DETECTION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS* IN SOME LOCALLY ORAL PHARMACEUTICAL SAMPLES

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ABSTRACT

Contamination of oral pharmaceuticals with microorganisms may lead to deleterious effects on the therapeutic properties of the drug, and may potentially cause injuries to intended recipients. Thirty contaminated samples were detected representing 2% out of 1500 total collected samples. Out of 30 contaminated samples, only six samples (20%) revealed the presence of *Clostridium perfringens*. The biofilm assay was performed using the Microtiter Plate Method (MTP) for the six of the detected *C. perfringens* were three isolates biofilm forming. PCR multiplex was done for *C. perfringens* showed the presence of alpha toxin gene among all species. In spite of the current recommendation for a conventional detection method for *Closterium* spp., the application of multiplex PCR showed to be more, effective and able to detect *Clostridium* toxins genes.

INTRODUCTION

The use of contaminated pharmaceutical products has proved hazardous to the health of users. Contamination with microorganisms may lead to deleterious effects on the therapeutic properties of the drug **(Coker, 2005)**. Several factors can influence the microbiological quality of the non-sterile pharmaceutical products like; nature and origin of the material, the technique of production which may lead to the potential for microbial contamination **(USP, 2018; BP, 2018)**. The microflora in the final product may represent contaminants from the raw materials, the equipment used, persons handling the product or from the container used for packaging **(Denyer and Baird, 2007).**

Non-sterile oral medications contaminated with microflora are warning of the making risk to public health measures, particularly when exceeding the acceptable limit of $>10^2$ CFU/g or ml **(USP, 2018).** Moisture and high amount of sugar in the oral liquid drugs, in particular, can support the microbial growth. These microbial contaminations may ultimately contribute to secondary bacterial infections in pediatric patients **(Cabañas Poy**

***et al*., 2016).** Even a few numbers of opportunistic pathogens may become infectious in patients with the severe underlying disease or immunocompromised **(Mugoyela and Mwambete, 2010).** In addition, the presence of pathogenic or opportunistic microorganisms or bacterial toxic metabolites and enzymatic activity can result in the inactivation of the product **(Gimenez-Bastida *et al*., 2018).**

Pharmacovigilance (PhV) has been defined by the World Health Organization (**WHO,2004**) as activities are done to monitor detection, assessment, understanding, and prevention of any obnoxious adverse reactions to drugs at a therapeutic concentration in animals and humans **(Ibrahim *et al*., 2016).** As part of Good Manufacturing Practices (GMP), the United States Pharmacopoeia (USP) Microbial Limits Test provides methods for determining the safety of a product through the absence of indicator microorganisms, which can be considered a hazard to consumers and indicative of contamination. For this purpose, the USP specifies 5 bacterial indicators: *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Clostridia* (**USP, 2018).**

Clostridia species are members of normal intestinal and are ubiquitous in soil. They are Gram-positive spore-forming bacilli that are responsible for up to 10% of anaerobic infections with a wide range of clinical sites. Several species have been described (*C. perfringens*, *C. novyi*, *C. septicum*, *C. bifermentans*, *C. sordellii*, etc.), among which *C. perfringens* has been reported to be the most frequent cause of gas gangrene (**Bryant and Stevens, 2010; Stevens *et al*., 2012**). The virulence of this Gram-positive, spore-forming anaerobe is largely attributable to its ability to produce at least 17 different toxins **(McClane *et al*., 2006)**. However, there is considerable variability in the toxin armamentarium of different *C. perfringens* strains, which provides the basis for a toxinotyping classification system that divides *C. perfringens* isolates into five types (A–E) depending upon their ability to produce alpha, beta, epsilon, and iota toxin **(McClane *et al*., 2006; Freedman *et al*., 2016).**

The presence of biofilm constitutes a major factor in the ability of the organism to resist the external unfavorable condition facilitating its growth and survival. (**Gad *et al*., 2011)** mentioned that 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 micro-colonization and formation of biofilm with significant resistance to antimicrobial agents **(Craven *et al*., 1981)**. Also, preservative availability may decrease by the interaction with packaging material or by volatilization during opening and closing of containers; hence, preserved medicines should be packed in sealed, impervious containers during storage **(Denyer *et al*.,2004)**.

