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Research Article

Microbial Evaluation of Some Non-sterile Pharmaceutical Preparations Commonly Used in the Egyptian Market

Gamal Fadl M Gad[1], Reham A Ibrahem Aly[1*] and Mohamed S El-din Ashour[2]

[1]Microbiology Department, Faculty of Pharmacy, Minia University, Minia, [2]Microbiology Department, Faculty of Pharmacy, Modern Science and Arts University, Cairo, Egypt

Abstract

Purpose: *To determine the type and incidence of predominant microorganisms in certain non-sterile pharmaceuticals immediately after collection and one year later.*
Methods: *All pharmaceutical samples were subjected to the following examinations: total bacterial count and presence of microbial pathogens, using conventional techniques. Attempts were also made to identify the isolates. The bioburden rate of some of the syrups and oral drops after storage for 0, 6 and 12 months were evaluated in order to assess the effect of storage on microbial contamination level.*
Results: *Microbial load varied among the pharmaceutical preparations with the highest microbial load in suspensions and the lowest in tablets. Bacterial counts ranged from 10 to more than 10^3 CFU per ml or g. The bacterial count at 6 and 12 months were significantly different from that at 0 month ($p < 0.05$). The isolated organisms were either of human flora types, essentially Gram-positive bacteria, or air-borne fungi.*
Conclusion: *The isolated organisms were either of human flora types, principally, Gram-positive bacteria, or air-borne fungi and the stored preparations lack an effective preservation. Several measures, including equipment automation, monitoring programs and post-marketing surveillanceare required to reduce the level of microbial contamination of non-sterile pharmaceutical products.*

Keywords: *Microbial contamination, Bacteria, Fungi, Non-sterile pharmaceuticals*

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Corresponding author:* ***E-mail:* *Rehamee_micro@yahoo.com;* ***Tel:*** *+020106804684*

INTRODUCTION

The use of contaminated pharmaceutical preparations has proved hazardous to the health of the users. There have been reports of drug-borne human infections worldwide [1]. Contamination of pharmaceuticals with microorganisms can also bring about changes in their physical characteristics, including breaking of emulsions, thinning of creams, fermentation of syrups, appearance of turbidity or deposit, and changes in odor and color [2].

The incidence of microflora in non-sterile preparations generally is influenced by the nature of the ingredients (whether natural or synthetic), the quality of the vehicle and the care and attitude of personnel involved in their handling [3]. An antimicrobial (preservative) may be included in a formulation to minimize the risk of spoilage and preferably to kill low levels of contaminants introduced during storage or repeated use of multi-dose preparations, but should never be added to mask poor manufacturing processes [4].

Due to the increasing number of immuno-compromised patients, increased attention is paid to the identification and quantitation of microorganisms in oral pharmaceutical products. Therefore, a systematic approach is required by manufacturers of non-sterile oral pharmaceuticals to evaluate the significance of microbial isolates other than primary pathogens and/or those in product monographs taking into account the number of organisms present, the type of dosage form, and the potential hazard to the user. Limits for objectionable microorganisms in oral products intended for use by immuno-compromised patient populations, such as children and cancer sufferers, should be more stringent than the limits for oral products intended for treating patients with diseases or conditions that do not affect the immune system because patients with deficient immune systems are more at risk of microbial infections. Smaller numbers of opportunistic pathogens become infectious when resistance mechanisms are impaired, either by severe underlying disease, or by use of immunosuppressive drugs [5].

Microbial contamination of non-sterile pharmaceuticals may be controlled by (a) enforcement and upgrading of GMP rules; (b) manipulating physicochemical factors that affect the fate of contaminants; and (c) incorporating a preservative in the pharmaceutical formulation not for the purpose of masking bad manufacturing practice but to ensure that the product remains satisfactory [6]. The objective of this study was to evaluate the type and incidence of microbial contamination of some oral pharmaceutical products in the Egyptian market.

EXPERIMENTAL

Materials

A total number of 300 non-sterile pharmaceutical samples were tested. The samples comprised of 120 syrups, 20 suspensions, 60 oral drops, 80 tablets and 20 nasal drops, and included locally manufactured and imported products. Samples were randomly purchased from 20 private community pharmacies in Egypt. The average number of samples per pharmacy was approximately 15. Some were imported and others were manufactured). In addition, 30 of the preparations (15 each of oral drops and syrups) were tested for storage effect on the level of contamination.

