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ISOLATION AND IDENTIFICATION OF SOME ENTEROPATHOGENIC BACTERIA IN SHEEP WITH SPECIAL REFERENCE TO *CLOSTRIDIUM PERFRINGENS* AND THEIR SUSCEPTIBILITY TO DIFFERENT ANTIMICROBIALS AND GARLIC OIL

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ABSTRACT

Sheep and lambs are threatened by a number of infectious diseases, among which enterotoxaemia that caused by *Clostridium perfringens* and other aerobic bacteria. A total of 54 faecal swabs (10 diarrhoeic, 44 apparently healthy) were collected from sheep of different ages in different localities in Alexandria governorate and examined bacteriologically. The results revealed that *Clostridium perfringens, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* could be isolated in a percentage of 44.5, 31.5, 11.1 and 9.3% respectively. Pathogenicity tests were carried out for aerobic bacteria and toxigenic typing of *C. perfringens* by multiplex PCR was performed which revealed that 25% of isolated *C. perfringens* were type A. An *in vitro* sensitivity test of *C. perfringens type A* was carried out against different antimicrobials and garlic oil, the results showed that it was sensitive to Ciprofloxacin (5µg), Amoxicillin/Clavulaniacid (10µg), Erythromycin (15 µg), Flumequine (30µg), Gentamycin (10µg), Chlormphenicol (30µg), Vancomycin (µg); Nitrofurantoin (300µg), intermediately sensitive to tetracycline (30µg), Enerofloxacin (5µg), and resistant to Amoxicillin (10µg), Neomycin (30µg) Streptomycin (10µg), Trimethoprim / sulfamethoxazole (25µg) and garlic oil. Proper hygienic measures in the sheep farms, Vaccination of dams at last stage of parturition against *Clostridium perfringen* type A for control of enterotoxaemia in sheep, feeding animals with balanced ration are recommended to avoid the infection of sheep with such enteropathogens.

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Key words: *Sheep, C. perfringens, E. coli, S. aureus, Ps. Aeruginosa, garlic oil, antimicrobials.*

INTRODUCTION

Small ruminants play an important role in nutrition and income of people around the world, they serve primarily as a source of meat, milk, skin and wool (Mbilu, 2007).

In Egypt, sheep are reared all over the country in close association with man in both rural and urban areas where sheep population was reported to be almost 3.5 million (FAO, 1996). Sheep and lambs are threatened by a number of infectious diseases, among which enterotoxaemia is believed to be the most important disease resulting in heavy economic losses (Lewis, 2007). Enterotoxaemia is also called ‘ over eating disease’ as the infection almost follows changes in diet from relatively poor diets to rich diets or over eating in fattening small ruminants and sudden death is the most common symptoms of this infection (Gyles and Thoen, 2004). It is not caused by over eating itself but it is caused by the toxins produced by *Clostridium perfringens* and other aerobic bacteria (Browning, 2007). *Clostridium perfringens* is an anaerobic Gram positive spore forming bacilli found universally in soil, manure and present in certain amounts in the intestinal tract, when animal over eats more than ¾ pound/head/day will lead to excessive bacterial growth and allow the bacteria to produce lethal amounts of toxins which absorbed into the animal systems (Nibal, 2012). Strains of *C. perfringens* are divided into five types (A, B, C, D and E), all types producing a different set of toxins (α, β, ε, ι) (McDonel, 1986).

C. perfringens type A is an anaerobic spore forming rod that can exist for several months in soil after being discharged in the faeces and present in small numbers in the digestive tract of healthy animals causing enteric disease in sheep (Songer, 1996; Quinn *et al.,* 2000 and Ahsani *et al.,* 2011). *C.perfringens* type A produce α toxin, this toxin is a phosphor-lipase in nature which is lethal and necrotizing. It causes lysis and disrupting cell membranes leads to cell death. Also causes increase vascular permeability through endothelial damage and necrosis at the tips of villi of intestine (Feldman, 2000).

