are usually marketed as a salt form with the active constituent - gentamicin sulphate 0.3% in 10 mL packs. It causes microbial cell death by inhibiting protein synthesis and/ or production of defective proteins. This is done by binding to the 30S subunit of the bacterial 70S ribosome [12]. Gentamicin eye drop is widely used, commonly abused and easily purchased as an over-the-counter drug for the treatment of most eye infections especially corneal ulcers in Nigeria. There is need therefore to conduct quality assessment on these eye products because poor quality eye product can be a source of potential danger to the user and due to the global distribution of the pharmaceutical industry, unsafe medicines can spread rapidly round the world without barrier [13,14]. The aim of this study was therefore to determine the sterility, antimicrobial effectiveness of the preservative and to limit the bacterial endotoxin content of nine brands of gentamicin eye drops sold in registered retail pharmacies in Port Harcourt, Rivers State.

EXPERIMENTAL

Procurement of eye drops. Nine different brands of gentamicin eye drops were purchased from different registered pharmacies in Port Harcourt, Rivers State. The nine different brands were labelled Genta A, Genta B, Genta C, Genta D, Genta E, Genta F, Genta G, Genta H, Genta I for the purpose of the study. The primary and secondary packages of the eye drops were carefully examined for proper packages and sealing. The product's active ingredient and percent content, manufacturing and expiry date, % preservative used, batch number, manufacturer's address and country of origin, NAFDAC (National Agency for Food and Drug Administration and Control) number were all noted.

Organoleptic examination. The eye drops were examined visually for particulate matter, colour and clarity against a visual inspection board with black and white background under sufficient illumination. Black particles are made visible using white background whereas any white particles are made visible against a black background.

Assessment of the pH of eye drops. The pH of the different brands of the eye drops were determined using a properly calibrated pH meter (pH Universal meter. PEC medical, USA). The calibration was done using standard buffer solutions of known pH (4 and 8). A volume of 10 mL of the particular eye drop was poured into a sterile 20 mL beaker and the sensitive bulb (probe) of the pH meter was dipped into it and this was allowed to stabilize for 20 seconds before taking the reading.

Sterility testing of the eye drops. This experiment was performed by direct inoculation method in a laminar air flow cabinet under an aseptic condition. This is to avoid accidental contamination of eye product during testing. Approximately, 1 mL of different brands of gentamicin eye drops was transferred aseptically into 20 mL of different media (fluid thioglycollate agar for anaerobic bacteria and Soyabean Casein Digest medium for aerobic bacteria) using a sterile pipette and then incubated at 37°C for 48 h. Approximately 1 mL of the same gentamicin eye drop was also transferred into Sabouraud Dextrose broth and incubated at 25 °C for 72 h for colony forming units of fungi. For positive controls: 20 mL of fluid thioglycollate media in a sterile universal bottle was inoculated aseptically with 0.1 mL of *Staphylococcus aureus* (adjusted using McFarland standard) to serve as a positive control for the anaerobic bacteria while 20 mL of Soyabean Casein Digest medium inoculated with 0.1 mL of *Pseudomonas aeruginosa* (adjusted using McFarland standard) served as positive control for aerobic bacteria. Finally, 20 mL of Sabouraud dextrose agar inoculated with 0.1 mL of *Candida albicans* (adjusted using McFarland standard) also served as positive control for fungi. These were done in triplicates.

Microbial challenge test. The preservative efficiency to support the gentamicin eye drop resist microbial contamination during in-use by patients was determined by challenging the eye drops with four different microorganisms comprising of three bacterial species and a fungi [15].

Preparation of the inoculum for the challenge test. The strains of the organisms (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*) were obtained from the culture collections maintained in Pharmaceutical Microbiology Laboratory, University of Port Harcourt. A loop full of each organism was sub-cultured into a sterile Peptone water contained in a sterile universal bottle. This was diluted further until it matches the McFarland standard (1×10^5 CFU/mL).

