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Microbiological Studies on Clostridium Perfringens Type (A)

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ABSTRACT

Background:*Clostridium perfringens*, a rapid-growing pathogen known to secrete an arsenal of >20 virulent toxins, has been associated with intestinal diseases in both animals and humans throughout the past century. Gas gangrene is synonymous with myonecrosis and is a highly lethal infection of deep soft tissue, caused by *Clostridium* species, with *Clostridium perfringens* being the most common. Clostridium perfringens is one of the most common cause's gas gangrene that is associated with broadspectrum antibiotic treatment. The objectives of the present article were focusing on the diagnosis of Clostridium perfringens from wound swabs from patients suffering from gas gangrene associated with antibiotics treatment and determining the best antibiotics that can inhibit the growth of Clostridium perfringens.Materials and methods: Four hundred isolates were collected from Fever Abbassia hospital in Cairo, Egypt. Clinical samples were collected from swab wounds of limbs. Samples were taken during the period of the first of Oct 2017 till the end of Oct 2018. From four hundred cases collected, Two hundred twenty-eight identified as gas gangrene (57%), One hundred twenty-eight identified as Clostridium perfringens type(A) (56.1%) confirmed by biochemical tests (Gram stain, Catalase test, Motility test, Skim milk test, Urease test, Indole test, Nagler reaction.) and Multiplex PCR. In this study, we are used different media such as (Thioglycalate broth, Reinforced Clostridial Agar, Blood agar, TryptoseSulfiteCycloserine Agar, Robertson's cooked meat media). The antibiotic sensitivity against CL.perfringens is assayed by two methods, disc diffusion Method and Determination of (MIC) using E-Test by following the reference standards established by the Clinical and Laboratory Standards Institute (CLSI) and British Society for Antimicrobial Chemotherapy (BSAC). The antimicrobial susceptibility test was performed for all of the isolates (128) by disc diffusion method using antibiotics (Chloramphenicol (30 µg /disc), Cefoxitin (10 µg /disc), Clindamycin (2 µg /disc),

Metrondiazole (5 µg /disc), Tetracyclin (30 µg /disc), Rifampin (5 µg /disc), Penicillin (10 µg /disc) and Piperacillin (30 µg /disc) and by E- test (MIC) (0.016-256µg/1ml) method using antibiotics (Chloramphenicol, Cefoxitin, Clindamycin, Penicillin G, Tetracycline and Piperacillin) on blood agar and incubate anaerobically in Anaerobic jar for 24 hrs. at 37 ˚C. As Rifampin and Metrondiazole breakpoints (E-Test) for *Clostridium perfringen*have not yet been established by either EUCAST or CLSI.Results and conclusion: In this study, isolates Clostridium perfringens from selective media TSC Agar or Blood agar and doing biochemical tests, the antibiotics susceptibility test showing that disc diffusion method and MIC method give the same result. Susceptibility tests were done on (128) isolates of CL.perfringens by E-Test to determine MIC.Our results revealed that the percentage of sensitivity of CL.perfringens to(Chloramphenicol , Cefoxitin, Clindamycin, Rifampin, Penicillin G, Metrondiazole and Tetracyclin) are *(53.9% , 53.1% , 52.3 % , 41.4 % , 35.9 % , 21.1 % , 14.1 %)* respectively . On the other hand, the data revealed that CL.perfringens was resistant to PRL (100%). Future advanced research should participate in the prevention of C. perfringens gas gangrene outbreaks.

INTRODUCTION

Clostridium perfringens (formerly known as *C. welchii,* or *Bacillus welchii*) is a gram-positive, rod-shaped, anaerobic, forming bacterium of the genus *Clostridium*(*Kiu R., Hall L. J, 2018*). Infections were due to *CL. Perfringens* are tissue necrosis, bacteremia, emphysematous cholecystitis, food poisoning and gas gangrene, which is also known as clostridial myonecrosis. *C. perfringens* can participate in polymicrobial anaerobic infections. *Clostridium perfringens* are commonly encountered in infections as a component of the normal flora. In this case, its role in disease is minor. ((Kiu*et al., 2017*) The virulence of this Gram-positive, spore-forming anaerobe is largely depending on its ability to produce at least 17 different toxins.

However, there is considerable variability in the toxin of different *C. perfringens* strains, *C. perfringens* isolates are divided into seven types (A–G) depending upon the toxin that produced such as alpha, beta, epsilon, and iota toxin. (Rood J. I, *et al.,* 2018). Gas gangrene is most often caused by bacteria called *Clostridium perfringens.* It also can be caused by group A streptococcus, *Staphylococcus aureus,* and *Vibrio vulnificus*, it results from unhygienic surgical practices and contaminated wounds with the bacteria. Most Individuals susceptible to gas gangrene are suffering from diabetes, or disease that obstruct blood flow such as atherosclerosis, multiple sclerosis (MS), (Onderdonk and Garrett, 2015). The protein synthesis inhibitors (eg, Clindamycin, Chloramphenicol, Rifampin and Tetracycline) may be more effective because they inhibit the synthesis of clostridial exotoxins and inhibit the local and systemic toxic effects of these proteins. There is improved efficacy of combination therapy of penicillin and clindamycin this combination can utilize in serious *Clostridial myonecrosis* (Gas gangrene) due to the ability of clindamycin's to inhibit toxins production (Giri B., Kole L., 2015).