Escherichia coli (*E. coli*) is enteric Gram-negative, rod-shaped, flagellated, motile, oxidase negative, facultative anaerobe and is classified under the family Enterobacteriaceae (Buxton and Fraser, 1977), it produces epticaemia and diarrhoea in a wide range of hosts including lambs (Paul *et al.,* 2010).

The *E. coli* infection is a disease of economic importance in sheep as the infection leading to dramatically decline in wool and meat production (Purkayastha, 2010).

Al- Mashat and Taylor (1983) isolated (*clostridia perfringens* type A and *E. coli* from the necrotic haemorrhagic small intestine of ewe that died after developing diarrhea. Nibal (2012) isolated *C. perfringens* mixed with *Staphylococcus aureus* from lambs showing symptoms of enterotoxaemia. Sharif *et al.* (2005) documented that *E. coli, C. perfringens* and *S. aureus are* the bacterial causes of neonatal mortalities in sheep and goat.

Pseudomonas aeruginosa is the most pathogenic species of *Pseudomonas* as it produce an enterotoxin that is responsible for gastroenteric disorder such as darrhoea and enteritis in animals (Quinn *et al.,* 2002).

PCR has been applied in several areas since the late 1980s, this method has been highlighted as a rapid and accurate method for the detection of low copy numbers of genes. Also, the sensitivity and specificity of this method were confirmed by amplification of specific target DNA under a unique conditions. This method is more accurate and faster than identification of *C. perfringens* by seroneutralization with mice or guinea pigs (Daube, 1994).

The development and spread of resistant microbes diminishes the effectiveness of the drugs (WHO, 1999), so searching of natural antibacterial is of great value.

Allium vegetables, particularly garlic (Allium sativum L.) exhibit a broad antibiotic activity against both Gram-positive and Gram-negative bacteria (Whitemore and Naidu, 2000). The raw juice of garlic was effective against many common pathogenic bacteria (Kumar and Sharma, 1982), against the strains that have become resistant to antibiotics (Jezowa *et al.,* 1966) and even toxin production by some pathogenic strains was prevented by garlic (Dewitt *et al.,* 1979). Therapeutic effect of garlic is possible because of its oil- and water- soluble organosulfur compounds (Thiosulfinates, e.g. allicin), which are responsible for its typical odour and flavour; and play an important role in the antibiotic activity of garlic (Srinivasan *et al.,* 2009). Feldberg *et al.* (1988) showed that allicin exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis.

There is extensive literature on the antibacterial effects of garlic juice, aqueous and alcoholic extracts, lyophilized powders, steam distilled oil and other commercial preparations of garlic (Deresse, 2010).

The present study was aimed to investigate some enteropathogenic bacteria in sheep, isolation and toxigenic typing of *C. perfringens* by multiplex PCR and *in vitro* studying of its susceptibility to different antimicrobials and garlic oil.

MATERIALS and METHODS

Collection of samples:

Fifty four faecal swabs were collected from diarrhoeic (10) and apparently healthy (44) sheep of different ages in different localities of Alexandria governorate by using sterile cotton swabs immersed in nutrient broth as a transported media for aerobic bacteria and another swabs immersed in thioglycolate broth as a transported media for *C. perfringens* kept in an ice box and transported to the lab with a minimum time of delay for bacteriological examination.

Isolation and identification of bacteria:

The faecal swabs maintained in nutrient broth was inoculated into trypticase soya broth, Brain heart infusion broth, and Selenite F broth and incubated aerobically at 37ºc for 18 – 24 hours and the faecal swabs maintained in thioglycolate broth was inoculated into cooked meat broth for isolation of *Clostridium perfringens* and incubated anaerobically with an anaerobic gas-pack system (Oxoid) at 37ºc for 48 hrs. A loopful from aerobically incubated liquid media was sub cultured on 5% sheep blood agar, Brain heart infusion agar (BHIA), MacConkey agar, Salmonella Shigella (S.S), Eosin methyline blue agar (EMB) and Mannitol salt agar (MSA) and incubated aerobically at 37ºc for 18–48 hrs. Another loopful was taken from anaerobically incubated cooked meat medium subcultured on tryptose sulfite cycloserine agar (TSC) and 5 % sheep blood agar then incubated anaerobically at 37ºc for 24-48 hrs.