Inoculation of the eye drop with the organisms. With the use of a sterile syringe, 0.06 mL of the challenging microorganism prepared to McFarland standard was transferred into 6 mL of each eye drop contained in sterile universal bottle. This was mixed thoroughly to determine the rate of microbial kill. Each organism was tested separately and the inoculated products was maintained at 20 - 25 °C throughout the test period. At days 1, 7, 14, 21 and 28 post inoculation, 1.0 mL of the mixture was withdrawn and diluted into three 10 fold serial dilutions. The last dilution was then plated out in duplicate and spread on the nutrient agar for bacteria and Sabouraud dextrose agar for fungi. This was incubated at 37 °C for 18 – 24 h for bacteria and 20 - 25 °C for 7 days for fungi.

Pyrogen test. Commercial reagent Kit (GenScript ToxinSensor™. Chromogenic

LAL Endotoxin Assay Kits (32 r×ns) Cat. No. L00350C Lot No. C50091512) for the measurement of bacterial endotoxin concentration in samples was used without any modification. The different components of the reagent such as: lyophilized Limulus amebocyte lysate (LAL), chromogenic substrate, Stop solution, buffer S for colour stabilizer #1, colour stabilizer # 2 and colour stabilizer # 3 and standard endotoxin solution were all reconstituted using LAL reagent water according to the manufacturer's protocol in the user's manual.

Preparation of standard endotoxin solution for calibration curve. The lyophilized endotoxin standard was dissolved using 2 mL of LAL reagent water in endotoxin free vials, vortexed for 15 minutes and incubated at 37 ± 1 °C for 12 min in a water bath. A 0.1 mL of reconstituted Chromogenic substrate solution was added to each vial and swirled gently. The contents in the vials were incubated for 6 min at 37 ± 1 °C, then was added 0.5 mL aliquot of reconstituted stop solution (colour stabilizer # 1) and vortexed gently. To the mixture again, 0.5 mL of reconstituted colour stabilizer # 2 was added and mixed. Finally, 0.5 mL of reconstituted colour stabilizer # 3 was added, swirled gently for 3sec to mix well. To obtain the endotoxin stock solution for calibration, 1.0 EU/mL of the standard stock solution was further diluted to obtain: 0.5, 0.25, 0.125, and 0.05 EU/mL solutions. The absorbance reading of these concentrations were read using a UV/VIS spectrometer (Techmel & Techmel, USA) at 545nm wavelength. A standard calibration curve was obtained from a plot of absorbance against the corresponding concentration.

Test procedure for sample eye drops. The test procedure involves taking 0.1 mL of test samples of the eye drops and also preparing them according to the protocol stated above and their absorbance readings also taken at wavelength 545nm. The concentration of the bacterial endotoxin in the different gentamicin eye drops was obtained by extrapolation of the absorbance reading on the standard calibration curve taking note of the dilution factor.

RESULTS

Properties of the eye drops. All the products had a polyethylene containers built with a dropper and a closure that had an intact seal. The date of manufacture, expiry date, NAFDAC registration number, batch number and country of origin were clearly written on the package (Table 1). The eye drops were also within the expiry dates which ranged from 2- 5years. The volume of each eye drop was about 10.0 – 15.0 mL with an advice to use within a month after first opening.

The physicochemical properties of eye drops. The physical characteristics of the eye drops, which includes: clarity, particulate matter, pH and colour are shown in Table 2.

Sterility testing. The sterility testing for the different brands of eye drops cultured in three different nutrient media that support and encourage the growth of aerobic, anaerobic bacteria and fungi respectively (Table 3), shows no growth.

Microbial challenge test. The ability of the preservative incorporated into the eye drop formulation to resist contamination by microorganisms during usage was demonstrated by the preservative challenge test (Table 4). This sampling for surviving microbes occurred weekly over a 28-day period post inoculation to test the robustness of the preservative system.

Pyrogen testing using Limulus Amebocyte Lysate (LAL) protocol. The standard calibration curve (Figure 2) shows the equation of the straight line as $y = 0.1435x – 0.0141$ and the linear regression coefficient of 0.9816. This graph shows a direct linear relationship between concentration of standard bacterial endotoxin and absorbance

according to Beer- Lamberts law. The concentrations or the levels of bacterial endotoxin for each eye drop sample was calculated from this regression equation and displayed as bar chart (Figure 3).