MATERIALS AND METHODS

Microorganism: Four hundred isolates were collected from Fever Abbassia hospital in Cairo, Egypt. Clinical samples were collected from swab wounds of limbs. Samples were taken during the period of first of (Oct 2017 till the end of Oct 2018). Two hundred twenty-eight identified as gas gangrene (57%), One hundred twenty-eight identified as Clostridium perfringens.

Media Used to Isolate Bacteria:

Thioglycolyate Broth: is used for sterility testing of biologicals and for the cultivation of anaerobes, aerobes and microaerophiles, it had the following composition (g /L): Pancreatic digest of casein, 15.000**;** Yeast extract, 5.0; Glucose, 5.500; Sodium

Chloride, 2.500; L-Cystine, 0.500; Sodium thioglycollate, 0.500; Resazurin sodium, 0.001; Agar, 0.750 and PH 7.1 (Tille and Forbes, 2014).

Robertson's Cooked Meat:is used for the cultivation of aerobic, microaerophilic, and anaerobic microorganisms, especially Clostridium species. It supports the growth of both spore-forming and non-spore-forming obligate anaerobes. The Food and Drug Administration recommends its use in the enumeration and identification of *Clostridium perfringens* from food. it had the following composition (g /L): Cooked meat medium, 250.0**; Peptic** Digest of Animal tissue, 17.5; Glucose, 5.0; Sodium Chloride, 5.0; Yeast extract, 5.0; Iron fillings, 10.0; Hemin, 10.0; Vitamin K, 10.0 and PH 6.9 (Robertson, M., 1916).

Blood Agar*:* is used to growing a wide range of pathogens particularly those that are more difficult to grow. It is also required to detect and differentiate haemolytic bacteria and can differentiate between Clostridium spp. ,it had the following composition (g /L) : Beef extract, 10.0 **;**Peptone, 10.0 ; Glucose, 5.0 ; Sodium Chloride, 5.0 ; Agar, 15.0 ; Sheep blood, defibrinated 50.0 ml and PH 7.3 (Rebecaa Buxton, 2012).

Media Used for Bacterial Identification:

Reinforced Clostridial Medium(RCA): is a medium used for the cultivation and enumeration of anaerobes, especially Clostridium spp., it had the following composition (g /L) : Beef extract, 10.0 **;**Peptone, 10.0 ; Yeast extract, 3.0; Soluble Starch, 1.0; Glucose Monohydrate, 5.0 ; Cystine Hydrochloride, 0.5 ; Sodium Chloride, 5.0 ; Sodium Acetate, 3.0; Agar, 15.0 and PH 6.9 (Brian.Byrne*,et al.,* 2008).

Tryptose Sulfite Cycloserine Agar (TSC Agar): Solid selective and differential medium for isolation and presumptive identification of Clostridium perfringens, according to ISO standards(International Organization for Standardization), it had the following composition (g /L) : Tryptose, 15.0 **;**Soya Peptone, 5.0 ; Yeast extract, 5.0; Sodium metabisulfite, 1.0; Ferric ammonium citrate, 1.0 ; Agar, 18.0; D-Cycloserine 200 mg per vial; Egg Yolk Emulsion 100 mL per vial and PH 6.9 (Anwar Zuha*et al.,* 2019).

Skim Milk medium*:* used to detect the stormy fermentation produced by Clostridium perfringensm it had the following composition (g /L): Skim milk powder, 100.000 **(**Das *et al.,* 2012).

Tests Used for Bacterial Identification (Shahdadnejad Neda *et al.*, 2016):

Indole Test: To determine the ability of organism for the production of indole by the action of tryptophanase enzyme, it had the following composition (g /L): Pancreatic digest of casein, 10.0**;** Sodium Chloride, 5.0; Indole kovacs reagent **(**p-Dimethylaminobenaldehyde, 50.0 gm; Hydrochloric Acid 37% 250.0 ml; Amyi Alcohol, 750.0 ml) and PH 7.5 (Michael and Burton, 2011).

Motility Test:To detect the bacterial motility, it had the following composition (g /L):Tryptose, 10.0**;** Sodium Chloride, 5.0;Agar, 5.0and PH 7.2 (Michael and Burton, 2011).

Urease Test*:*is used to determine the ability of an organism to split urea, through the production of the enzyme urease, it had the following composition (g /L): Urea, 20.0**; Peptone**, 1.0; Monopotassium phosphate, 2.0; Glucose, 1.0; Phenol red, 0.012; Sodium Chloride, 5.0; Agar, 15.0 and PH 6.8 (Michael and Burton, 201).