The media used for the microbiological analysis include: casein soya bean digest agar, nutrient broth, nutrient agar, MacConkey agar, mannitol salt agar, blood agar, thioglycollate medium and ***Sabouraud*** dextrose agar. They were manufactured by either Oxoid (Cambridge, UK), Difco (USA) or Britannia (Buenos Aires, Argentina). The media were prepared according to the manufacturers' instructions.

Test sample preparation

For tablets, five units were dispersed in 10 ml of sterile normal saline. The dispersion was mixed in a vortex mixer for 5 min to dislodge possible microbial cells. The solid particles were allowed to sediment and the supernatant was used for microbial test [7].

For liquid samples (syrups, suspensions, oral and nasal drops), 10 ml of the product examined was diluted in sterile buffered solution with the following composition: peptone (1.0 g/L), potassium dihydrogen phosphate (3.7 g/L) , disodium hydrogen phosphate (7.2 g/L), and sodium chloride (4.3 g/L) . Generally, ten-fold dilutions were prepared as described in British Pharmacopoeia [8].

Total viable aerobic bacterial count

Viable aerobic mesophilic bacterial count was evaluated using pour plate method [8]. In this method, 1 ml of the sample was added to 20 ml of the liquefied casein soya bean digest agar at about 45 ^{0}C in a Petri dish. At least 2 Petri dish for each level of dilution were used. The plates were incubated at 30 – 35 ^{0}C for 5 days, unless a reliable count was obtained in a shorter time. Suitable dilutions yielding < 300 colonies were counted. The arithmetic mean of the counts was taken and number of colony forming units per gram or milliliter (cfu/g or mL) was calculated.

Detection, isolation and identification of potential aerobic bacteria:

An aliquot (1 ml) of the supernatant (for tablets) or 1 ml of each liquid dosage product was spread on nutrient agar, blood agar, MacConkey agar and mannitol salt agar plates. All plates were incubated at 37 ^{0}C for 24 h. The colonies produced were examined morphologically, microscopically and biochemically. Morphological identification was based on size, diameter, elevation, translucency, color, etc of the colonies formed while microscopical identification was achieved by spreading the bacteria on a microscope slide and examined under a microscope after Gram staining to identify Gram- and –negative bacteria. For biochemical identification, a number of biochemical tests including carbohydrate utilization, catalase production, oxidase production, tests, methyl red, Voges-Proskauer, nitrate reduction, starch hydrolysis, tryptophan hydrolysis, hydrogen sulfide production, and citrate utilization were carried out.

Detection, isolation and identification of *Clostridium* species

Five grams (for tablets) or 5 ml (for liquids) of each of the samples sample were mixed with 50 ml of thioglycollate medium, heated at 80 ^{0}C for one minute, and then incubated at 37 ^{0}C for 48 h in a carbon dioxide incubator (model Nu-4500/E, NuAire Inc, USA). The resulting growth (if any) was sub-cultured on the surface of reinforced clostridial agar medium plates.

Detection, isolation and identification of fungi

One milliliter of the test supernatant (for tablets) or of the test liquid product was spread on ***Sabouraud*** agar plates. The plates were incubated at 25 – 27 ℃ for 72 – 96 h and fungal growth were examined both macroscopically and microscopically.

Effect of storage on microbial load

Microbial contamination of 30 of the sample preparations (15 syrups and oral drops each) were evaluated for microbial contamination (as described above) over a storage period of one year at room temperature. Samples were withdrawn from the products at 0, 6 and 12 months, and their total aerobic bacterial count and identification of contaminants were made.

Statistical analysis

In order to assess the statistical difference between bacterial counts for the storage test, Spearman test for correlation coefficient was carried out using SPSS software (version 10). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Microbial contents of non-sterile preparations

All the samples tested were free from anaerobic bacteria, *coliforms* and *Pseudomonas aeurginosa*. Bacterial counts in the samples, shown in Table 1, indicate that only syrups and suspensions had counts greater than 1000 CFU/ml. The other dosage forms did not show counts in this range.

Table 2 shows the microbial species isolated from the non-sterile pharmaceutical products. Again, syrups and suspensions, and also oral drops, showed the highest number of microbial isolates while tablets and nasal drops showed the lowest.