DISCUSSION

Quality control tests on eye drops using pharmacopoeial standards ensures that proper regulation and harmonization of products are maintained.

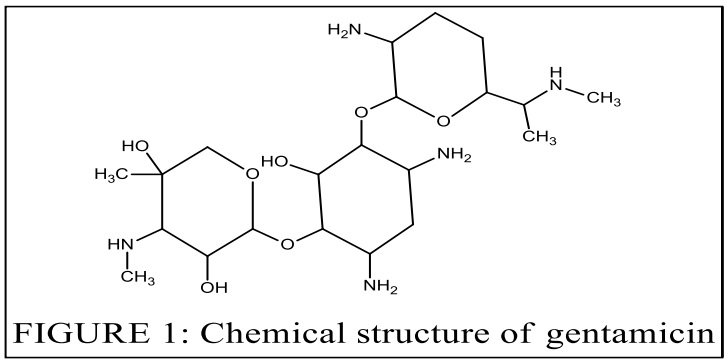

FIGURE 1: Chemical structure of gentamicin

Table 1. Some relevant information on the package of the different brands of gentamicin eye drops

Sample code/ batch	Preservative (% w/v)	Country of manufacture	Manufacture date	Expiry date	Batch number	NAFDAC* status	Volume (mL)
Genta A	Benzalkonium chloride (0.01)	India	10/2015	09/2018	Yes	Registered	10
Genta B	Benzalkonium chloride (0.01)	India	06/2015	05/2018	Yes	Registered	10
Genta C	Phenylmercuric nitrate (0.002)	India	07/2015	06/2018	Yes	Registered	10
Genta D	Benzalkonium chloride (0.01)	Nigeria	06/2013	05/2018	Yes	Registered	10
Genta E	Benzalkonium chloride (0.01)	India	09/2015	08/2017	Yes	Registered	10
Genta F	Benzalkonium chloride (0.01)	India	04/2014	03/2017	Yes	Registered	10
Genta G	Benzalkonium chloride (0.01)	India	10/2015	09/2018	Yes	Registered	10
Genta H	Benzalkonium chloride (0.01)	India	05/2014	04/2017	Yes	Registered	10
Genta I	Benzalkonium chloride (0.01)	India	06/2015	05/2018	Yes	Registered	10

NAFDAC* = National Agency for Food and Drug Administration and Control.

Table 2: Physicochemical properties of the eye drop samples

Sample Code	pH	Colour and Clarity
Genta A	6.95	Colorless and clear
Genta B	7.26	Colorless and clear
Genta C	6.80	Colorless and clear
Genta D	5.40	Colorless and clear
Genta E	7.13	Colorless and clear
Genta F	6.45	Colorless and clear
Genta G	5.40	Colorless and clear
Genta H	5.68	Colorless and clear
Genta I	7.01	Colorless and clear

Table 3: Evaluation of microbiological quality of different samples of freshly opened gentamicin 0.3% eye drops

Sample Code	Liquid thioglycollate medium	Soyabean casein digest medium	Sabouraud dextrose broth
Genta A	NG	NG	NG
Genta B	NG	NG	NG
Genta C	NG	NG	NG
Genta D	NG	NG	NG
Genta E	NG	NG	NG
Genta F	NG	NG	NG
Genta G	NG	NG	NG
Genta H	NG	NG	NG
Genta I	NG	NG	NG

NG = No colour change and no growth of organism in the medium

Table 4: Antimicrobial preservative efficacy for eye drops challenged with microorganisms