Catalase Test*:*Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme, (Michael and Burton, 2011).

LecithinaseTest or NaglerReaction: is used for the identification of alpha-toxin of *Clostridium perfringens* ,it had the following composition (g /L) : Pancreatic Digest of Casein, 10.0 **;** Beef Extract, 3.0 ; Yeast extract, 1.0; Sodium chloride, 5.0; Egg Yolk Emulsion, 100ml ; Agar, 15.0 and PH 7.4 (Gamal A. Younis *et al.*, 2018).

Collection of Samples: Clinical samples were collected from patients by a sterile swab

of wound taken from the leg or hand. The swabs were cultured first on thioglycolyate broth and placed on an incubator for 24 hrs. at 37°C and then subcultured on cooked meat broth for 48 hrs. at 37°C under anaerobic condition.

Isolation of Bacteria:

On Blood Agar: After incubation of cooked meat broth then cultured on blood agar and incubate for 24 hrs. at 37°C under anaerobic conditions (Anaerobic jar).

On Reinforced Clostridial Agar*:* After incubation of cooked meat broth cultured on reinforced clostridial agar and incubate for 24 hrs. at 37°C under anaerobic conditions (Anaerobic jar).

On Tryptose Sulfite Cycloserine Agar (TSC Agar)*)*: After incubation of cooked meat broth cultured on TSC agar and incubate for 24 hrs. at 37°C under anaerobic conditions. (Anaerobic jar).

Identification of Cl.perfringens:

Cell Morphology*:* Bacterial cells were stained with Gram stain. After staining, the morphology of bacterial cells including shape and staining features was examined by optical light microscope.

Skim Milk Medium: Incubate skim milk tubes with a separate colony from TSC medium and incubate for 7 days at 37°C under anaerobic conditions (Anaerobic Jar).

Indole test*:* Inoculate the tryptophan broth with an isolated colony of the test organism (on TSC agar medium) in tryptophan broth. Incubate at 37°C for 24-28 hours under anaerobic conditions (anaerobic Jar), add 0.5 ml of Kovac's reagent to the broth culture.

Motility Test*:* Inoculate (an isolated colony of TSC agar medium) with a straight wire, making a single stab down the center of the tube to about half the depth of the medium. Incubate under the anaerobic condition (Anaerobic jar) incubate at 37°C for 24 hrs.

Urease Test: The broth medium is inoculated with a loopful of a pure culture of the test organism (from TSC agar medium); the surface of the agar slant is streaked with the test organism. Leave the cap on loosely and incubate the test tube at 35 °C under anaerobic conditions for 18 to 24 hours; unless specified for longer incubation.

Catalase Test: Transfer a small amount of bacterial colony to a surface of a clean dry glass slide using a loop or sterile wooden stick, Place a drop of 3% H_2O_2 on to the slide and mix.

NaglerReaction: Label and dry the egg yolk media plate and mark the plate into two halves. Inoculate 60 µl of *Clostridium perfringens* type A antitoxin in half of the plate, spread over the surface of agar using a spreader and allow to absorb and dry.

1. Mark the side of the plate in which the antitoxin is inoculated.
2. Streak the test organism in a straight line from the toxin-free agar half of the plate to toxin containing side. Repeat the same procedure with control strains on the same plate.
3. Incubate anaerobically at 37°C for 24 hrs.
4. Examine the plate for an opalescent halo around the inoculum and inhibition by antitoxin

Molecular Identification of the Selected Bacterial Isolates by Multiplex PCR (Polymerase chain reaction) (Tao,. **J *et al.*, 2020):**

Multiplex-PCR was applied on isolates of C. perfringens using primers mixture of alpha, beta, epsilon and iota toxins genes (Table 1) to investigate gene encoding Alpha toxins.

Table 1: Oligonucleotide primers sequences

Toxin	Primer	Sequence	Amplified product	Reference
Alpha toxin	*F*	GTTGATAGCGCAGGACATGTTAAG	402bp	**YOO *et al*., 1997**
	R	CATGTAGTCATCTGTTCCAGCATC		
Beta toxin	*F*	ACTATACAGACAGATCATTCAACC	236 bp	
	R	TTAGGAGCAGTTAGAACTACAGAC		
Epsilon toxin	*F*	ACTGCAACTACTACTCATACTGTG	541 bp	
	R	CTGGTGCCTTAATAGAAAGACTCC		
Iota toxin	*F*	GCGATGAAAAGCCTACACCACTAC	317 bp	
	R	GGTATATCCTCCACGCATATAGTC		

Source: Midland Certified Reagent Company- oilgos(USA).

Effect of Physical And Culture Conditions on Growth of CL.Perfringens:

Incubation Temperature*:* using well diffusion method, make well on blood agar plates & put 0.5 ml of a suspension of the pure colony (CL.perfringens) on the well, then incubated in anaerobic condition at different temperatures (30˚C, 37˚C, 40˚C, 45˚C and 50˚C) for 24 hrs. to study the effect of different temperatures on bacterial growth.