The isolated colonies of aerobic bacteria were picked up, purified and streaked on slope agar as stock culture for identification (morphologically by microscopical examination and cultural characteristics; and biochemically by Oxidase, Catalase, motility, Indole, Citrate, Methyl red, Voges Proskauer, Haemolysis on sheep blood agar, slide coagulase, sugar fermentation, reaction on triple sugar iron agar) identification according to CruickShank *et al.* (1975), *Escherichia coli* was identified biochemically with API A12 (Oxoid-Microbact GNB-Australia) The pure colonies were picked up and inoculated in semisolid agar for preservation and motility.

The typical colonies of *C. perfringens* showing dual hemolysis on blood agar and black colonies on TSC were picked up and subcultured. The subcultured colonies were identified morphologically by microscopical examination (after Gram staining) and cultural characteristics (shape, size, colour and pattern of haemolysis on blood agar that showing an

inner, complete zone of hemolysis is caused by PFO (perfringolysin O) and the less complete outer zone is caused by CPA (*C. perfringens* α toxin)); and biochemically by motility, nitrate reduction and gelatine / lactose fermentation (Cruick Shank *et al.*, 1975), then the purified colonies were inoculated in cooked meat media for preservation and further identification by polymerase chain reaction technique (multiplex PCR).

Detection of pathogenicity of isolated aerobic bacteria:

In vitro pathogenicity of the isolated *E. coli* was detected by inoculation of the identified isolates on Congo red agar medium which differentiated between pathogenic and non pathogenic *E. coli*, as pathogenic *E. coli* grew as red colonies while non pathogenic *E. coli* grew as white colonies (Berkhoff and Vinal, 1986).

Pathogenic *Pseudomonas aeruginosa* were identified by cyanine (blue) pigment produced on nutrient and MacConkey agar, alpha haemolysis on blood agar and growing of red colonies on Congo red agar medium.

Pathogenic *S. aureus* were identified by its growth on (MSA) and, beta-haemolysis on blood agar and positive slide coagulase test using rabbit plasma with EDTA.

Detection of *Clostridium perfringens* types by Multiplex PCR of toxin genes:

DNA extraction, specific primers and PCR technique were carried out according to Yoo *et al*. (1997).

DNA extraction:

Boiling method was followed as four to five colonies of *C. perfringens* grown on a blood agar plate were suspended in 0.5 ml of distilled water and the mixture was boiled for 10 min. The pellets were removed by centrifugation at 12000 x *g* for 10 min, and the supernatant was used as template DNA in PCR.

Primers:

Specific primers corresponding to each toxin were tabulated in table (1).

PCR technique:

PCRs were performed in 50 µl mixture containing 25 µl Fermentas Dream Taq Green Master Mix, 0.5 µl of each primer, 4µl DNA template and molecular DNase-RNase free water to complete a final reaction of 50 µl. Amplification was carried out in thermocycler using the following program:

5 min at 94ºC, followed by 30 cycles consisting of 1 min at 55ºC, 1 min at 72ºC, and 1 min at 94ºC and then an extra extension step of 72ºc for 2 min The amplified products were separated by electrophoresis in a 1.5% agarose gel added with 0.5 µg/ml ethidium bromide (Sigma). Gels were visualized under UV transilluminator.

Table 1: Nucleotide sequences of primers and length of amplified products of *C. perfringens* toxin genes.