Sample code	Organism	Sampling time/viable load ($CFUmL^{-1}$)				
		Day1	Day 7	Day 14	Day 21	Day 28
Genta A	*E. coli*	117×10^3	88×10^3	32×10^3	0	0
	P. aeruginosa	40×10^3	20×10^3	10×10^3	0	0
	S. aureus	72×10^3	32×10^3	16×10^3	0	0
	C. albicans	80×10^3	62×10^3	22×10^3	0	0
Genta B	*E. coli*	52×10^3	67×10^3	44×10^3	0	0
	P. aeruginosa	60×10^3	32×10^3	15×10^3	0	0
	S. aureus	72×10^3	52×10^3	23×10^3	0	0
	C. albicans	61×10^3	40×10^3	23×10^3	0	0
Genta C	*E. coli*	40×10^3	20×10^3	16×10^3	0	0
	P. aeruginosa	48×10^3	22×10^3	6×10^3	0	0
	S. aureus	67×10^3	30×10^3	10×10^3	0	0
	C. albicans	98×10^3	70×10^3	22×10^3	0	0
Genta D	*E. coli*	120×10^3	64×10^3	12×10^3	0	0
	P. aeruginosa	100×10^3	80×10^3	30×10^3	0	0
	S. aureus	80×10^3	50×10^3	22×10^3	0	0
	C. albicans	63×10^3	32×10^3	15×10^3	0	0
Genta E	*E. coli*	114×10^3	90×10^3	40×10^3	0	0
	P. aeruginosa	98×10^3	70×10^3	30×10^3	0	0
	S. aureus	120×10^3	80×10^3	15×10^3	0	0
	C. albicans	87×10^3	60×10^3	28×10^3	0	0
Genta F	*E. coli*	117×10^3	80×10^3	40×10^3	22×10^3	22×10^3
	P. aeruginosa	90×10^3	50×10^3	30×10^3	20×10^3	20×10^3
	S. aureus	70×10^3	40×10^3	15×10^3	15×10^3	10×10^3
	C. albicans	135×10^3	90×10^3	50×10^3	38×10^3	35×10^3
Genta G	*E. coli*	98×10^3	50×10^3	38×10^3	0	0
	P. aeruginosa	92×10^3	70×10^3	28×10^3	0	0
	S. aureus	123×10^3	70×10^3	22×10^3	0	0
	C. albicans	114×10^3	80×10^3	40×10^3	0	0
Genta H	*E. coli*	83×10^3	60×10^3	15×10^3	0	0
	P. aeruginosa	55×10^3	35×10^3	10×10^3	0	0
	S. aureus	72×10^3	50×10^3	28×10^3	0	0
	C. albicans	90×10^3	40×10^3	20×10^3	0	0
Genta I	*E. coli*	77×10^3	58×10^3	18×10^3	0	0
	P. aeruginosa	63×10^3	60×10^3	20×10^3	0	0
	S. aureus	111×10^3	80×10^3	12×10^3	0	0
	C. albicans	88×10^3	78×10^3	28×10^3	0	0