Initial pH: using well diffusion method, make well on blood agar plate & put 0.5 ml of a suspension of the pure colony (CL.perfringens) on the well, then incubated in anaerobic condition at different pH using 0.1 M HCL or 0.1 M NaOH and PH test strip (5.0, 6.0, 7.0 and 8) for 24 hrs. at *37°C*. to study the effect of different PH on bacterial growth.

Incubation Period: using well diffusion method, make well on blood agar plate & put 0.5 ml of a suspension of the pure colony (CL.perfringens) on the well, then incubated in anaerobic condition at different incubation periods(24hrs. , 36hrs. , 48hrs. and 72hrs.) at *37°C* to study the effect of different incubation period on bacterial growth.

Antimicrobial Test: The antimicrobial susceptibility test was performed for all of the isolates(128) by disc diffusion method using antibiotics (Chloramphenicol(30mcg), Penicillin(10U/mcg) , Metronidazole (5 mcg), Tetracyclin (30 mcg) , Cefoxitin(10 mcg) , piperacillin (30 mcg) , Rifampin (5 mcg) and Clindamycin (2 mcg) , the entire diameter of the zone was measured including the diameter of the disk and the results were interpreted according to the recommendations of (BSAC(British Society for Antimicrobial Chemotherapy))and (EUCAST). (European Committee on Antimicrobial Susceptibility Testing) and by MIC (Minimum inhibitory concentration).

By (E-test) method using antibiotics (Chloramphenicol, Penicillin, tetracyclin, Cefoxitin, piperacillin, Clindamycin). As Rifampin and Metrondiazole breakpoints for *Clostridium perfringen*have not yet been established by either EUCAST (European Committee on Antimicrobial Susceptibility Testing) or CLSI (Clinical and laboratory standards institute). Antibiotic Combinations therapy was done of all possible pairs of antibiotics were tested by placing antibiotic disks at a distance of 20 mm from each other (center to center). After 24 hours of incubation, if a synergistic effect was present among two antibiotics, an inhibition zone was formed between their disks, (EUCAST ,2017), (BSAC, 2015).

Statistical Analysis:

All experiments were repeated two times, the data shown in tables and figures were the mean values of the experiments. Data were analyzed using SPSS version 16 (SPSS Inc., Chicago, IL, USA). Qualitative variables were presented as no. & %.

RESULTS

Out of 400 samples, age (16-30 years) was 36 (9%), age (31-50 years) was 86 (21.5 %), age (51-70years) was 170 (42.5%), age (71- 85 years) was 108 (28%). Out of 400 samples, Male was 177 (44.3%), Female was

223 (55.8%), inpatient was 178 (44.5), outpatient was 222 (55.5%).

Two hundred twenty-eight (57%) from 400 samples were identified as gas gangrene**.** Out of two hundred twenty-eight, one hundred twenty-eight (56.1%) identified as Clostridium perfringens type (A).

Identification of Clostridium Perfringens: By using different media and biochemical tests, we were able to identify Clostridium perfringens type (A) as shown in Table (2).

Table 2: Show identification of Clostridium perfringens based on morphological, cultural and biochemical properties.

Tests	Clostridium perfringens
Growth on cooked meat medium	Ferment meat (carbohydrate)and produce acid & gas
Growth on blood agar	Large, smooth and convex colony with a double zone of haemolysis (beta haemolysis effect)
Growth on TSC agar	Black colony (due to reduction of sulfite) with opaqe zone(due to lecthinase effect)
Growth on reinforced clostridial agar	White convex colony
Catalase	Negative (no bubbles after adding H2O2)
Indole	Negative (No red color, do not produce tryptophanase enzyme)
Skim milk	Stormy fermentation (due to lactose fermentation and hydrolyze casein)
MIO	Non-motile (growths at a straight line, have sharply defined margins and leave the surrounding medium clearly).
Urease	Negative (No pink color, do not produce urease enzyme)
Nagler reaction	Positive (Lecithinase)
Gram stain under a microscope	G (+) rod shape with a rounded end

Identification of CL.perfringens type (A) by multiplex PCR:

Multiplex-PCR was applied on isolates of C. perfringens to investigate genes encoding the alpha-toxin. All C. perfringens type(A) contains the alpha-toxin gene only. Fig (1).

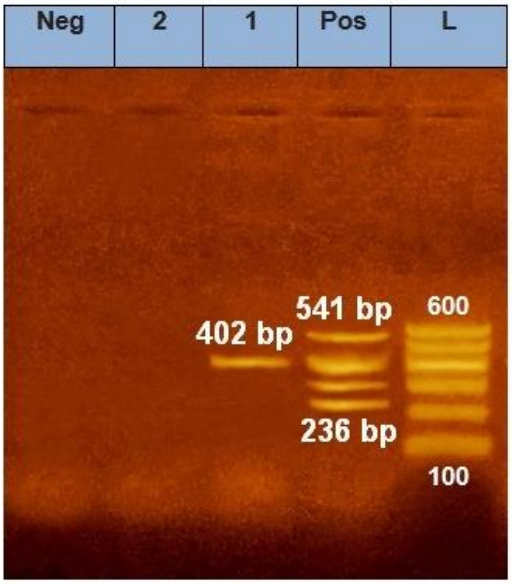

Fig 1: The multiplex-PCR showing the detection of Alpha toxin encoding genes of C. perfringens type (A) isolates.

