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Presence of Antibiotic Resistant Bacteria along the Pharmaceuticals Production Line

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Abstract

The antimicrobial susceptibility of forty-five bacterial isolates obtained from different pharmaceutical raw materials of several companies in Cairo, Egypt during June to august, 2008 was determined using agar diffusion method. Only fourteen out of forty-five antibiotic resistant bacterial isolates were selected according to their sensitivity to equal or more than two antibiotics used. Isolates were subjected to antimicrobial susceptibility testing. The percent of resistance to seven antibiotics were Colis. (16.7%), Azithr. (78.6%), Oxytet. (0.0%), Amox. (21.4%), Kan. (42.8%), Cipro. (0.0%), and Spictin. (100.0%). Active potency of the raw materials was determined by incubating the drugs with and without microbes using HPLC and UV spectrophotometer. Among 14 selected isolates two were found to contain plasmids that are probably related to their drug resistance.

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Keywords: Antibiotic resistant bacteria, Pharmaceuticals, Hospital drain.

1. Introduction

Pharmaceutical products of various forms and dosage are susceptible to contamination by a variety of microorganisms during manufacturing and use. Such products are considered microbiologically unsafe, if low levels of pathogenic or higher levels of opportunistic pathogens are present or toxic microbial metabolites persist even after death or removal of all microorganisms present or detectable physical and chemical changes have occurred in the products. The use of such products, even where the level of contamination is low may present potential health hazards to patients. In addition, such spoiled products constitute waste and may have serious economic implication for the manufacture. Orally administered drugs often contain non-pathogenic microorganisms [1].

Antibiotic resistance has become a major clinical and public health problem within the life time of most people living today [2]. Confronted by increasing amounts of antibiotics over the past 60 years, bacteria have responded to the deluge with the propagation of progeny no longer susceptible to them. While it is clear that antibiotics are pivotal in the selection of bacterial resistance, the spread of resistance genes and of resistant bacteria also contributes to the problem [2].

In recent years, several reports from the scientific community have raised concerns that antibacterial drug development

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will not adequately address the problems posed by antibiotic resistance amongimportant bacterial pathogens [3]-[10]. The recent emergence, in European hospitals and globally, of bacteria that are totally, or almost totally, resistant to currently available antibiotics is even more threatening since treatment options for infected patients are extremely limited [11]-[13].

In a recent joint technical report, ECDC and the European Medicines Agency (EMA) in collaboration with Action on Antibiotic Resistance (ReAct) estimated that at least 25,000 patients die each year in the EU from an infection due to multidrug resistant bacteria (Centre for Disease Prevention and Control ECDC/European Medicines Agency EMEA, 2009).

Possible mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria include the deliberate or accidental introduction of antibiotics, resistant bacteria and resistance genes into the environment. Antibiotics exert a selection in favor of resistant bacteria by killing or inhibiting growth of susceptible bacteria; resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance [14], [15].

This study focused on isolation, purification and identification of microbial contamination from pharmaceutical raw materials in Egypt and attempted to generate original local data and examine the possibility of contaminated pharmaceuticals contributing to the resistance problem. Also, to provide, as accurately and as comprehensively as possible, an account of the status of the antibacterial drug development pipeline by documenting and characterizing the activity of new agents that have entered clinical development. Particular attention was given to antibacterial agents for systemic administration.

2. Material and Method

2.1 *Samples Collection and Storage*

Samples of pharmaceutical products were collected within three months from different pharmaceutical companies in Cairo, Egypt (Up, Ep, Ap, A, Adp, Np, IMG, Ico, Alp, Eg, FA, and Mp). Samples were collected in 250 ml glass pre-sterilized bottles pre-sterilized and transported to the laboratory in a cooler and stored at 4°C in the chiller.

2.2 Antibiotics used against isolates from pharmaceutical drugs

The following antibiotics were used to determine patterns of resistant bacteria and the stock solutions were prepared according to USP [16], [17]**.** Colistin sulphate from cyclic polypeptide group; Azithromycin dihydrate from macrolides group; Oxytetracyclin HCl from tetracycline group; Amoxycillin trihydrate from β- lactam group; Kanamycin acid sulphate from Aminoglycosides group; Ciprofloxacin HCl from Fluoroquinolones group; and Spectinomycin HCl from Amino cyclitols group.