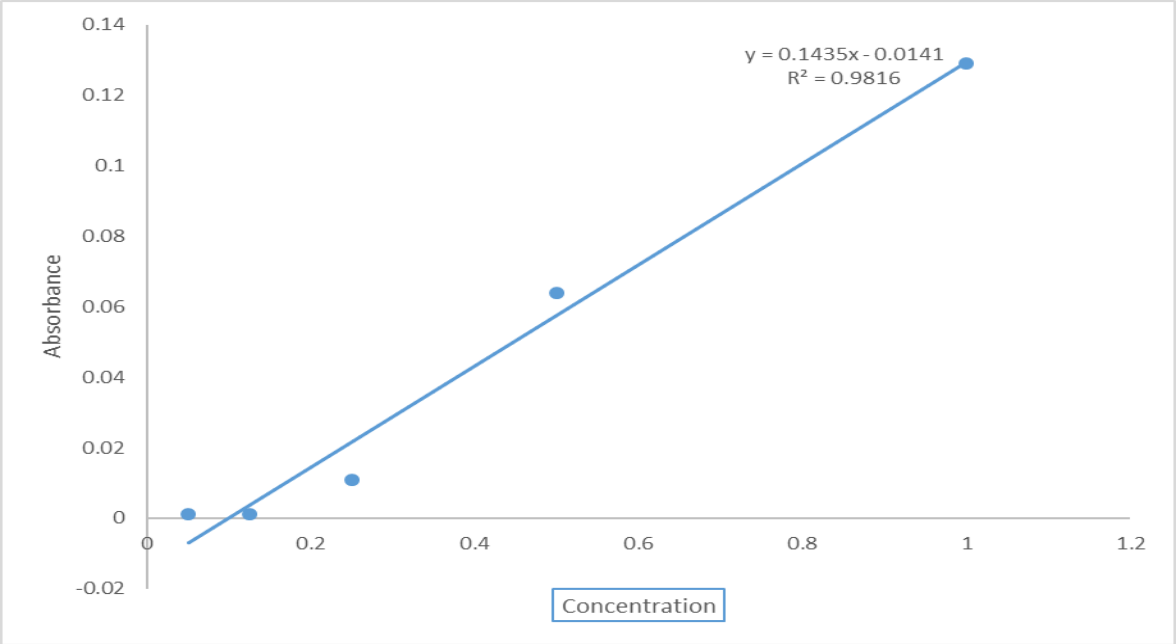

Figure 2. Standard calibration curve for the standard bacterial endotoxin

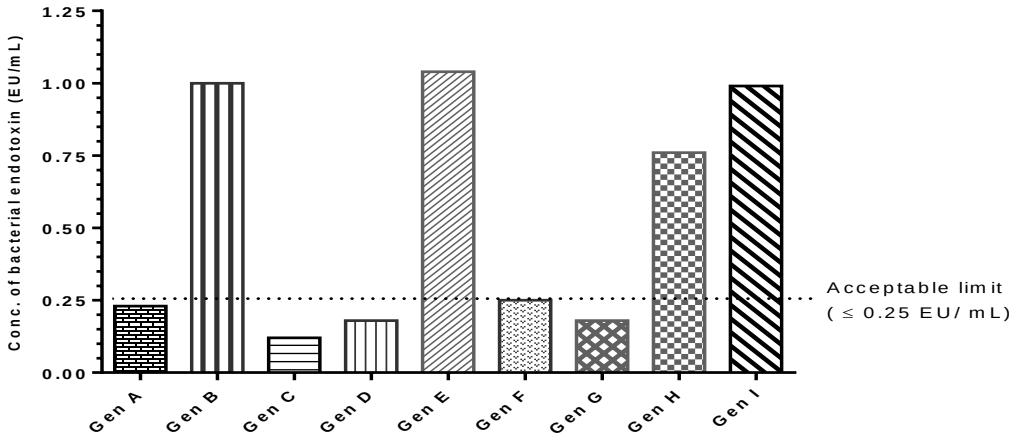

Figure 3. Bar charts representing the concentrations of bacterial endotoxin in the gentamicin eye drop samples

This work was therefore executed to investigate the physicochemical, sterility, preservative effectiveness and pyrogen testing on nine brands of gentamicin eye drops purchased from registered retail pharmacies in Port Harcourt. Eye drops are sterile preparations administered directly to the eyes, which is the most sensitive, delicate sensory organ responsible for vision. Though there is the problem of rapid tear wash out, limited penetration of drug and poor patient compliance with the topical applications, eye drops remain the most convenient, successful and non-invasive method of drug application to the eyes due to their local therapeutic effect [16]. The absorption of eye drops depends on the physicochemical properties of the drug in the eye drop such as molecular weight, viscosity, osmolality, hydrophilic/ lipophilicity, ionization/ unionization, pH, concentration and the presence of additives [17]. The aminoglycoside gentamicin is an

effective, safe, ophthalmic preparation widely used for conjunctivitis, keratitis and other superficial eye infections.