The aim of this study was to determine the prevalence of *Clostridium* species in oral non-sterile pharmaceutical products investigated at National Organization for Drug Control & Research (NODCAR) and to detect toxin typing genes by the multiplex Polymerase Chain Reaction (PCR).

MATERIALS AND METHODS

Nutrient agar Oxoid UK, Columbia agar base, Reinforced liquid medium and Fluid Thioglycolate Medium from Lab M(UK), Casein hydrolysate from Duchefa Biochemie

(Netherlands), Bile esculin agar from Biolife Italiana. Chemicals were purchased from Sigma Aldrich, USA; El Nasr pharm co. Egypt. Standard reference *Clostridium sporogenes* ATCC-11437 from American Type Culture Collection available at microbiology lab. NODCAR.

Sample collection and preparation

Samples were collected from Oct. 2016 till Nov. 2017 from pharmaceutical products delivered at NODCAR laboratories. Under aseptic conditions, a sample was taken using sterile tools and sterile containers. The sample container was labeled in details. Microbial enumeration tests were done with a Total viable aerobic count for all collected samples. Also, for monitoring the safety of non-sterile pharmaceutical preparations, the USP microbial limit tests require the absence of the following species; *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli*, *Clostridia* and *Candida albicans.* Tests for specified microbial contaminants were conducted according to the United States Pharmacopeia, USP40 **(USP, 2018)**. A total number of 1500 of non-sterile samples comprised into different available classes of preparations including 406 syrup, 530 tablets,169 suspensions, 30 oral drops, 25 oral gel,320 capsules and 20 mouthwash which included locally manufactured and imported products were collected and examined for Total viable aerobic count.

Samples were prepared by dissolving or diluting 10g or 10ml of product in 90ml Soybean-casein digests broth (SCD) to make 100ml prepared sample of (1:10) dilution as described in the (Microbiological Examination of non-sterile products) (**USP, 2018)**. The samples were mixed thoroughly in a rotatory shaker for the time period of 15 min at a speed of 120 rpm. To verify testing conditions, a negative control was performed using the chosen diluents in place of the test preparation and a reference strain *C. sporogenes* ATCC-11437 was used as a standard reference.

Isolation and identification of *Clostridia* spp.

Since May 2009 some changes in chapter 62 in USP was done about clarification of Clostridia test, therefore our research focused on the detection of this particular contaminant. The sample prepared in SCD broth was incubated at 37°C for 24h. The sample was divided into two portions of at least 10 ml then one of the portions was heated at 80°C for 10 min and cooled rapidly while the other portion set without heat. One milliliter aliquot of the sample in SCD broth of both portions was inoculated in the reinforced medium for *Clostridia.* The broth was incubated under anaerobic conditions at 35°C for 48 h. Subsequently, a loopful of each broth tube were separately streaked onto the surface of Columbia blood agar plate and incubated under anaerobic conditions using Oxoid ThermoFisher Scientific Jar System with BR0055 AneroGen indicator, incubation was done at 30 to 35°C for 48 -72 h. The occurrence of anaerobic growth of rods (with or without endospores) with negative catalase reaction indicated the presence of Clostridia. The presumed positive isolates were then confirmed by biochemical tests.

Biochemical tests

Identification of the potential bacteria was carried out using several biochemical tests. Members of the *Clostridium* species were classified according to the keys of **Ellner *et al.* (1973); Willis (1977); Dowell and Hawkins (1987); Jorgensen *et al.* (2015)**. The biochemical tests performed for anaerobic gram-positive rods that are suggestive of *Clostridium perfringens* were; spore staining, Catalase test, Oxidase test, Motility test, Nitrate reduction test, Lipase-lecithinase test, Double zone hemolysis test**,** Iron Milk test, Indole production test, Urease test, Bile esculin test, Lactose fermentation-gelatin hydrolysis test and Starch hydrolysis test. Confirmation was carried out for the anaerobic bacterial isolates using the Vitek2 ANC card system **(Rennie *et al*., 2008).**

Spore Staining

Spore Stain (Schaeffer-Fulton Method) is a technique designed to isolate endospores by staining with malachite green, and any other vegetative cells with safranin as mentioned by **Cappuccino and Sherman (2014).**

Detection of some virulence factors

1-Biofilm assay for ***Clostridium perfringens***

Clostridium perfringens was grown anaerobically on Reinforced Clostridial Agar or in fluid thioglycollate medium at 37°C.