Effect of storage on microbial load

All the preparations showed growth after storage except one oral drop product**.** The bacterial count of the products increased after storage for 6 and 12 months, respectively. For most of the preparations, the number of isolated contaminants increased with duration of storage. It was observed that preparations with high nutritive

Table 1: Total bacterial count (CFU/ml or g) in the different dosage forms

Dosage form (quantity)	< 100 CFU/ml or g		100 - 1000 CFU/ml or g		> 1000 CFU/ml or g	
	n	%	*n*	%	*n*	%
Syrup (120)	42	35.0	14	11.7	7	5.9
Suspension (20)	3	15.0	5	25.0	2	10.0
Oral drops (60)	20	33.3	5	8.3	0	0
Tablet (80)	4	20.0	2	10.0	0	0
Nasal drops (20)	15	18.8	1	1.3	0	0

Table 2: Microorganisms isolated from different non-sterile dosage forms

Microbial species	Syrup		Suspension		Oral drop		Nasal drop		Tablet	
	no.	%	no.	%	no.	%	no.	%	no.	%
Staphylococcus epidermidis	33	27.5	7	35	13	21.7	3	15	8	10.0
Staphylococcus aureus	31	25.8	1	5	10	16.7	2	10	5	6.3
Bacillus subtilis	10	8.3	1	10	6	10.0	1	5	3	3.6
Aspergillus niger	48	40.0	6	35	20	33.3	4	20	11	13.6
Aspergillus fumigatus	20	16.7	3	15	11	18.3	1	5	7	8.6
Aspergillus flavus	16	13.3	3	15	5	8.3	0	0	3	3.5
Penicillium sp.	11	9.2	0	0	3	5.0	1	5	4	5.0
Rhizopus	7	5.8	0	0	2	3.3	0	0	0	0
Cladosporium	3	2.5	0	0	1	1.7	0	0	0	0
Alternaria	6	5.0	1	5	0	0	0	0	0	0

Note: *No. and % represent the number and percent of samples of the dosage form contaminated with the microbe*

ingredients (oral drops and syrups) showed not only the highest increase in bacterial count and also produced fungal species during the storage period.

Table 3: Effect of shelf storage (at room temperature) on microbial profile of syrup samples

Parameter	**At Month 0**	**At Month 6**	**At Month 12**	***P* value**
Bacterial count	-	2 × 10	8 × 10	**< 0.05**
Isolated bacteria	-	*S. aureus*	*S. aureus*	
Isolated fungi	*A. flavus*	*A. flavus*	*A. flavus*	
Bacterial count	2 ×10	8.1 ×10	2.5×10^2	**< 0.05**
Isolated bacteria	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	
Isolated fungi	-	*A. flavus*	*A. niger* *A. flavus*	
Bacterial count	-	-	3 ×10	**0.33**
Isolated bacteria	-	-	*S. aureus*	
Isolated fungi	-	-	*A. niger*	
Bacterial count	1 ×10	4 ×10	1×10^2	**< 0.05**
Isolated bacteria	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	
Isolated fungi	*A. fumigatus*	*A. niger* s *A. fumigatu*	*A. niger* *A. fumigatus*	
Bacterial count	1×10^3	5×10^3	1.2×10^4	**< 0.05**
Isolated bacteria	*S. epidermidis* *B. subtilis*	*S. epidermidis* *B. subtilis*	*S. epidermidis* *B. subtilis*	
Isolated fungi	*A. niger* *A. fumigatus*	*A. niger* *A. fumigatus* *A. flavus*	*A. niger* *A. fumigatus* *A. flavus*	
Bacterial count	6 × 10	9 × 10	2×10^2	**< 0.05**
Isolated bacteria	*S. aureus*	*S. aureus*	*S. aureus*	
Isolated fungi	*A. niger* *A. flavus*	*A. niger* *A. flavus*	*A. niger* *A. flavus*	
Bacterial count	8 × 10	3×10^2	9×10^2	**< 0.05**
Isolated bacteria	*S. aureus*	*S. aureus*	*S. aureus*	
Isolated fungi	*A. fumigatus* *Penicillium sp.*	*A. fumigatus* *Penicillium sp*	*A. fumigatus* *Penicillium sp*	
Bacterial count	-	-	-	**-**
Isolated bacteria	-	-	-	
Isolated fungi	*A. niger* *A. flavus*	*A. niger* *A. flavus*	*A. niger* *A. flavus* *Alternaria*	
Bacterial count	-	6 × 10	2×10^2	**< 0.05**
Isolated bacteria	-	*S. aureus*	*S. aureus*	
Isolated fungi	-	-	*A. fumigatus*	
Bacterial count	2 × 10	-	-	**0.333**
Isolated bacteria	*S. aureus*	-	-	
Isolated fungi	*A. niger* *A. flavus*	*A. niger* *A. flavus* *A. fumigatus*	*A. niger* *A. flavus* *A. fumigatus*	