From the inspection of the packaging, all the eye products had package integrity though they were in multi-dose, plastic containers with a single application dropper. Proper wholesome packaging with intact closure and seals are important to avoid microbial contamination thereby making the eye drop a reservoir for further eye infections. The manufacturing and expiry dates of the products were disclosed on the packages indicating the shelf-life of product when the quality of the drug is assured. Only one of the nine brands of eye drops was manufactured in Nigeria while others were foreign but all of them had a NAFDAC registration number indicating proper registration by the country's regulatory body. It is important to note that though there is globalization of pharmaceutical industry [18], local industries in poor developing countries should be encouraged in manufacturing and local content to save foreign exchange and create more employment for their population. Also good quality, safe and effective medicines are made possible due to proper regulation by the National regulatory bodies like National Agency for Food and Drug Administration and Control in Nigeria (NAFDAC) [19] and will ensure public health and safety in the country.

The most common preservative in the gentamicin eye drops was benzalkonium chloride 0.01% w/v though one brand (Genta C) from India contained Phenyl mercuric nitrate 0.002% w/v. Though these preservatives hinder the growth of micro-organism in the eye drop thereby ensuring continued sterility and stability during storage and usage, the type of preservative and the concentration should be monitored due to possible toxicities that could limit their chronic use has been established in literature [15,20].

The pH of the tested brands of gentamicin eye drops (5.40 -7.13) were within the tolerable and acceptable pH range for eye formulations (physiological pH of tear fluid is 7.4). Extremities of pH in an eye drop causes stinging and irritation when applied to the eye directly because the tear fluid may not washout its irritant effect quickly and there may also be possible drug degradation due to altered pH of the eye drop solution [17]. The clarity, colour and absence of particulate matter in the eye drops could be attributed to an efficient membrane filtration process in the production that excludes every visible particulate matter.

The test for sterility showed no visible growth of any microorganism in all the products showing that they were sterile products and therefore devoid of microbial (bacteria and fungi) contamination and can therefore be declared safe for use.

The microbial challenge test is also known as preservative efficiency test because it is an indicator of the ability of the eye product to resist possible contamination by microorganism during use since the eye drop is multi-dose. The result of the challenge test (Table 4) showed that only one product (Genta F) which contained benzalkonium chloride 0.01% w/v failed the test. This suggests that the preservative in that particular brand of eye drop could not withstand microbial contamination resulting in turbidity indicating growth of microbes. This may be that the concentration of the preservative (benzalkonium chloride 0.01% w/v) in the eye drop could be less than the stipulated amount or there was loss of its antimicrobial activity due to binding with an additive or the container [21].

The pyrogen testing (Bacteria endotoxin test) using Limulus Amoebocyte Lysate (LAL) has been an official USP test since 1985 [19] and can be done using the end point chromogenic method or the turbidimetric kinetic method. Bacteria

endotoxins are contaminants from Gram-negative bacteria and are the most common cause of pyrogenicity in pharmaceutical products especially parenteral products. The basis of the test is by measuring colorimetrically a chromophoric substance produced during the LAL-endotoxin reaction [22]. This test has high sensitivity and specificity and the reagent kit is easy with ready-to-use reagents and materials. From our study (Figure 3), five of the products (Genta A, C, D, F, G) passed the test by having concentration within the acceptable limit permitted for sterile products (≤ 0.25 EU/mL) while products (Genta B, E, H, I) failed the test. The product Genta F, passed the sterility test, failed the preservative challenge test however passed the limit test for endotoxin (pyrogen test) while Products Genta B, E, H, I passed the sterility and preservative challenge test but failed the limit test for endotoxin. This means that sterility of a product does not automatically mean pyrogen-free solution. Thus after confirming sterility of products, the limit test for pyrogen should also be conducted to establish that the quantity of pyrogen-producing materials and organisms available in the product are not more than the acceptable limit permitted in the Pharmacopoeia.

Conclusion. Gentamicin eye drop is an antibiotic pharmaceutical preparation, which ought to be self-sterilizing, but from this study, a good preservative system is also required to maintain a robust, effective antimicrobial activity of the product during use. The compound used for preservation is of special interest as some of them have been banned due to ocular toxicity. Sterility of an eye product also does not mean exclusion of bacterial endotoxin, as endotoxins are thermostable substances. Eye drops should be properly regulated because substandard antibiotics can precipitate bacterial resistance and finally negative clinical outcome.

Conflict of interest. The authors declare that they have no conflict of interests.

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