Microtiter Plate Method (MTP) was performed according to **Vidal *et al*. (2015)**. Strains diluted of (1:10) were inoculated into fluid thioglycolate medium (FTG) and grown overnight at 37°C to produce biofilms. Although FTG free of cells was added to three wells of a sterile 96-well flat-bottomed plastic tissue culture plate and incubated at 37°C under anaerobic conditions.

Biofilm was washed three times with phosphate-buffered saline (PBS) and then allowed to dry for 15 min. Crystal violet (0.4%) was then added, and the biofilms were incubated for 15 min and washed with deionized water to remove excess dye. After washing, crystal violet-stained biofilms were further dried at room temperature for 15 min. To quantify biofilm biomass, crystal violet was removed by adding 33% acetic acid solution. The Optical density (OD) of stained adherent bacteria was determined with a microplate reader (Biotech ELX 800 Power Wave Xs, USA) at a wavelength of 570nm (OD570). Each strain occupied three wells, and negative controls (media blanks) were included in at least 6 wells. All experiments were repeated three times.

For a comparative analysis of biofilm assay in MTP results, the adherence capability of the bacteria was classified into four categories: nonadherent, weakly adherent, moderately adherent and strongly adherent, based upon the ODs of the bacterial films **(Stepanovic *et al.*, 2000)**.

2-Detection of four characterization toxin genes of *C. perfringens* by multiplex PCR

DNA extraction

Selected isolates were subjected for toxin investigation as a virulence factor. To extract template DNA from *C. perfringens* for multiplex PCR, pure colonies were grown overnight in 5ml brain heart infusion at 37°C for 24h anaerobically. Then a rapid boiling procedure was used to prepare DNA template from *C. perfringens* according to **Sheedy *et al*. (2004).**

Multiplex PCR of *C. perfringens* by four characteristic genes

The multiplex PCR assay was used to detect the presence of genes encoding alpha-toxin (cpa), beta-toxin (cpb), epsilon-toxin (etx) and iota-toxin (iap). Primer sequences were reported by **Van Asten *et al*. (2009)** and illustrated in table 1. The protocol of amplification was performed as follows: 12.5 µl of 2× Green PCR master mix (DreamTaq™, Fermentas, Thermo scientific, USA), 4 µl DNA template, 0.5 µM of each primer (10 pmol/µl) then add PCR-grade water up to 25µl total reaction volume. The PCR reactions were performed using a Biometra PCR thermal cycler, Germany, with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of, denaturation at 94°C for 1min, annealing at 55°C for 1min then extension at 72°C for 1 min and the final extension was at 72°C for 10 min **(Van Asten *et al*., 2009).** The amplified samples were then analyzed by agarose gel electrophoresis using 1.5 % agarose gels (Fischer, Scientific®, UK)containing 0.5X TBE and prestained with ethidium bromide (Fischer Scientific, Canada). Electrophoresis was done at 70v for 60min in the presence of 100bp DNA ladder (Fermentas Life Science, EU) according to **Sambrook *et al*. (1989)**. A negative control PCR mixture with no template DNA was included.

Table (1). Oligonucleotide primers sequences for the four toxin genes of *C. perfringens* used in multiplex PCR.

Toxin gene	Primer sequence (5'-3')	Amplified product size (bp.)	Reference
cpa	F. 5` GCTAATGTTACTGCCGTTGA 3` R. 5` CCTCTGATACATCGTGTAAG 3`	**324**	Van Asten *et al*.,2009 and Hamza *et al*., 2017
cpb	F. 5` GCGAATATGCTGAATCATCTA 3` R. 5` GCAGGAACATTAGTATATCTTC 3`	**196**	
etx	F. 5` GCGGTGATATCCATCTATTC 3` R. 5` CCACTTACTTGTCCTACTAAC 3`	**655**	
iap	F. 5` ACTACTCTCAGACAAGACAG 3` R. 5` CTTTCCTTCTATTACTATACG 3`	**466**	