Primer (direction)	Nucleotide sequence Location	Size(bp) of amplified products
CPA (alpha toxin)		
Forward	5'-GTTGATAGCGCAGGACATGTTAAG-3'	402
Reverse	5'-CATGTAGTCATCTGTTCCAGCATC-3'	
CPB (beta toxin)		
Forward	5'-ACTATACAGACAGATCATTCAACC-3'	236
Reverse	5'-TTAGGAGCAGTTAGAACTACAGAC-3'	
CPE (epsilon toxin)		
Forward	5'-ACTGCAACTACTACTCATACTGTG-3'	541
Reverse	5'-CTGGTGCCTTAATAGAAAGACTCC-3'	
CPI (iota toxin)		
Forward	5'-GCGATGAAAAGCCTACACCACTAC-3'	317
Reverse	5'-GGTATATCCTCCACGCATATAGTC-3'	

***In vitro* sensitivity test of isolated *C. perfringens* to antimicrobials:**

Agar disc diffusion method was carried out according to **(*Bauer et al., 1966)*** with some modifications:

enerofloxacin (5µg), ciprofloxacin (5µg), flumequine (30µg), chlormphenicol (30µg), gentamycin (10µg) and trimethoprim-sulfamethoxazole (25) obtained from Oxoid laboratories were distributed over the surface of TSC agar plates swabbed with the inoculum of each isolate separately and incubated at 37ºc for 18-24 hours then the diameter of inhibition zone was measured and interpreted according to **Neo-**Sensitabs (2007) as shown in table (4).

***In vitro* sensitivity test of the isolate to garlic oil:**
The garlic oil used in the study was obtained from El- Captain Company (Cairo- Egypt). Disc diffusion assay was followed according to Hood *et al*. (2003)

with some modifications:

1. Ten ml Tween 80 in sterile TSC agar (final concentration of Tween 80 of 0.1% and 1%) were poured on to a 10 ml prepared TSC agar plate.
2. An over night culture of bacteria (0.1 ml) was spread over the surface of the agar plate using a sterile glass rod and incubated at 37ºc for 30 min.
3. Tween 80 (final concentration of 0.5%, 1%, 2.4% or 5%) was added to the oil prior to application to the susceptibility disc.
4. Ten µl of oil treated with Tween 80 was added on susceptibility discs that forming from sterile blotting paper of 6 mm diameter.
5. The oil impregnated discs were placed on the surface of the agar plate.
6. The agar plate was incubated over night at 37ºc and the zones of bacterial inhibition were recorded.

RESULTS

Table 2: Percentage of pathogenic bacteria isolated from faecal swabs of examined sheep (n= 54).

Isolate	Diarrhoeic (10)		Apparently healthy (44)		Total (54)	
	Positive	%	Positive	%	positive	%
E. coli	6	60	11	25	17	31.5
Ps. aeruginosa	1	10	4	9.1	5	9.3
S. aureus	2	20	4	9.1	6	11.1
C. perfringens	8	80	16	36.4	24	44.5

Table 3: Toxigenic types of isolated *C. perfringens* resulted from multiplex PCR.

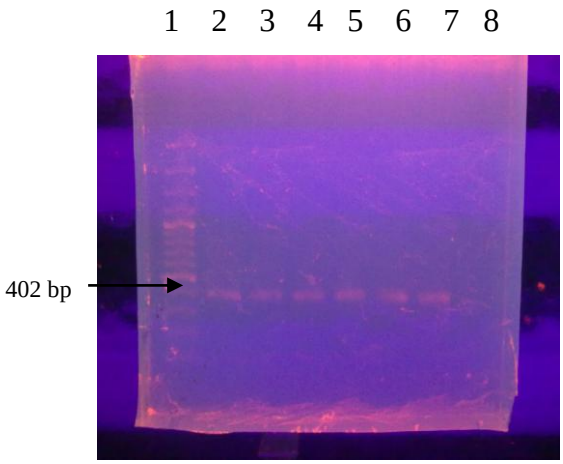
Strain	*C. perfringens* toxin-encoding genes					Isolated type	
	+ve	%	cpa (α toxin)	cpb (β toxin)	cpe (ε toxin)	cpi (ι toxin)	
Toxigenic	6	25	0	0	0	0	A
Non toxigenic	18	75	0	0	0	0	-