2.3 Media Used

Three media were used to collect bacterial samples other than selective merdia: Peptone tween water (1gm**:** 10ml to 1000ml deionized water) in pharmaceutical drugs and peptone water (1 gm to 1000ml deionized water) in water dilutions; Tryptic soya agar for bacterial count; Muller Hinton agar for determination of antibiotic resistant bacteria; and different selective media used (Difco Laboratories): Mueller Hinton Agar pH 7.4; Vogel Johnson Agar pH 7.2; BBL™ Mannitol Salt Agar pH 7.4; Baird-Parker Agar Base; EY Tellurite Enrichment pH 6.8; Soya Casein Digest agar media pH 7.3; Bacto™ Tryptic Soy Broth; Cetrimide agar pH 7.2; *Pseudomonas* agar pH 7.2 for detection of fluorescin; *Psudomonas* agar medium pH 7.2 for detection of pyocyanin; BBL™ Lactose Broth pH 6.9; Selenite Cystine Broth pH 7.0; Tetrathionate broth base pH 8.4; Brilliant Green agar 6.9; Scharlau Xylose Lysine Deoxycholate agar pH 7.4; Bismuth Sulfite agar pH 7.5; BBL™ TSI Agar pH 7.3; MacConkey agar pH 7.1; BBL™ Eosin Methylene Blue Agar, Levine pH 7.1; Blood Base agar pH 7.0; and Brain Heart Infusion Broth.

2.4 Assay of Pharmaceutical materials using UV Methods:

Quantitative tests of Aspico tablet, Piracetam in Cervitam capsule, Dafrex tablet, Unizithrin capsule, Unocron tablet, Vincamine HCL 22.1 mg in Cervitam capsule were determined using UV Double Beam, (UV-160-1pc, UV visible spectrophotometer, SHIMADZU) at different wave lengths with and without bacterial isolates for Standard Working Solution and Test Working Solution using Ethanol (96%) as a blank and calculated the drug recovery (%) using the following formula according to **USP,** [18], [17].

$$\text{Drug Recovery (\%)} = A_T / A_S \text{ X } 100 \quad (1)$$

Where**:** A_T is a drug absorbance response in test working solution; A_S is a drug absorbance response in standard working solution.

2.5 Assay of Pharmaceutical Drugs using HPLC Methods

Quantitative Test for Unocron MR 30mg (white tablet) was determined with and without bacterial isolates using HPLC method (Hypersile BDS C18, 250 X 4.6 mm 5 µm), mobile phase degassed and filtered mixture of acetonitrile and the buffer solution (60: 40); flow rate, 1.5 ml/min; Injection volume, 20 µl; Glicalzide, having the same retention time (RT) as that of reference standard and detected by UV at 235 nm. Unocron recovery (%) was done using the same formula as in section *D*.

2.6 Detection of Microorganisms in pharmaceutical products

The detection of microorganisms in pharmaceutical products was done using USP, [18] . These include test for *Staphylococcus aureus* and *Pseudomonas aeruginosa*; Coagulase test for *Staphylococcus aureus*; Oxidase and pigment tests (for *Pseudomonas aeruginosa*). Also, the tests for *Salmonella* species and *E. coli* were examined.

2.7 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined by means of the agar diffusion method, according to guidelines established by National Committee for Clinical Laboratory Standards (NCCLS) [19].

2.8 Plasmid isolation

Plasmid isolated according to [20] was electrophors using Hofer HE 99Xmax submarine electrophorasis unit on 0.7 agarose gel and 1X TBE buffer at consistent 85 volt for about 3hr. The different bands sizes determined against lambda ECOR1 (marker) (21.226, 7.421, 5.804, 4.878, and 3.530 kb) and separated bands were stained by 0.1 µg/ml ethidium promide and photograph was taken using gel document unit.

3. Results

3.1 Isolation of bacteria from pharmaceutical products

In this study, isolation, purification, and identification of antibiotic resistant bacteria from several pharmaceutical products were done. About 45 bacterial strains were isolated from 117 different pharmaceutical products selected from several companies (Up, Ep, Ap, A, Adp, Np, IMG, Ico, Alp, Eg, FA, and Mp) in Egypt. All samples were collected on general and specific media used. Screening forty-five isolates was done against seven different antibiotics (colistin sulphate, azithromycin dihydrate, oxytetracyclin HCl, Amoxycillin trihydrate, Kanamycin acid sulphate, Ciprofloxacin HCl, and Spectinomycin HCl) using disc diffusion method.

