Microbiological quality control of some non-sterile preparations commonly used in Pakistan

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Abstract: The quality of medicines in some developing countries including Pakistan is not very satisfactory regarding safety and efficacy. In addition to sterile preparations, the microbial contamination of non sterile preparations should also be monitored according to USP microbial limits for these preparations. This study was designed to check the microbial quality of some commonly used non-sterile preparations available in Pakistan. Total 133 samples containing national and multinational brands of different dosage forms were collected from retail setups of Sargodha, Khushab and Chakwal cities. The total aerobic bacterial count and fungal contamination was tested by pour plate method. The absence of objectionable microorganisms was confirmed by using selective media, biochemical testing and microscopy. Bioburden of these preparations was also tested after a storage period of six months. The bio burden varied among all the selected non-sterile preparations whether of local or multinational brands. The highest load was observed in syrups, among which syrup number 1 showed maximum aerobic count (8.4×10^6). Lowest count was observed in tablets, among which tablet preparation number 1 contained 1.5×10^3aerobic bacteria. Creams and capsules produced no recovered bacteria. The fungal contaminants were also observed in all dosage forms except tablets. The isolated organisms included Gram-negative bacteria which contained objectionable ones such as *Salmonella*, *Shigella*, *Pseudomonas* and *E.coli* and some airborne moulds including *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp. and *Acremonium* spp. Several measures such as GMPs, monitoring programs and SOPs should be followed by the pharmaceutical companies to reduce the microbial contamination level in the non sterile preparations. The regulatory agencies have to implement strict analysis strategy to check the microbial quality of the medicines before their release for sale in the market.

Keywords: Non sterile pharmaceutical formulations, bioburden, total aerobic count, fungal contaminant.

INTRODUCTION

Drugs such as antimicrobials, anti-inflammatory, cough preparations and multivitamins etc are mostly used to treat the ailments but if these are contaminated, then they can further worse the patient condition (Bashir *et al.*, 2013).The use of these contaminated non-sterile preparations can cause hazards in majority of ways as they can cause the economic loss to the industrialists, alter the therapeutic effect of the drug and affect the health of the critically ill patients especially old and infants whom immunity is already compromised (Stephen and Rosamund, 2007).

The microbes attacks the formulation ingredients such as active ingredients, polymers, surfactants, oils, sweetening agents, flavoring agents, coloring agents and preservatives (Denyer *et al.*, 2004). They causes the change in the physical characteristics such as turbidity of liquid formulations for example syrups and solutions, cracking of emulsions, thinning consistency of creams and change in color and texture of tablets and powders (Kamil and Lupliasa, 2011). The source of contamination may be from starting materials and excipients, water used during manufacturing, operational equipment, untidy surrounding environment and through workers (Denyer *et al.*, 2007). The USP provides acceptance criteria for the non-sterile preparations. According to these criteria oral aqueous preparations should not contain more than 10^2 TAMC (total aerobic microbial count) and not more than 10^1 TYMC (total yeast and mold count) per mL (USP, 2013).

MATERIALS AND METHODS

Sample collection

A total of 133 different samples were collected from various retail pharmacies from the 3 different cities of Pakistan i.e. Sargodha, Khushab and Chakwal. These included 50, 50, 12, 12 and 9 different preparations each of tablets, capsules, syrups, suspensions and creams, respectively. These samples included local and multinational brands.

The media used for the microbial analysis included Nutrient agar, Sabouraud dextrose agar, Mannitol salt agar, *Salmonella Shigella* agar, *Pseudomonas* cetrimide agar and Eosin methylene blue agar (Oxoid Hampshire, England). The media were prepared according to manufacturer specifications.

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Chemicals

Hydrogen peroxide (Merck Pvt. Ltd. Pakistan), Kovac's reagent (BDH laboratory supplies, England) and Oxidase reagent (Oxoid Basingstoke, Hampshire, England) were purchased. All the chemicals used were of analytical grade.