Factors Effecting On The Growth of CL.perfringens:

This study revealed that the optimum condition for the growth of CL.perfringens at PH 7 , incubation period 24 hrs. and temperature 37°C as shown in Tables(3,4, &5).

Table 3: Effect of different temperatures on the growth of CL.perfringens**.**

Incubation temperature	Growth of Bacteria (cm)
30°C	2.3
37°C	4.7
40°C	4.0
45°C	0
50°C	0

Table 4: Effect of different PH on the growth of CL.perfringens.

PH	Growth of Bacteria (cm)
5	0
6	0
7	4.7
8	0

Table 5: Effect of different incubation periods on the growth of CL.perfringens**.**

Incubation period	Growth of Bacteria (cm)
24 hrs	4.7
36 hrs.	2.3
48 hrs.	2.2
72 hrs.	0

Antimicrobial Susceptibility Test:

First Method, Disc Diffusion:

Susceptibility tests were done on (128) isolates of CL.perfringensby disc diffusion.Our results revealed that the percentage of sensitivity of CL.perfringens to C , FOX, DA, RA, PG, MET and TE are (53.9% , 53.1% , 52.3 % , 41.4 % , 35.9 % , 21.1 % , 14.1 %) respectively . On the other hand, the data revealed that CL.perfringens was resistant to PRL (100%) as shown in (Table 6) and (Fig 2) (Control antibiotics wereClindamycin dosage of 2 mcg , Penicillin G dosages of 10 mcg and Metrondiazole dosage of 5 mcg)**.**

Table 6: Show the effect of antibiotics on the growth of Clostridium perfringens by Disc diffusion.

Antibiotic	Sensitive		Resist	
	No	%	No	%
Chloramphenicol(C)	69	53.9	59	46.1
Cefoxitin (FOX)	68	53.1	60	46.9
Clindamycin (DA)(Control)	67	52.3	61	47.7
Rifampin (RA)	53	41.4	75	58.6
Penicillin G(PG)(Control)	46	35.9	82	64.1
Metrondiazole (MeT)(Control)	27	21.1	101	78.9
Tetracyclin (TE)	18	14.1	110	85.9
Piperacillin (PRL)	0	0	128	100

Each value represents the mean (n=128)

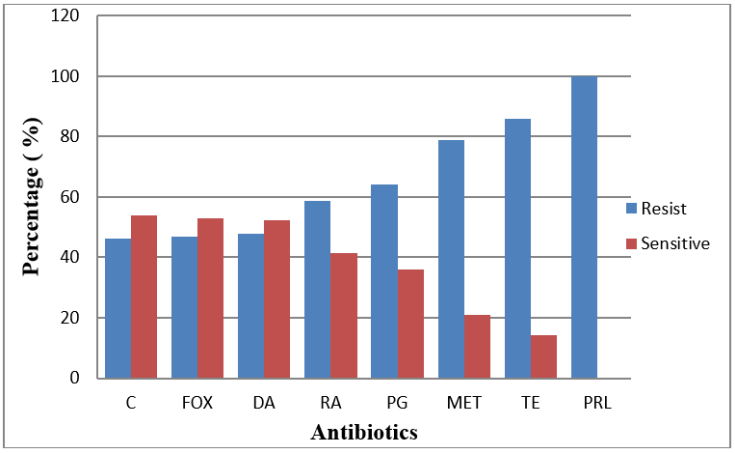

Fig. 2: The percentage susceptibility and resistance of antibiotics (C, FOX, DA, RA, PG, MET, TE, PRL) used in this study for *Clostridium perfringens.*

Second Method, E-Test (Determination of MIC):

Susceptibility tests were done on (128) isolates of CL.perfringens by E-Test to determine MIC .Our results revealed that the percentage of sensitivity of CL.perfringens to(C , FOX, DA, PG and TE) are (53.9 % , 53.1 % , 52.3 % , 35.9 % , 14.1 %) respectively . On the other hand, the data revealed that CL.perfringens was resistant to PRL (100%) as shown in Table (7) and Fig (3). As Rifampin and Metrondiazole breakpoints for Clostridium perfringen have not yet been established by either EUCAST (European Committee on Antimicrobial Susceptibility Testing) or CLSI (Clinical and laboratory standards institute).

Table 7: Show the effect of antibiotics on the growth of Clostridium perfringens by MIC (E – Test).