Only fourteen out of forty-five bacterial isolates selected according to their sensitivity for equal or more than two antibiotics used, and expressed with **(38.89%).** The results indicated that all selected isolates were susceptible to both Oxytetracyclin HCl and Ciprofloxacin HCl. On the other hand, the same isolates without exceptions were resistant to Spectinomycin HCl. The percent of resistance to seven antibiotics were as the following: Colis. (16.7%), Azithr. (78.6%), Oxytet. (0.0%), Amox. (21.4%), Kan. (42.8%), Cipro. (0.0%), and Spictin. (100.0%). Results are given in **Table 1.**

Table 1. Antibiotic sensitivity test for bacterial isolates from pharmaceutical products

Isolate Code	**Antibiotics used**						
	Colis	***Azithr***	***Oxytet***	***Amox***	***Kan***	***Cipro***	***Spictin***
8D	S	***R***	S	S	***R***	S	***R***
9D	S	S	S	**R**	S	S	***R***
10D	***R***	***R***	S	S	S	S	***R***
13D	S	***R***	S	S	S	S	***R***
15D	S	***R***	S	S	***R***	S	***R***
21D	S	***R***	S	S	***R***	S	***R***
25D	S	***R***	S	S	S	S	***R***
28D	S	***R***	S	S	S	S	***R***
29D	S	S	S	S	***R***	S	***R***
30D	S	***R***	S	S	S	S	***R***
33D	S	***R***	S	S	S	S	***R***
37D	S	***R***	S	**R**	***R***	S	***R***
38D	***R***	S	S	**R**	S	S	***R***
43D	S	***R***	S	S	***R***	S	***R***

(***R***): Resistant, (**S**): Susceptible.

3.2 Characterization and identification of antibiotic

Only three antibiotic resistant bacterial isolates were characterized and identified according to morphological characterization, microscopic examination and biochemical tests. The results are shown in **Table (II)**. These results indicated that the microorganisms were *Alcalignes xylosoxidans* for 9D; *Staphyllococcus xylosus* for 13D; and *Staphyllociccus sciuri,* for 21D bacterial isolates according API 20.

3.3 Determination of active potency in pharmaceutical products

Determination of active potency of pharmaceutical products using HPLC and UV analysis by incubation of these products with and without microorganism. Not all isolates were analyzed because of some limited facilities, for this reason HPLC analysis was made once for Unocron containing Gliclazide as active ingredient for 9D bacterial isolate and UV done for the same bacterial isolate, Aspico containing acetylsalcilic acid for 13D and Unizithrin antibiotic contain azithromycin dihydrate as active ingredient of 21D bacterial isolates

The UV analysis results revealed that Unizithrin antibiotic after incubation with 21D isolate (grown on Vogel Johnson agar medium) decreased the concentration compared to that without microorganism Fig. (1 a,b). Also, the concentration of Aspico product decreased after incubation with 13D isolate (grown on mannitol salt agar medium) compared to that without microorganism Fig. (2 a,b). The UV result revealed that concentration of Unocron after incubation with 9D isolate (grown on MacConkey agar medium) did not change compared to that without microorganism Fig. (3 a,b).

The result obtained from HPLC analysis revealed that the concentration of Unocron after incubation with 9D bacterial isolate (grown on MacConkey agar medium) did not change compared to that without microorganism Fig. (4 a,b).

Table 2. Morphological and biochemical characteristics of antibiotic resistant bacterial isolates

Product name	**Unocron**	**Aspico**	**Unizithrin**
Company	Ep	Up	Up
Isolate no.	**9D**	**13D**	**21D**
Morphological characteristics			
Colony shape	punctiform	punctiform	punctiform
Colony colour	Y-W	white	White-metallic chain
Consistency	mucoid	non mucoid	non mucoid
Elevation	flat	flat	flat
Margin	entire	entire	entire
Microscopic examination			
Cell shape	Bacilli (long rods)	Cocci (staph)	Cocci (staph)
Gram reaction	Gram -ve	Gram +ve	Gram +ve
Growth medium	Growth on MacC	Manitol Salt agar	Vogal Jonson agar
Biochemical Reactions			
Oxidase test	+	ND	ND
Catalase test	+	+	+
G. MacC	+	-	-
KOH 3%	+	-	-
D-fructose	AG	A	A
Starch	-	-	-
Lactose	AG	A	-
Maltose	AG	A	-
Mannitol	AG	A	-
Glugose	AG	A	A
Mannose	AG	A	A
Sucrose	AG	A	A

ND: Not detected; AG: Acid & Gas; A: Acid; Y-W: Yellowish-white; G. MacC.: Growth on MacConkey