RESULTS

Our results indicated that thirty samples (2%) out of total 1500 samples were over microbial limit 10^2 CFU/g or /ml according to the USP 40 specified acceptance criteria of a total viable count for non-sterile products. Accordingly, table (2a) illustrates that 30 different trade products (10 syrups, 2tablets, 4capsules,12 suspensions and 2 oral drops) were found to exceed the specified limits. Only six contaminated products were rejected for indicator pathogens, according to the recommended criteria of oral pharmaceutical products in the USP. Also, microbial contaminants recovered from different collected dosage forms of oral pharmaceutical samples were illustrated in (table2b).

Biochemical tests for *C. perfringens* were shown a positive result with starch hydrolysis test, double zone hemolysis, nitrate reduction test lipase-lecithinase test, iron Milk test, lactose fermentation-gelatin hydrolysis test and glucose, sucrose, lactose and maltose fermentation tests. While negative results illustrated in urease test, indole production test, lipase production, catalase test and motility test. However, bile-esculin hydrolysis fluctuated in results among *C. perfringens.*

Table (2a). Microbial load in oral pharmaceutical products

Serial	Brand Name	Batch no.	*S. aureus*	*Salmonella*	*E.coli*	*P.aeruginos*	*C.*	*Aspergillus.s* pp	*Candida*. spp	*Total bacterial count*	*Total fungal count*
1	**Tribulus terrestris extract capsule**	1502296	+							$2.8x10^2$	
2	**Prednisolone syrup**	4984	+						*C. pelliculosa*		$2 x10^2$
3	**Calcium with magnesium, zinc & vitamin D3 suspension**	51434	+							$2.5x10^2$	
4	**Tribulus terrestris extract capsule**	1402314	+							$1.2x10^3$	
5	**Chlorpheniramine maleate syrup**	515087	+							$2.3x10^2$	
6	**Raw materials extracted from Sweet Potato Fiber,Cyamoposis Gum, and Amor Phallus Konjac**	20140529	+	+							

	capsule										
7	**Bromhexine syrup**	25883098	+							$2.1x10^2$	
8	**Albenzole 3.5% suspension**	ATB1215-2	+							$4.2x10^2$	
9	**Ambroxol oral drop**	525007	+								
10	**Garlic tablet**	T02716	+					*A. niger*			$2 x10^2$
11	**Alfacalcidol capsule**	622401	+								
12	**Garlic tablet**	TO2816	+							$2.9x10^2$	
13	**Ivy Leaf dry extract syrup**	201116	+							$3.1x10^2$	
14	**Salbutamol syrup**	5530013			+						
15	**Dexamethasone syrup**	630500			+						
16	**Ibuprofen suspension**	4211020				+				$1.1x10^3$	
17	**Ibuprofen suspension**	4211068				+				$1.3x10^3$	
18	**Bromhexine syrup**	2583099				+	+	*A. flavus*		$2.1x10^3$	
19	**Caraway oil, Fennel, Ginger oil and inactive zinc sulphate syrup**	130022				+				$4x10^2$	
20	**Calcium with magnesium, zinc & vitamin D3 suspension**	15101044				+				$2.3x10^3$	
21	**Ibuprofen suspension**	4211062					+			$4.3x10^2$	
22	**Ginkgo Biloba Leaves dry extract oral drop**	1640127					+	*A. niger*		$2.1x10^2$	
23	**Bromhexine syrup**	2583098					+			$2.5x10^2$	

24	**L – Carnitine syrup**	2050416					+				
25	**Ibuprofen suspension**	4211066					+			$2.7x10^2$	
26	**Amoxicillin and Clavulanic acid 230 mg suspension**	152062							*C. lusitaniae*		*2.5x10 2*
27	**Amoxicillin and Clavulanic acid 312 mg suspension**	152050						*A. niger*	*C. krusei*		*2.8x10 2*
28	**Ibuprofen suspension**	5211001						*A. flavus*			3.2x10 2
29	**Nifuroxazide suspension**	161430								$4.8x10^2$	
30	**Nifuroxazide suspension**	1501124								$2.1x10^3$	