Antibiotic	Sensitive		Resist	
	No	%	No	%
Chloramphenicol(C)	69	53.9	59	46.1
Cefoxitin (FOX)	68	53.1	60	46.9
Clindamycin (DA)(Control)	67	52.3	61	47.7
Penicillin G(P)(Control)	46	35.9	82	64.1
Tetracyclin (TE)	18	14.1	110	85.9
Piperacillin (PRL)	0	0	128	100

Each value represents the mean (n=128)

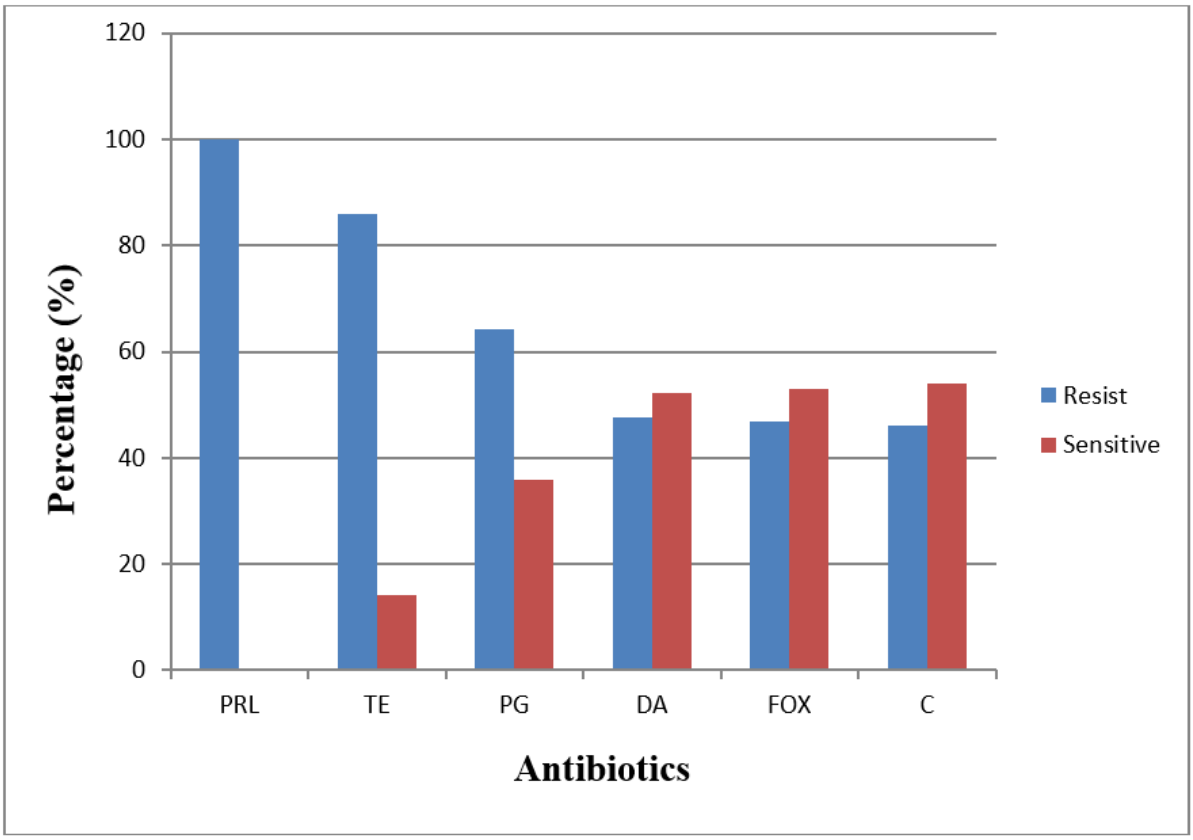

Fig.3: The percentage susceptibility and resistance of antibiotics (PRL, TE, PG, DA, FOX, C) used in this study for *Clostridium perfringens* by E-Test.

The Combination Therapy:

This experiment was carried out showing the effect of Antibiotic combination therapy on CL.perfringens. Antibiotic Combinations therapy was done of all possible pairs of antibiotics were tested. In a few cases that were resistant to all antibiotics were used, the combination was the good treatment for Cl.perfringens(C+DA, C+RA, C+FOX and C+TE). But using one antibiotic was more effective than a combination of two antibiotics for C.perfringens. Tables (8), (9).

Sample (1) & Sample (2): Two patients have C.perfringens type (A) that caused gas gangrene (as examples).

Table 8: The evaluation of inhibition zone diameter (mm) of Sample (1) in antibiotic susceptibility test and antibiotics combination test.