Sample dispersion and dilution

All the samples were diluted in the sterile water (Obi and Nwannunu, 2010). Five units of tablets and capsules (should be 10gm each), 10mL of syrups and suspensions and 10g of cream samples were added into the sterile water, and their dispersion was prepared by vortex mixer. Further dilutions were prepared from these dispersions by serial dilution method (Obi and Nwannunu, 2010; Gad et *al.,* 2011; Rana *et al.,* 2014).

$$\text{Count} \frac{\left(\frac{\text{CFU}}{\text{mL}} \text{or} \frac{\text{CFU}}{\text{g}}\right)}{\text{Dilution factor} \times \text{Volume of sample}} \text{number of colonies in plate}$$

Viable bacterial count

From each dilution of every dosage form 1mL of the supernatant was taken and plates were prepared by using pour plate method. After solidification the plates were placed in an incubator at 37^{o}C for two to three days. After the desired period the plates were observed for any visible growth and the colonies were counted by the following formula: (Yousef and Carlstrom, 2005). Water for injection was used as negative control.

The morphology of the growth colonies were observed on the basis of size (pinpoint, smaller, intermediate or larger), Color, shape (circular, wavy, rhizoid), margin (entire, undulate, filaments like) and elevation (convex, raised or flat) (Cappuccino and Sherman, 2005).

Identification of specific organisms by using selective media

The specific organisms were detected by selective media such as *Pseudomonas* cetrimide agar for *Pseudomonas*, mannitol salt agar for *Staphylococcus*, eosin methylene blue agar for *E.coli* and *Salmonella Shigella* agar for *Salmonella* and *Shigella* (Daniyan *et al.,* 2011).

Confirmation by biochemical testing

The bacteria obtained by selective media were further confirmed by using the biochemical tests such as oxidase, catalase, coagulase and indole tests (Daniyan *et al.,* 2011).

Storage effect on microbial load and statistical evaluation

The microbial load of the above contaminated samples was also evaluated after a storage period of 6 months. The statistical differences between the bacterial growth count before and after the storage period, the p-value was determined by using the SPSS software (version 10). The p-values less than 0.05 were considered statistically significant. samples were examined after 6 months for the presence of bacterial and fungal contaminants.

RESULTS

Among all the samples tested, creams and capsules showed no bacterial count, while only one brand of tablets was contaminated with the bacterial count. Syrups and suspensions showed maximum total aerobic bacterial count and showed presence of objectionable contaminants. The bacterial count, colony morphologies of bacteria isolated on nutrient agar, characteristics of isolated organisms on selective media, and biochemical testing, microscopic properties are shown in tables 1, 2, 3 and 4 respectively. Except tablets, nearly all dosage forms were contaminated with fungal spores. The colony morphology and identified fungal species are shown in table 5.

Effect of storage on contamination

All the selected preparations showed increase in growth count after the storage period of 6 months. Fungal species were also detected as shown in table 6.

Table 1: Total bacterial count in the selected brands on nutrient agar plates

Sample code	TAMC (CFU/mL)
Sus1	5×10^{3}
Sus2	3.8×10^{4}
Sus3	5×10^{3}
Sus4	7×10^{2}
Syp1	8.4×10^{6}
Syp2	Nil
Syp3	4.5×10^{4}
Syp4	3.5×10^{6}
Cream1	Nil
Cream2	Nil
Cream3	Nil
Tab1	15×10^{2}
Tab2	Nil
Tab3	Nil
Tab4	Nil
Tab5	Nil
Cap1	Nil
Cap2	Nil
Cap3	Nil
Cap4	Nil
Cap5	Nil

DISCUSSION

In the present study, suspensions and syrups were highly contaminated with aerobic bacterial count, while creams and capsules showed no bacterial count. This was most probably that liquids formulations are more prone to microbial contamination compared to solid one. Only one national brand of tablet showed bacterial count. Except tablets all selected dosage forms showed contamination with fungal spores.