+ presence of organism

Table (2b). Microbial contaminants recovered from different collected dosage forms

Dosage form	Total number of samples	No. of contaminated samples	
		Total number	%
Syrup	406	10	2.46
Suspension	169	12	7.1
Tablet	530	2	0.37
Capsule	320	4	1.25
Oral drop	30	2	6.6
Mouthwash	20	0	0
Oral gel	25	0	0
Total	1500	30	2

As continuing to our investigation for detecting the *Clostridia*. Six samples were recovered with *Clostridia* spp. (20%) out of 30 contaminated samples (table 3). Those samples were in classified form as four syrups, one oral drop and one capsule. Following their isolation, the *Clostridia* spp. were subjected to biochemical identification as illustrated in and confirmed identity by Vitek2 identification systems ANC ID card. Those six species

were selected for further toxins detection as a virulence factor using multiplex PCR-method.

Table (3). The incidence of *C. perfringens* among collected samples.

Serial Number	Dosage form	Batch number	Active constituents
1	Suspension	4211062	**Ibuprofen**
2	Syrup	2583099	**Bromhexine**
3	Oral drop	1640127	**Ginkgo Biloba Leaves dry extract**
4	Syrup	2583098	**Bromhexine**
5	Capsule	2050416	**L - Carnitine**
6	Suspension	4211066	**Ibuprofen**

Table (4) illustrates the detection of biofilm formation among the six *C. perfringens* strains. Our results indicated that three strains had the ability to produce biofilm. One sample of *C. perfringens* in oral drops vivid a moderate biofilm formation followed by two weak biofilm formation represented in syrup products with different batch number. However, the least three batches revealed a non-adherent biofilm. The calculation was done as the Cut off was equal to 0.082512(mean of sterile medium + 3 SD).

Table (4). Detection of biofilm among six isolates of *C. perfringens* recorded from collected samples

Serial no.	generic Name	Batch no.	Biofilm formation
1	Ibuprofen suspension	4211062	Non-Adherent
2	Bromhexine syrup	2583099	**Weak**
3	Ginkgo Biloba Leaves dry extract oral drop	1640127	**Moderate**
4	Bromhexine syrup	2583098	**Weak**
5	L - Carnitine cap.	2050416	Non-Adherent
6	Ibuprofen suspension	4211066	Non-Adherent

Performing PCR with four sets of specific primers for detecting the presence of α, β, ε, and ι encoding genes of *C. perfringens*. The fragment of 324 bp belonging to the α toxin gene was detected in all samples. While β, ε and ι encoding genes were not represented among the six *C. perfringens.*

DISCUSSION

Our results vivid that six *C. perfringens* were detected among 30 contaminated pharmaceuticals with four different brands this was consistent with a study done by **Ashour *et al*. (1989)** on 36 of talcum powder examined for anaerobic bacteria and they mentioned that the count of more than 100 CFU/g was not obtained from any product examined in the survey. However, qualitative tests for the presence of *Clostridium* spp. were carried out by conventional methods resulted in one of each brand contained *C. perfringens* and two samples from the third brand.

In a related work, **El-Houssieny *et al*. (2013)** investigated the microbial contaminant in non-sterile pharmaceuticals over 280 samples and they reported that Gram-positive rods spore-bearers were the most commonly isolated bacteria from oral and topical medicaments and the tested oral preparations showed a higher incidence of contamination (33.75%) in comparison to the topical preparations (19.1%). Also, these findings were consistent with those of previous studies, where the majority of microbial contaminants in non-sterile pharmaceuticals are Bacillus spp. **(Mwambete,2010 and Al-Charrakh ,2012**).

In contrast to our results, **Gad *et al*. (2011)** reported that a total number of 300 non-sterile pharmaceutical samples were tested for microbial contamination and all the tested samples were free from anaerobic bacteria, coliforms, and *P. aeruginosa*. Bacterial counts in the samples indicate that only syrups and suspensions had counts greater than 1000 CFU/ml.