Table 3 *(contd)*: Effect of shelf storage (at room temperature) on microbial content of syrup samples

Parameter	At Month 0	At Month 6	At Month 12	*P* value
Bacterial count	9 × 10	4×10^2	1×10^3	
Isolated bacteria	*S. epidermidis*	*S. epidermidis* *S. aureus*	*S. epidermidis* *S. aureus*	**< 0.05**
Isolated fungi	*Penicillium sp.*	*A. niger* *Penicillium sp.*	*A. niger* *Penicillium sp.*	
Bacterial count	4×10^2	8×10^2	2.2×10^3	
Isolated bacteria	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	
Isolated fungi	*A. niger*	*A. niger* *Penicillium sp*	*A. niger* *A. fumigatus* *Penicillium sp*	**< 0.05**
Bacterial count	5 × 10	1×10^2	6×10^2	
Isolated bacteria	*S. aureus*	*S. aureus*	*S. aureus*	
Isolated fungi	-	*A. niger* *A. flavus*	*A. niger* *A. flavus* *A. fumigatus*	**< 0.05**
Bacterial count	-	5 × 10	8 × 10	
Isolated bacteria	-	*S. aureus*	*S. aureus*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger* *A. fumigatus*	*A. niger* *A. fumigatus*	
Bacterial count	-	2×10^2	8×10^2	
Isolated bacteria	-	*S. epidermidis*	*S. epidermidis*	**< 0.05**
Isolated fungi	-	*A. niger*	*A. niger*	

Table 4: Effect of shelf storage (at room temperature) on microbial profile of oral drop samples

Parameter	At Month 0	At Month6	At Month 12	*P* value
Bacterial count	-	3 × 10	-	
Isolated bacteria	-	*S. aureus*	-	**1**
Isolated fungi	*A. niger*	*A. niger*	*A. niger* *A. flavus*	
Bacterial count	-	2 × 10	2×10^2	
Isolated bacteria	-	*S. epidermidis*	*S. epidermidis*	**< 0.05**
Isolated fungi	*A. fumigatus*	*A. niger* *A. fumigatus*	*A. niger* *A. fumigatus*	
Bacterial count	-	4 × 10	4×10^2	
Isolated bacteria	-	*S. epidermidis*	*S. epidermidis*	**< 0.05**
Isolated fungi	*A. niger* *A. flavus*	*A. niger* *A. flavus*	*A. niger* *A. flavus*	
Bacterial count	1 ×10	7× 10	4×10^2	
Isolated bacteria	*B. subtilis*	*B. subtilis*	*S. epidermidis* *B. subtilis*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger*	*A. niger*	

Table 4 *(contd)*: Effect of shelf storage (at room temperature) on microbial profile of oral drop samples

Parameter	At Month 0	At Month 6	At Month 12	*P* value
Bacterial count	-	-	3 × 10	
Isolated bacteria	-	-	*S. epidermidis* *B. subtilis*	**0.33**
Isolated fungi	-	-	*A. niger*	
Bacterial count	-	1 ×10	9 ×10	
Isolated bacteria	-	*B. subtilis*	*B. subtilis*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger* *A. fumigatus*	*A. niger* *A. fumigatus*	
Bacterial count	1 ×10	4×10^2	3×10^3	
Isolated bacteria	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger* *A. flavus*	*A. niger* *A. flavus*	
Bacterial count	1×10^2	4.7×10^2	8×10^2	
Isolated bacteria	*S. aureus*	*S. aureus*	*S. aureus*	**< 0.05**
Isolated fungi	-	-	*A. niger*	
Bacterial count	-	1×10^2	9×10^2	
Isolated bacteria	-	*S. epidermidis*	*S. epidermidis*	
Isolated fungi	*Rhizopus*	*A. niger* *A. flavus* *Rhizopus*	*A. niger* *A. flavus* *A. fumigatus* *Rhizopus*	**< 0.05**
Bacterial count	4 × 10	9 × 10	5×10^2	
Isolated bacteria	*S. aureus*	*S. aureus*	*S. aureus*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger* *A. flavus*	*A. niger* *A. flavus*	
Bacterial count	-	-	3 × 10	
Isolated bacteria	-	-	*B. subtilis*	
Isolated fungi	*A. niger*	*A. niger* *A. fumigatus* *A. flavus*	*A. niger* *A. fumigatus* *A. flavus*	**< 0.05**
Bacterial count	2×10^2	6×10^2	1.2×10^3	
Isolated bacteria	*S. epidermidis*	*S. epidermidis*	*S. epidermidis*	**< 0.05**
Isolated fungi	*A. niger*	*A. niger* *Rhizopus*	*A. niger* *Rhizopus*	
Bacterial count	-	-	-	
Isolated bacteria	-	-	-	**-**
Isolated fungi	-	*A. niger*	*A. niger* *Alternaria*	
Bacterial count	-	-	-	
Isolated bacteria	-	-	-	**-**
Isolated fungi	*A. niger* *A. flavus*	*A. niger* *A. flavus*	*A. niger* *A. flavus*	
Bacterial count	-	-	2 ×10	
Isolated bacteria	-	-	*S. aureus*	**0.33**
Isolated fungi	-	-	*A. fumigatus*	

