Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

ISSN 2278-4136
JPP 2014; 3 (2):34-38
Received: 09-05-2014
Accepted: 17-05-2014

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Microbiological Quality Investigation of Eye and Ear Ointments Available in Bangladesh

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Abstract
With a previous observation of the microbial load in the ear and eye medicaments in sterile dosage forms, present investigation further incremented the microbiological condition along with anti-bacterial traits of ointment products used for the treatment of eye and ear infections. Fifteen (15) different types of eye and ear ointments manufactured in different pharmaceutical industries were assessed to demonstrate and quantify the microbiological propagation using cultural and biochemical methods. The *in vitro* anti-bacterial activity was also examined employing the agar well diffusion method. Almost all the samples were found to harbor bacteria while in 3 samples the bacterial bio-burden exceeded the United States Pharmacopeia (USP) or British Pharmacopeia (BP) limit ($<10^2$ cfu/g). Fungal proliferation was also observed in most of the samples. Among the specific bacterial pathogens, *Staphylococcus* spp. and *Bacillus* spp. were found to be dominant, *Escherichia coli* and *Klebsiella* spp. were detected in 6 samples; however, all of the samples were completely free from fecal coliforms. The study of antibiogram revealed that 13 samples possessed the anti-bacterial activity with variations while 2 were devoid of such attribute. As has been found earlier in the eye and ear drops, the ointment dosage forms in the current study also revealed a huge microbial contamination. Absence of anti-bacterial feature in 2 samples is also likely to lead to the product ineffectiveness against bacterial infections. Thus, routine microbiological validation of the finished products is of importance for the sake of public health management.

Keywords: Eye & ear ointments Microbiological quality Anti-bacterial activity Public health safety.

1. Introduction
The extent of microbiological contamination of the pharmaceutical drugs, especially those with liquid and semi-solid formulations are not unlikely due to several discrepancies in the good manufacturing practice (GMP), presence of microorganisms in the raw materials or in the manufacturing water, lack of microbiological monitoring of the equipments and manufacturing environment, packaging defect, personal unhygienic casualty, improper storage temperature and humidity, etc. [1-12]. Among the pharmaceutical drugs, including the oral and topical dosage forms, the presence of contaminating total viable bacteria exceeding the acceptable limit of $<10^2$ cfu/g especially in the eye and ear ointments , brings a major threat in public health measures [2-4, 8, 9, 11]. The contaminating microorganisms including *Clostridium tetani, Pseudomonas aeruginosa*, fungi, viruses, etc. may trigger the sustainable spoilage of the finished products with the loss of its therapeutic properties and hence serious infections are likely to arise during medication [3, 6].
The evidence of microbial contamination is reflected through the market complaints for a number of sold finished products [13]. In Bangladesh the onset of a number of diseases with the complications due to the microbiological spoilage of in different pharmaceutical drugs has also been noticed earlier [4, 10, 12, 18]. In the aspect of sterile liquid products used to treat eye and ear infections, huge bacterial and fungal bio-burden was observed as well as the presence of specific pathogenic bacteria has also been demonstrated [18]. Another important facet lies on the increasing instigation of drug-resistance properties of the contaminating bacteria which further necessitates the detection of the sustainability of the associated anti-bacterial activity of the finished products [18]. In the international perspective, these factors are of significance in the light of public health management since such problems may lead to the medication oriented complications not only locally but also in the communities in the other developing countries.
Along these lines, the present study (1) assessed the bacterial and fungal load of the topical products commonly used to eradicate eye and ear related complications, and (2) also attempted to examine the *in vitro* anti-bacterial activity of the samples microbiologically tested.

2. Materials and Methods

2.1 Sample Collection, Processing, and Microbiological Analysis

Fifteen (15) different eye and ear ointment samples labeled with manufacturing and expiry dates were collected from different retailer drug stores located within the city of Dhaka during November 2013 - March 2014, and were subjected to microbiological examinations; i.e., the total viable bacteria and fungi were quantified and the presence of specific pathogens was detected as well.

The microbiological bio-burden was demonstrated as described previously [4, 10, 12, 18]. Briefly, 10 ml of samples were homogenized with 90 ml of buffer peptone water (BPW) and serial dilutions were prepared up to 10^{-4}. An aliquot of 0.1 ml of each suspension from the 10^{-2} was spread onto Nutrient agar (NA) plate to enumerate the total bacteria (TVB) and on Sabouraud dextrose agar (SDA) plate for the estimation of fungal load. The NA and SDA plates were incubated at 37 ^{o}C for 24 hours and at 25 ^{o}C for 24 to 48 hours, respectively.