Table 2: Colonial morphological features of bacteria on nutrient agar plates

Sample	Colony no.	Shape	Color	Margin	Elevation	Size (cm)	Surface
Sus1	1	Circular	White	Entire	Raised	1.2	Smooth
Sus2	1	Circular	White	Entire	Convex	0.2	Smooth
	2	Circular	Orange	Entire	Convex	0.7	Smooth
	3	Irregular	Off white	Undulate	Raised	1.3	Smooth
	4	Irregular	Yellow	Undulate	Raised	1.2	Smooth
	5	Circular	Brown	Entire	Convex	1.5	Smooth
Sus3	1	Circular	White	Entire	Convex	1	Smooth
	2	Irregular	White	Undulate	Raised	0.3	Rough
Sus4	1	Circular	White	Entire	Convex	0.6	Smooth
Syp1	1	Circular	Off white	Entire	Convex	0.1	Smooth
	2	Wavy	Off white	Entire	Flat	0.5	Smooth
Syp3	1	Circular	Off white	Entire	Convex	0.1	Smooth
	2	Irregular	White	Entire	Flat	0.3	Smooth
Syp4	1	Circular	Off white	Entire	Convex	0.2	Smooth
Tab1	1	Circular	White	Entire	Raised	0.1	Smooth
	2	Irregular/wavy	White	Entire	Raised	1.5	Smooth
	3	Rhizoid	Off white	Undulate	Flat	0.5	Rough

TAMC, total aerobic microbial count; Sus, suspension; Syp, syrup; Cream, cream; Tab, tablet; Cap, capsule

Table 3: Growth morphology of bacteria on selective media

Sample	Growth on selective media			
	EMB agar	SSA	MSA	Cetrimide agar
Sus1	Nil	Nil	Off white	Nil
Sus2	Pink colonies With metallic sheen	1.Colorless colonies 2.Dark color Colonies with transparent margin	Nil	Off white
Sus3	Pink colonies With metallic sheen colonies	Nil	Off white	Nil
Sus4	Pink colonies With metallic sheen	Nil	Off white	Nil
Syp1	Pink colonies With metallic sheen	Black color colonies	Nil	Creamy yellow
Syp3	Pink colonies With metallic sheen	Black color colonies	Nil	Creamy white
Syp4	Pink colonies With metallic sheen	Black color colonies	Nil	Creamy white
Tab1	Pink colonies With metallic sheen	1.Colorless colonies 2.Dark color Colonies with transparent margin	Off white to cream Color	Off white

EMB, eosin methylene blue; SSA, *Salmonella Shigella* agar; MSA, mannitol salt agar

Table 4: Biochemical testing and microscopic features of detected bacteria

Sample	Detected organism	Biochemical tests				Microscopy
		Cat	Ind	Coag	Oxd	
Sus1, 2, 3	*Salmonella*	+	-	-	-	Gram negative rod
	Shigella	-	-	-	-	Gram negative rod
	E. coli	-	+	-	-	Gram negative rod
	Pseudomonas	+	-	-	+	Gram negative rod
	Staphylococcus	-	-	+	-	Gram positive cocci
Syp1, 3, 4	*Salmonella*	+	-	-	-	Gram negative rod
	E. coli	-	+	-	-	Gram negative rod
	Pseudomonas	+	-	-	+	Gram negative rod
Tab1	*Salmonella*	+	-	-	-	Gram negative rod
	Shigella	-	-	-	-	Gram negative rod
	E. coli	-	+	-	-	Gram negative rod
	Pseudomonas	+	-	-	+	Gram negative rod
	Staphylococcus	-	-	+	-	Gram positive cocci

Cat, catalase test; Ind, indole test; Coag, coagulase test; Oxd, oxidase

Table 5: Morphology of the isolated fungal species from the selected dosage forms