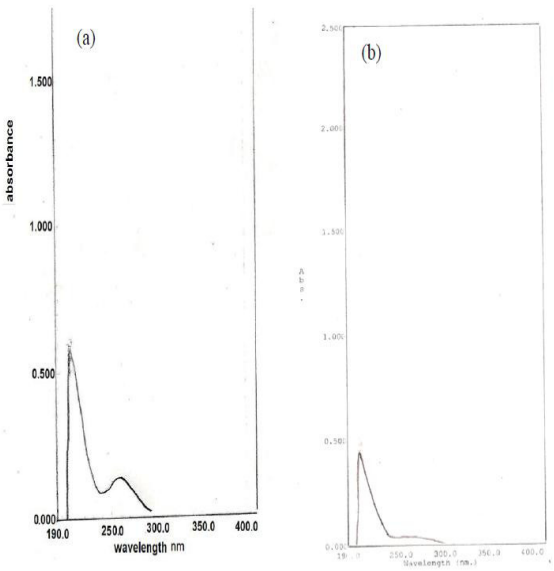

Fig. (1): a) UV showing Unizithrin antibiotic contain azithromycin dihydrate without 21D isolate; b) with the same isolate.

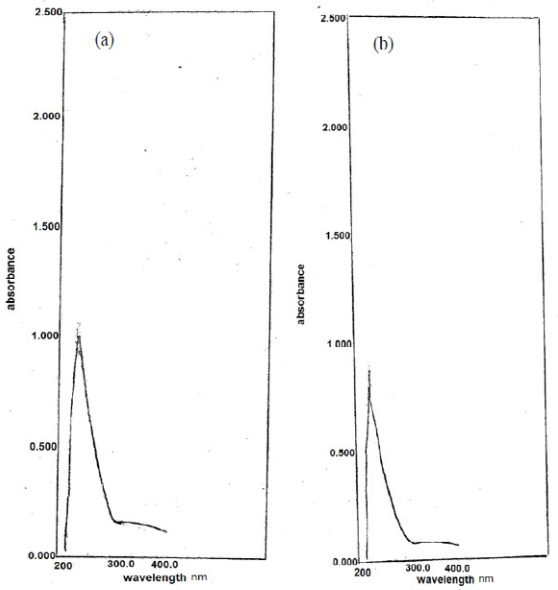

Fig. (2): (a) UV showing Aspico contains acetylsalcilic acid after incubation without microorganism; (b) with 13D isolate.

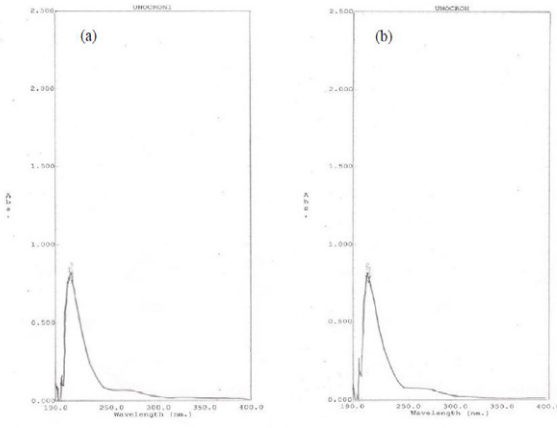

Fig. (3): (a) UV showing Unocron contains Gliclazide as active material after incubation without microorganisms; (b) with 9D isolate.

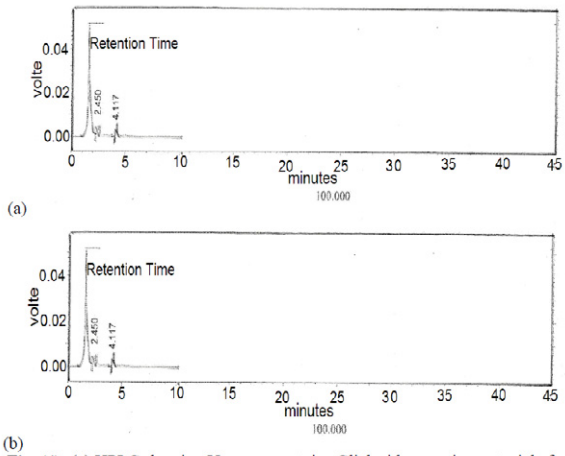

Fig. (4): (a) HPLC showing Unocron contains Gliclazide as active material after incubation without microorganism; (b) with 9D isolate.

3.4 Plasmid detection of six antibiotic resistant bacteria

The antibiotic resistant bacteria isolated were examined to determine the plasmid which may be responsible for the resistant towards antibiotics that explain the relationship between plasmid and antibiotic resistant bacteria **Fig. 9.** the result indicated that the isolates (9D), (13D) and (21D) obtained from pharmaceutical products were possess two plasmids.