2.2 Detection of Specific Pathogenic Bacteria

An aliquot of 0.1 ml from the 10^{-2} dilution of each sample was spread onto Membrane fecal coliform (MFC), Mac Conkey agar, Mannitol salt agar (MSA), Pseudomonas agar, S-S (Salmonella-Shigella) agar and Mannitol egg yolk polymyxin (MYP) agar base media for the enumeration of total fecal coliform, *Escherichia coli*, *Staphylococcus* spp., *Pseudomonas* spp., *Salmonella* spp, *Shigella* spp., and *Bacillus* spp., consecutively [4, 10, 12, 18]. All the plates were incubated at 37 ^{o} C for 24 hours except MFC agar which was incubated at 45 ^{o} C for 18-24 hours. Confirmative identification of the specific pathogens was accomplished through the biochemical tests [19].

2.3 Evaluation of Anti-bacterial Activity

The anti-bacterial activity of the samples was tested as described earlier [18, 21]. All the samples were evaluated for anti-bacterial activity against the 8 laboratory isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus* spp., *Salmonella* spp., *Vibrio* spp., *Listeria* spp., *Staphylococcus* spp. and *Klebsiella* spp. in Muller Hinton Agar (MHA) plates [20, 21]. A fraction (100 µl) of the suspension of the test microorganisms (adjusted with the McFarland standard OD of around 0.5) was added on the plates with a sterile cotton swab and bacterial lawns were prepared [21]. Wells (8 mm^3) were prepared using a cork borer into which 0.1 ml (11µg/µl) of sample was poured aseptically [18]. Gentamicin10µg is used as the positive control. Presence of clear zone around the sample solution (if any) was indicative of the presence of antibacterial activity of the samples tested and the diameter of inhibition zone was recorded [21, 22].

3. Results and Discussion

Pharmaceutical sector is technologically the most developed manufacturing industry in Bangladesh and the third largest industry in terms of contribution to government's revenue [12]. Besides the in-process microbiological quality control during manufacturing, packaging, distribution and storage, the microbiological analysis both in terms of the examination products' microbial condition and of their effectiveness as anti-bacterial agent is of the essence to ensure the credibility of public health management as previously concluded by our earlier research on the pharmaceutical drugs in Bangladesh [4, 12, 14, 15, 18].

Table 1: Prevalence of Pathogenic Microorganisms in Eye & Ear Ointment

Sample No.	Samples	Total viable bacteria (TVB) (cfu/g)	Total fungal load (cfu/g)	Fecal coliform (cfu/g)	*Escherichia coli*	*Bacillus* spp.	*Klebsiella* spp.	*Salmonella & Shigella* spp.	*Pseudomonas* spp.	*Staphylococcus* spp.
01	Acyvir	1.3×10^2	1.0×10^1	0	-	+	-	-	-	+
02	Aristen	1.2×10^2	-	0	-	-	-	-	-	+
03	Atropine-OSL	2.2×10^4	1.2×10^2	0	-	-	-	-	-	+
04	Methasol-N	8.1×10^1	1.0×10^1	0	-	-	-	-	-	+
05	Pevitin	3.6×10^2	-	0	-	+	-	-	-	+
06	Terbifin	9.1×10^2	1.2×10^2	0	+	+	-	-	-	+
07	Aprocin	1.1×10^2	2.5×10^2	0	-	+	+	-	+	-
08	Dexagen	2.2×10^4	1.1×10^1	0	-	+	+	-	-	-
09	Cloram	1.4×10^2	-	0	-	-	-	-	-	+
10	Betnovate-N	4.7×10^4	-	0	-	-	-	-	-	+
11	Aristocort	2.4×10^2	2.2×10^2	0	-	+	+	-	+	+
12	Micosone	1.8×10^2	-	0	-	+	-	-	-	-
13	Polydex-N	6.0×10^2	4.8×10^2	0	-	-	-	-	+	-
14	Genacyn	1.9×10^2	5.1×10^2	0	+	-	+	-	+	+
15	T-Mycin	2.0×10^2	2.2×10^1	0	-	+	+	-	+	+

USP Limit:
Total viable bacteria $<10^2$cfu/g
Total fungal load $<10^1$ cfu/g.
+ Presence of bacteria
- Absence of bacteria

3.1 Prevalence of Microorganisms in Eye and Ear Ointments

In cohort with our earlier findings with the eye and ear drops, the ointment dosage forms in the current study also revealed a huge bacterial and fungal prevalence (Table 1). Out of 15 samples studied, 3 samples were found to harbor the total bacterial load exceeding the USP limit ($<10^2$ cfu/g). Rest 12 samples were also found to be populated with bacteria; however, the bio-burden was assessed to be within the acceptable limits recommended by USP or BP [2, 18]. Thus 80%

of the samples studied were found to be microbiologically controlled while the minor but significant fractions of the finished products revealed consumer risk upon usage.