Antibiotic (µg /disc)	Inhibition zone diameter (mm)	Antibiotics Combinations (µg /disc)	Inhibition zone diameter (mm)
RA	32	RA+C	15
		RA+MET	15
		RA+TE	11
		RA + FOX	10
		RA + DA	0
		RA + PRL	0
		RA+PG	0
PG	27	PG + MET	18
		PG+C	16
		PG+FOX	13
		PG+TE	11
		PG+DA	9
		PG+ RA	0
		PG + PRL	0
TE	23	TE+DA	11
		TE+RA	11
		TE+PG	11
		TE+FOX	10
		TE+C	9
		TE+MET	0
		TE+PRL	0
C	22	C +PG	16
		C +MET	16
		C + RA	15
		C + FOX	15
		C +PRL	10
		C + DA	10
		C + TE	9
FOX	20	FOX+C	15
		FOX+PG	13
		FOX+DA	12
		FOX + TE	10
		FOX + MET	10
		FOX + RA	10
		FOX+PRL	0
MET	20	MET + PG	17
		MET+C	16
		MET+RA	15
		MET+FOX	10
		MET+DA	10
		MET+ TE	9
		MET + PRL	0
DA	14	DA+FOX	11
		DA+TE	11
		DA+MET	10
		DA+C	10
		DA+PG	9
		DA+RA	0
		DA+PRL	0
PRL	5	PRL +C	0
		PRL +FOX	0
		PRL + DA	0
		PRL + RA	0
		PRL +PG	0
		PRL + MET	0
		PRL + TE	0

Each value represents the mean (n=2)

Table 9: The evaluation of inhibition zone diameter (mm) of Sample (2) in antibiotic susceptibility test and antibiotics combination test

Antibiotic (µg /disc)	Inhibition zone diameter (mm)	Antibiotics Combinations (µg /disc)	Inhibition zone diameter (mm)
C	23	C+RA	30
		C+DA	24
		C+TE	23
		C+FOX	21
		C + PG	5
		C + MET	5
		C+PRL	5
FOX	18	FOX + C	21
		FOX + PG	11
		FOX + DA	10
		FOX + TE	10
		FOX + MET	9
		FOX + PRL	5
		FOX + RA	0
DA	9	DA+C	24
		DA+RA	11
		DA+PG	11
		DA+FOX	10
		DA+TE	7
		DA+MET	0
		DA+PRL	0
PRL	5	PRL+C	5
		PRL +FOX	5
		PRL + DA	0
		PRL+ RA	0
		PRL +PG	0
		PRL + MET	0
		PRL + TE	0
RA	0	RA +C	30
		RA+DA	11
		RA+FOX	0
		RA+PG	10
		RA+MET	10
		RA+TE	10
		RA+PRL	0
TE	0	TE+C	23
		TE+RA	10
		TE+FOX	10
		TE+DA	7
		TE+PG	0
		TE+MET	0
		TE+ PRL	0
PG	0	PG+FOX	11
		PG+DA	11
		PG+RA	10
		PG+C	5
		PG+MET	0
		PG+TE	0
		PG + PRL	0
MET	0	MET+RA	10
		MET+FOX	9
		MET+C	5
		MET+DA	0
		MET+PG	0
		MET+ TE	0

Each value represents the mean (n=2)

DISCUSSION

Clostridium perfringens, a rapid-growing pathogen known to secrete an arsenal of >20 virulent toxins, has been associated with intestinal diseases in both animals and humans throughout the past century. *Clostridium perfringens* (formerly known as *Bacillus aerogenes capsulatus, Bacillus perfringens, Bacillus welchii* , or *Clostridium welchii*) is a Gram-positive, spore-forming, anaerobic, rod-shaped bacterium. It was first isolated and identified as a novel bacterium in 1891 by William H. Welch from the autopsy of a 38-year-old man, where gas bubbles were observed within infected blood vessels. This gas-forming trait (the original name *Bacillus aerogenes capsulatus*, 'aerogenes' literally means 'air producing in Latin) was later linked to gas gangrene symptoms seen in British and French Soldiers during World War I (*Kiu R., Hall L. J, 2018*).

Gas gangrene is synonymous with myonecrosis and is a highly lethal infection of deep soft tissue, caused by *Clostridium* species, with *Clostridium perfringens* being the most common. (Takehara M., 2018.)

C.perfringens cause 80% to 90%, of gas gangrene cases, but other species can cause infection. In order of prevalence, they are *Clostridium novyi(40%), C.septicum (20%), C.histolyticum (10%), Clostridium btifermentans (10%), Clostridium fallax (5%) and C.sordelli* .

These organisms are in the soil and organic waste especially if contaminated with fecal material. The virulence of the organism depends on the exotoxins produced; Clostridium perfringens is the most pathologic with 17 known toxins, with the most toxic being the alpha-toxin, a lecithinase. Alpha toxin is a phospholipase (lecithinase) that breaks down cell membranes triggering platelet aggregation, thrombosis, and histamine release. Also present are collagenase, hyaluronidase, hemagglutinins, and hemolysins. (Leiblein,M *et al.,* 2020)