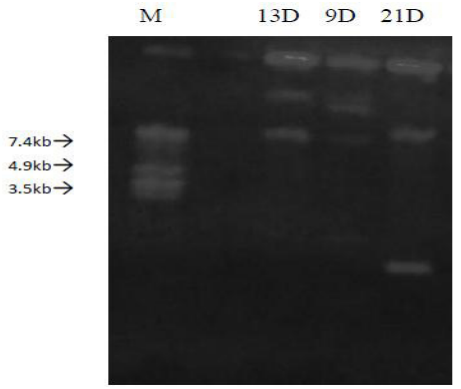

Fig. 5. Plasmid isolation by electrophoresis from six microorganisms, the lanes arranged from left to right: (M) Marker and bacterial isolate.

4. Discussion

The study aims on isolation, purification and identification of microbial contamination from pharmaceutical products under study.

In the past, consideration of microbiological problems and their means of control occurred only late in pharmaceutical product design; this has led to inevitable compromises in product quality at all stages of development, manufacture and use. In order to exploit these opportunities to the full, a clear appreciation of all aspect of pharmaceutical microbiology is required [21]. The awareness by medical and pharmaceutical personnel of the potential health hazards on the use of non sterile pharmaceutical contamination with microorganisms have give much interest and spurred research on the microbial contamination of pharmaceutical product. The adverse effects of contaminated pharmaceutical products are well documented [22].

The results of isolation of bacteria contaminated pharmaceutical products were agreed with [23], [24] reported that any pharmaceutical products whether manufacture in hospitals or industrial environment has potential to be contaminated with microorganisms which may include bacteria, yeast or moulds.

Some workers suggested that bacterial contamination may originate from the raw materials or may be introduced during production environment condition of equipment, personal and packaging materials, while in our study we found bacterial contamination of raw material namely (Avecil 101, Avecil 102, Talc, Hespeiridin, and Diosmin) have been isolated [25].

Also our study agreed with [26] reported that the product may also become contaminated during storage and use**.** The result of Gram negative bacterial isolate *Alcalignes xylosoxidans* from Unocron tablet was agreed with the study performed by [27] recorded the presence of Gram negative bacteria as the most frequently strains in pharmaceutical medications. By far the most common source of spoilage or pathogenic species is water or unpreserved stock solutions; for example, solution such as peppermint water may become heavily contaminated with Gram negative microorganisms if not properly prepared or if incorrectly stored. Typically Gram negative water borne species which may found in distilled water include *Acinetobacter*, *Achrmobacter*, *Enterobacter*, *Flavobacterium*, *Pseudomonas* and species of enteric organisms such as *E. coli* and *Salmonella*, may survive for substantial periods in polluted water [28].

In study of a hospital manufacturing unit by [1] *Pseudomonas aeruginosa* was isolated from products manufactured in the pharmacy. Isolation of some strain of the microorganism from environmental sites within the pharmaceutical indicated that this was source of contamination in many products. When strict environmental control procedure was implemented the product isolation rate for *Pseudomonas aeruginosa* falls to 2-3% [29].

Microbial contamination of pharmaceutical products represents a potential hazard for two reasons: first it may cause product spoilage, the metabolic versatility of microorganisms is such that any formulation ingredient from simple sugar to complex aromatic molecules may undergo chemical modification in the presence of suitable microorganism, spoilage will not only affect therapeutic properties but may also discourage patient [21]. Second, product contamination represents a health hazard to the patients although the extent of the hazard will vary from product and patient to patient depending on the type and number of microorganism present [30].

5. Conclusion

The results indicated that all selected isolates were susceptible to both Oxytetracyclin HCl and Ciprofloxacin HCl. On

the other hand, the same isolates without exceptions were resistant to Spectinomycin HCl. The percent of resistance bacteria to seven antibiotics were as the following: Colis. (16.7%), Azithr. (78.6%), Oxytet. (0.0%), Amox. (21.4%), Kan. (42.8%), Cipro. (0.0%), and Spictin. Only three antibiotic resistant bacterial isolates were characterized and identified and suggested to be *Alcalignes xylosoxidans* for 9D; *Staphyllococcus xylosus* for 13D; and *Staphyllociccus sciuri,* for 21D. The relationship between plasmid and antibiotic resistant bacteria explained from pharmaceutical products isolates and detected two plasmids.

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