Regarding fungal growth in the samples, all samples were found to be acceptable since the presence of fungi was within the recommended limit (Table 1). Among the specific pathogenic bacteria, the presence of *Staphylococcus* spp. were found to be present in 12 samples, *Bacillus* spp. were present in 10 samples, *Pseudomonas* spp. and *Klebsiella* spp. were present in 5 samples each, while *Escherichia coli* was detected only in 2 samples (Tables 1 & 2). Fecal coliforms, *Salmonella* spp. and *Shigella* spp. were completely absent in all the samples tested.

Table 2: Confirmative biochemical identification of the isolates.

Isolates	TSI				Motility	Indole production	MR test	VP test	Citrate utilization	Catalase test	Oxidase test	Confirmed bacterial isolate
	Slant	Butt	H_2S	Gas								
01	A	A	+	+	-	-	+	-	+	+	+	*Klebsiella*
02	K	A	-	-	-	-	-	+	+	+	+	*Pseudomonas* spp.
03	A	A	-	+	+	-	-	-	-	+	-	*Bacillus* spp.
04	K	A	-	-	-	-	-	+	-	+	-	*Staphylococcus aureus*
05	K	A	+	+	+	-	+	-	-	+	-	*E. coli*

A: Acidic reaction K: Alkaline reaction
MR= Methy Red VP: Voges-Proskauer
+ Positive - Negative

As has been stated earlier, the contaminated raw materials, manufacturing water, unhygienic handling together with a microbiologically uncontrolled production environment could be the main factors for the perceived microbial growths in the samples studied [4, 12, 18, 22-26]. The presence of *Staphylococcus* spp and *Bacillus* spp. in the samples indicates that the microbial quality of the products is not in line with the recommended standard [27-32].

3.2 *In Vitro* Anti-bacterial Activity of the Samples

Out of 15 drugs examined, 13 samples exhibited the anti-bacterial activity against the test bacteria (Table 3). Sample 10 was found to possess the anti-bacterial activity against almost all the test bacteria. Samples 4, 7 and 9 were also found to possess significant activities against test bacteria; whereas samples 1, 4-6, 13-15 were found to exhibit moderate activities. Samples 2, 3 and 11 were found to show very weak anti-bacterial activities while samples 8 and 12 were completely devoid of such attribute.

Table 3: Anti-bacterial activity of the Eye & Ear Ointments studied through the assessment zone of inhibition (mm^3) formed by test bacteria.

Test bacterial strains	Samples														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Acyvir	Aristen	Atropine-OSL	Methasol-N	Pevitin	Terbifin	Aprocin	Dexagen	Cloram	Betnovate-N	Aristocort	Micosone	Polydex-N	Genacyn	T-Mycin
1	0	0	0	12.3	0	16.7	36.2	0	17.4	11.8	0	0	0	11.0	0
2	10.4	0	0	18.8	12.2	9.6	22.6	0	17.8	13.4	0	0	7.7	0	0
3	0	0	0	11.5	0	0	14.3	0	12.9	20.6	0	0	18.7	20.3	22.8
4	10.8	0	13.0	20.1	18.3	8.6	17.5	0	10.3	19.5	14.5	0	17.1	22.1	19.1
5	0	0	0	0	0	0	0	0	0	10.4	0	0	0	0	0
6	8.2	11.2	0	16.3	11.6	24.7	20.8	0	9.2	17	13.1	0	0	0	0
7	0	0	0	0	0	0	28.3	0	23.2	6.0	0	0	0	0	0
8	0	0	0	14.4	0	0	16.6	0	7.7	12.3	0	0	9.1	7.9	7.3

Strain 1: *Escherichia coli* Strain 5: *Klebsiella* spp.
Strain 2: *Bacillus* spp. Strain 6: *Salmonella* spp.
Strain 3: *Staphylococcus* spp. Strain 7: *Vibrio* spp.
Strain 4: *Pseudomonas* spp. Strain 8: *Listeria* spp.

As the positive control, gentamicin 10 µg was used.

According to the results indicated in the anti-bacterial activity perspective, there is not a clear correlation between the bactericidal efficacy testing of eye and ear ointments and the abundance of their microbiological contamination level (Tables 1 and 3). Such discrepancy could be due to the in vitro study approach where microbial proliferation in the samples is measured apart from their anti-bacterial activity assessment.

4. Conclusion

Overall, our study revealed the presence of microorganisms with the harmful ones in more than 20% samples. The anti-bacterial activity of the drug samples was weak or even nil in around 35% samples. Such a condition is not that satisfactory for effective medication of eye and ear infections, which is a common clinical problem in the Bangladeshi community. Regular microbiological examination with legislative measures would be effective for the better management of public health and user safety.

5. Acknowledgements

We thank Stamford University Bangladesh, for providing us with the facilities for the experiments described here and also grateful for the ongoing support, interest, and technical expertise of the faculties in Department of Microbiology, Stamford University Bangladesh.

6. Conflict of Interest

Authors have no potential conflict of interests.

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