Károly Péter Sárvári and Dzsenifer Schoblocher,(2020) showed that C. perfringens and other gas gangrene forming Clostridium species are part of the human gut and vaginal microbiota, they can cause serious and sometimes fatal infections. They found that Amoxicillin/Clavulanic acid, Cefoxitin, Imipenem, Meropenem and Metronidazole had excellent activity against these isolates as had Tigecyclin(a tetracycline antibacterial) against C. perfringens. The rates of Penicillin and Clindamycin resistance were low for C. perfringens.In the present study, Cefoxitin and Clindamycin showed excellent activity against C.perfringens isolates with percentages (53.1%, 52.3%).On the other hand, Penicillin and Metronidazole are poorly active against C.perfringens with percentage(35.9%, 21.1%)

Clostridium perfringens was first discovered by William H. Welch, MD, in 1891 at The Johns Hopkins Hospital after an autopsy on a 38-year-old man and was initially named *Bacillus aerogenes capsulatus*. Later, it was known as *Bacillus welchii* before finally renamed to *Clostridium perfringens*, which is derived from Latin for "burst through. (Yao, Phil, and Pavan Annamaraju, 2020) showed that early antibiotic treatment with Penicillin G and Clindamycin, Tetracycline, or Metronidazole in combination with surgical debridement of necrotic tissue may help to prevent death. A suspicion of clostridial myonecrosis warrants a surgical consultation. Consultation should not be delayed waiting for laboratory or imaging results. Broad-spectrum antibiotics should be promptly administered. Our findings showed that antibiotics combination is a poor treatment choice for *C.perfringens* except in some cases antibiotics combination therapy as (C+RA, C+DA, C+TE and C+FOX) is better than using one antibiotic.

Many studies had been reported, (Mohiuddin, Mudassar, et al. 2020) showed that:

(a)-all isolates of C.perfringens type (A) (Isolated from feces of Sheep and goats in Pakistan) are susceptible to Penicillin (10

μg), Rifampin (5 μg) and Ceftiofur (30 μg) with percentage (100%).

(b) -(72%) of C.perfringens type (A) were found to be resistant to Tetracycline (30 μg).

(c)-Linezolid (30 μg) and Chloramphenicol (30 μg) showed significant susceptibility against (57%) of CL. perfringens isolates

(d)-While Ciprofloxacin (5 μg) and Erythromycin (15 μg) showed intermediate susceptibility.

In the present study Chloramphenicol (30 μg) showed significant susceptibility against (53.9 %) of C.perfringens while Rifampin (5 μg) and Penicillin (10 μg) wereshowed moderate susceptibility against (41.4%, 35.9%) of C.perfringens isolates, respectively. Tetracycline (30 μg) showed poor susceptibility with a percentage (14.1%).

Benard O Aliwa and Kaindi Dasel Wambua Mulwa (2019) reported that *CL.perfringens* (isolated from raw camel milk) was resistant to many antibiotics as Ampicillin 25µg (61.02%), Sulphamethazole 200µg (47.46%), Clotrimazole 25µg (45.76%), Streptomycin 10µg (44.07%), Chloramphenicol 30µg (42.37%), Kanamycin 30µg (40.68%), Tetracycline 25µg (37.29%), and Gentamycin 10µg (35.59%).

Antibiotic resistance testing was performed by the Kirby–Bauer disc diffusion method.Mehdi and Wannas(2017) in this study antibiotics disc diffusion methods were performed showing that *C.perfringens* is highly susceptible to Chloramphenicol (1µg\disc), Ampicillin (25µg\disc), Metronidazole (5 µg\disc) and Clindamycin (10 µg\disc) while was resistant to Gentamycinm (10µg\disc), Erythromycin (15 µg\disc) and Colistin (10 µg\disc).

Akhi et al., (2015) and Saadia et al., (2020) in these studies antibiotics susceptibility tests were determined by E-test strip according to CLSI guidelines for anaerobic susceptibility testing CL.perfringens (which is isolated from faeces) is highly susceptible to beta-lactam antibiotics because this bacterium does not produce beta-lactamase. CL.perfringens isolated in this study were 100% sensitive to Penicillin, Cefoxitin and Metronidazole.

Finally, the obtained results in the present study are in agreement with previously reported findings as (KárolyPéterSárvári and Dzsenifer Schoblocher, 2020), Mohiuddin, Mudassar, et al., 2020) and (Mehdi and Wannas, 2017) with few exceptions showing that the best antibiotics active for C.perfringens type (A) are Chloramphenicol, Cefoxitin, Clindamycin, Rifampin, Penicillin and Metronidazole, respectively.

Our results are in disagreement with (Yao, Phil, and Pavan Annamaraju, 2020) at which findings showed that antibiotic combination therapy is a poor choice of treatment for *C.perfringens.*

Conclusion

This study revealed that the gas ganrene is most often caused by Clostridium perfringens type (A). The results obtained from the present investigation indicate that the effective antibiotics that can inhibit the growth of *CL.perfringens*, thus help us for gas gangrene treatment (Chloramphenicol, Cefoxitin, Clindamycin, Rifampin, penicillin, Metronidazole, tetracycline). Future advanced research should explain the epidemiology of enterotoxigenic C.perfringens and also participate in the prevention of C.perfringens gas gangrene outbreaks.

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