Our results revealed the incidence of three *C. perfringens* capable to form biofilm this was agreed by **Varga *et al*. (2008) and Charlebois *et al*. (2014)** who reported that the atmospheric oxygen tolerance assays showed that the biofilm could protect *C. perfringens* cells from oxygen stress. Moreover, oxidative stress resistance and the general stress response might be important for the development of biofilms in *C. perfringens* **(Charlebois *et al*., 2016).**

Alpha-toxin (cpa) was present in the six *C. perfringens* isolates in our study. Hence, the importance of its existence is consistent with many previous studies as well as **Vidal *et al.* (2015)** mentioned that alpha-toxin (cpa) may help in the building of *C. perfringens* biofilms and incorporated into the matrix, where it was seen partially colocalizing with extracted DNA. Moreover, **Smedley *et al*. (2004)** had stated that *C. perfringens* is a major cause of enteric diseases due to the production of several toxins.

Pantaléon *et al*. (2014) mentioned that *C. perfringens* biofilms if formed in vivo, are able to produce toxins that may participate in virulence and produce spores that may promote new infections and may be involved in environmental adaptation and resistance, virulence and spreading. This was consistent with several studies done by **Rood and Cole (1991); Rood (1998) and Bryant (2003)** stated that *C. perfringens* type A produces nearly 90% of all gas gangrene cases.

Generally, the presence of potentially pathogenic opportunistic microbes, cannot be overemphasized, because they may cause a significant deterioration in the health status of patients, particularly in elderly, debilitated and chronically sick patients, those who are

immunologically compromised, and of infants with an immature immune system **(Mugoyela and Mwambete,2010).**

CONCLUSION

The indicator pathogens of bacteria were isolated in the current study. The use of the five pathogen indicator bacteria does not mean that the presence of other bacteria might not be a problem during quality evaluations. Therefore, the presence of any microorganism should be considered undesirable for all drugs. Thirty samples (2%) out of total 1500 oral pharmaceuticals had over microbial limit 10^2 CFU/g or /ml according to the USP 40. Six samples were recovered with *Clostridia* spp. (20%) out of 30 contaminated samples. Biochemical tests and Vitek2 confirmed the presence of *C. perfringens*, but are incapable of distinguishing different types of *C. perfringens.* The pcr-based method provided to be more rapid, with results obtained in a few hours, more reliable, cost-effective and more sensitive detection for classification of toxigenic *C. perfringens.*

Ethical considerations

This research project was carried out in accordance with regulations stipulated by the microbiology and immunology department of the faculty of pharmacy (Boys) at Al-Azhar University in Cairo, Egypt and National Organization for Drug Control and Research. Samples and facilities were coded to maintain anonymity. The coding information was kept confidential and available only to the researcher.

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الكشف عن الكلوستريديم بيرفينجينز وتحديده في بعض العينات الدوائية عن طريق الفم

للسادة الدكاترة

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قسم الميكروبيولوجى – هيئة الرقابة والبحوث الدوائية

قسم الميكروبيولوجيا والمناعة– كلية الصيدلة (بنبن)جامعة الأزهر

ان تلوث المستحضرات الصيدلانية بالكائنات المجهرية قد يؤدي الي اثار ضارة على الخصائص العلاجية كما يمكن ان يسبب أضرار للاشخاص المستفيدين من العلاج .لقد اجريت الدراسة على ١٥٠٠ عينه وظهر منها ٣٠ عينه فقط غير صالحة(ملوثة ميكروبيا) تمثل ٢٪ من اجمالي العدد.حيث تم تعريف عدد ٦ سلالات من الكلوستريديم بيرفيرنجينز(*Clostridium perfringens*) من الاجمالي ٣٠العينة الغير صالحة وتم التعريف عن طريق صبغة الجرام و اختبارات الكيمياء الحيوية. وكشف اجراء تقييم الغشاء الحيوي لتك السلالات عن وجود ثلاثة منها لها القدرة على انتاج الغشاء الحيوي(البيوفيلم).كما اجرى تفاعل البلمرة التسلسلي لهم على اساس تحديد أنواع الجينات السمية و ظهرت النتائج بوجود جين الفا فى السلالات الستة وعلى الرغم من التوصية الحالية بطرق الكشف التقليدية فقد اظهر تطبيق تفاعل البلمره التسلسلي فاعلية وكفاءة عالية.