DISCUSSION

The number of isolated microorganisms in this study is smaller than that reported earlier by other authors [9]. This may be to the introduction of better adherence to 'Good Manufacturing Practices' by pharmaceutical manufacturers in recent years. Some of the preparations were contaminated by *Staphylococcus* species, suggesting contamination from the equipment and/or raw material, or poor hygiene of the factory hands during production [10].

The proportion of the products containing viable aerobic microbial count (> 1000 CFU per ml or g) was small (5.8 % in syrups and 10 % in suspensions) which indicates that the microbiological quality of the examined products was, in general, adequate and, in most cases, excellent. On the other hand, the presence of some molds reflects the storage quality of the preparations. The presence of certain molds is harmful since they produce metabolites that may be toxic to consumers [10] and cause rapid deterioration of the product due to the biodegradation of the different components of formulations arising from the production of toxins, such as *Aspergillus flavus* and *A. parasiticus* [11].
In a related work, Okunlola *et al.* [12] investigated the microbial characteristics of 21 different herbal medicinal products of various dosage forms which were sourced from some traditional medicine sales outlets as well as retail pharmacy outlets in southwestern Nigeria. The aerobic bacterial count of the products ranged from 5.0×10^2 to 2.2×10^4 but their microbial load varied considerably. Ten (47.6 %) of the samples were contaminated by *E. coli*, 7 (33 % by *Salmonella,* 15 (71.4 %) by *Staphylococcus aureus* and 12 (57.1 %) by fungi. The values are considerably higher than those found in the present study, probably due to the fact that in contrast to our study, natural ingredients, which are likely to be more contaminated, were mainly used.

The presence of *Staphylococcus aureus* in the oral preparations may not necessarily constitute a potential hazard to users since not all strains of *S. aureus* produce the enterotoxin that causes poisoning and, in any case, the organism would have to grow to a density of several million cells/g for its toxin to constitute a problem [13]. *B. subtilis* has occasionally been implicated as a causative agent of food-poisoning. The infective doses, in this case, is estimated to be in the range $10^5 – 10^8$ CFU/g, which is far higher than the concentration of endospore-forming isolates found in any of the oral pharmaceuticals examined in this study [14].

In the present study, contamination rate was lower in tablets than the other dosage forms. This may be attributed to the lower water activity of tablets which is usually < 0.60) [15]. Physical preservation of syrups is facilitated by reduced water activity arising due to the addition of sugar [4]. Low water activity values normally inhibit the growth of bacteria such as members of the family, Enterobacteriaceae, as well as aerobic and anaerobic spore formers, but allow the growth of certain vegetative microorganisms, such as *staphylococci* and *micrococci*, especially *S. aureus* which grow below a water activity of 0.86 [6] . Hence *Staphylyloccoci* species survived and grew during the period of storage in this study.

Preservative effectiveness might have been modulated by changes in microbial cell envelope and glycocalyx; furthermore, the presence of slime layer under conditions of limited nutrition may promote microclonization and formation of biofilm with significant resistance to antimicrobial agents [16]. Also, preservative availability may be reduced by interaction with packaging material, (e.g., quaternary ammonium preservative levels are reduced by adsorption on plastic or glass containers) or by volatilization during opening and closing of containers; hence, preserved medicines should be packed in sealed, impervious containers during storage [4].

CONCLUSION

The isolated organisms were either of human flora types, essentially Gram-positive bacteria, or air-borne fungi. Furthermore, the stored preparations lacked effective preservation. Using production systems in which personnel are removed from critical zones, e.g., equipment automation, should improve the microbial quality of pharmaceuticals. Microbial monitoring programs are necessary to estimate the bioburden of the environment. Post-marketing surveillance should be put in place to detect any problem concerned with product instability, degradation, toxin production, etc.

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