Sample	Media	Color	Diameter (cm)	Spores	Texture	Expected Fungi
Sus1	SDA	Black spores with white margin	0.1	Moderate	Velvety	*A. niger*
Sus2	SDA	Green with white margin	0.5	Heavy	Velvety	*Penicillium spp.*
Sus3	SDA	Creamy white	0.6	Moderate	Velvety	*Fusarium spp.*
Sus4	SDA	Grayish black spores with white margin	0.2	Moderate	Velvety	*A. niger*
Syp2	SDA	Green spores with white margin	3	Heavy	Velvety	*Penicilliumnotatum*
Cream2	SDA	Black spores with white margin	2	Heavy	Velvety	*A. niger*
Cream3	SDA	Yellow spores	4	Heavy	velvety	*A. sulphureus*
		Black spores	3.5	Moderate	Powdery	*A. niger*
		White spores	2	Heavy	Velvety	*Fusarium*
Cap1	SDA	Black spores With white Margin	2	moderate	Powdery	*A.niger*
		Light brown spores with white margin	1	Moderate	Velvety	*A.vesicolor*
Cap2	SDA	Peach color spores	3.5	Heavy	Velvety	*Acremoniumkillense*
		Black color spores	1	Moderate	Powdery	*A. niger*
		Green to yellow spores	2	Moderate	Powdery	*Penicillium spp.*

Table 6: Effect of storage on microbial growth

Sample	After 0 month	After 6 months	p-value	Detected fungus
Sus 1	5×10^3	7×10^3	<0.05	*A. niger*
Sus 2	3.8×10^4	6×10^4	<0.05	*Penicillium spp.*
Sus 3	5×10^3	6×10^3	<0.05	*Fusarium spp.*
Sus 4	7×10^2	8×10^2	<0.05	*A. niger*
Tab 1	15×10^2	16×10^2	<0.05	-
Syp 1	8.4×10^6	9×10^6	<0.05	-
Syp 3	4.5×10^4	6×10^4	<0.05	-
Syp 4	3.5×10^6	6×10^6	<0.05	-

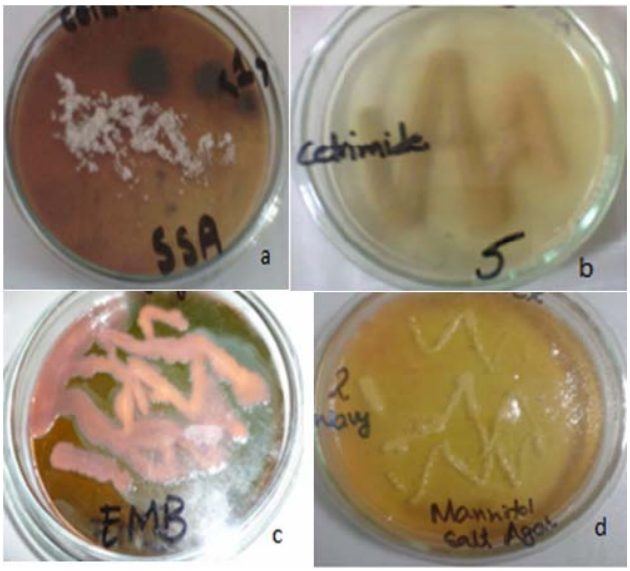

Fig. 1: Growth characteristics of specified contaminants on selective culture media a)*Salmonella* and *Shigella* growth on SSA (b) *Pseudomonas* growth on cetrimide agar (c) *E. coli* growth on EMB agar, d) *Staphylococcus* on MSA

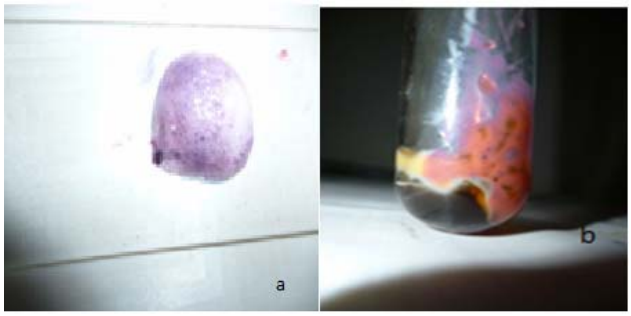

Fig. 2: Biochemical tests for identification of bacterial contaminants: a) Foam production in Catalase test, b) Indole test

The detected bacterial species included objectionable bacterial isolates such as *Salmonella, Shigella, E. coli, Pseudomonas* and *Staphylococcus* and fungal isolates included some *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp. and *Acremoniumkillense* (Maza *et al.*, 2005; Omer-Zahid, 2012; Corry *et al*., 2000) The detection of *E.coli* in oral aqueous preparations and *Staphylococcus* and *Pseudomonas* in topical preparations is very alarming, as they are objectionable microorganisms as stated by USP.

The presence of these objectionable organisms i.e. *E. coli* can be very dangerous for the patients that have weak immune system. *E.coli* can cause different types of diarrhea, nausea and high fever. *Shigella* causes high fever, ache in abdomen, and loose stool with pus. *Salmonella* causes typhoid, loose motion and food poisoning. *Pseudomonas* causes severe infections in burn patients (Maza *et al.*, 2005*).Staphylococcus aureus* is the most commonly isolated human bacterial pathogen and is an important cause of skin and soft-tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections and sepsis (Lowy, 1998).

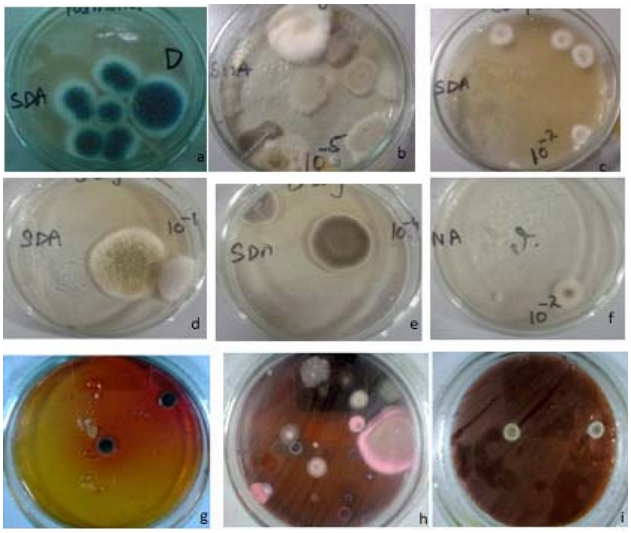

Fig. 3: Various fungal contaminants from tested dosage forms: a) *Penicillium notatum* in syrup, b) Fungal contaminant in cap, 1c) Fungal spp. in cap, 2d) Fungal species in cream, 2e) Fungal species in cream, 2f) Fungal species in cream, 1g) Fungal species in suspension, h) Fungal species in suspension, i) Fungal species in suspension.

Fusarium spp., *Penicillium* spp., and *Aspergillus* spp., are associated with the different health consequences such as allergies and toxic effects. *Penicillium* causes two types of toxins, one that affects hepatocytes and kidney cells. Second one affects the brain cells and results in jerks of whole body. *Aspergillus* causes aspergillosis which is a disease of the respiratory system (lungs) and results in cough, chest ache and increased body temperature (Jankiewicz *et al.*, 2008; Thompson *et al.*, 2008). *Acremonium Killense* produces toxic metabolite acrebol which causes the breakdown of the outer membrane of the mitochondria of the sperm cells and results in inability of the movement of sperm (Anderson *et al.*, 2009).

CONCLUSION

The microbial contamination was detected in all dosage forms except creams and capsules, while syrups were highly contaminated. Except tablets all tested dosage forms were contaminated out of the USP limits. This suggests the possible contamination by environment, water, personnel and starting materials. The preparations were also lack of effective preservation. Companies should follow monitoring programs, SOPs and GMPs in order to ensure the safety and quality. All regulatory

agencies should check the quality of the medicines before they are released in the markets.

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