Number of *C. perfringens* strains= 24

Table 4: *In vitro* sensitivity test of *C. perfringens* to antimicrobials and garlic oil

Antimicrobial	Inhibitory zone diameter (mm)	Interpretation
Amoxicillin (10µg)	0	R
Chlormphenicol (30µg)	38	S
Vancomycin (µg)	28	S
Enerofloxacin (5µg)	18	I
Flumequine (30µg)	38	S
Gentamycin (10µg)	18	S
Neomycin (30µg)	0	R
Nitrofurantoin (300µg)	28	S
Streptomycin (10µg)	0	R
Tetracycline (30µg)	12	I
Trimethoprim/ sulfamethoxazole (25µg)	0	R
Ciprofloxacin (5µg)	36	S
Amoxicillin/Clavulanic acid (10µg)	24	S
Erythromycin (15µg)	42	S
Garlic oil	0	R

S=Sensitive I=Intermediatly sensitive R=Resistant



Amplification of *C. perfringens* toxin genes by multiplex PCR. Lane 1, DNA size marker (100bp ladder); lane 2, 3, 4, 5, 6, 7 *C. perfringens* type A (alpha toxin); lane 8 negative control.

DISCUSSION

In sheep, enterotoxemia causes considerable economic losses to the sheep industry due to high fatality rates, increased treatment costs, and decreased productivity (Greco *et al.*, 2005). The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon, and iota toxins) and other toxins, including enterotoxin (Hatheway, 1990; Stubbings, 1990). Strains of *C. perfringens* are divided into five types (A, B, C, D and E), all types producing a different set of toxins (α, β, ε, ι) (McDonel, 1986). Alpha toxin is commonly produced by all five types and is a phospholipase C that can hydrolyze lecithin into phosphorylcholine and diglyceride and is believed to be a major factor responsible for the organism's tissue pathology (Awad *et al.*, 1995). This is the predominant product of *C. perfringens* type A. Therefore, type A exhibits several powerful toxicities, and infection with type A may result in myonecrosis, hemolysis, an increase in vascular permeability, and platelet aggregation; the major lethal effects associated with this toxin are necrotic enteritis and enterotoxemia in animals (Hatheway, 1990; Daube, 1994). *Clostridium perfringens* type A infection represents one of the most serious problems affecting sheep due to severe economic losses as a result of sudden death of sheep (Al-Humiany, 2012).

Table (2) revealed that *C. perfringens* could be isolated from faecal swabs of apparently healthy sheep, diarrhoeic sheep, and all examined faecal swabs with in percentages of 80, 36.4 and 44.5% respectively. Lower results were recorded by Sharif *et al.* (2005) and Hala *et al.* (2009) who isolated it with in a percentage of 33.3% from intestine of sheep, Nibal (2012) who could isolate it with in a percentage of 41.6% from faecal swabs of clinically suspected sheep and Heba and Hala (2009) who isolated it with in a percentage of 28.2% from affected kidney of sheep while higher results were recorded by Mahmoud (1991) and Kalender *et al.* (2002). *Clostridium perfringens* type A represented as 25% from all isolated strains of *C. perfringens* (table, 3) that were typing by multiplex PCR. The obtained results were lower than that recorded by Hala *et al.* (2009) and Mafruza *et al.* (2012) while higher than Heba and Hala (2009); Ahsani *et al.* (2010). Non toxigenic strains of morphologically and biochemically identified *C. perfringens* represented as 75% as shown in table (3) that was higher than that recorded by Hala *et al.* (2009); Heba and Hala (2009).

In vitro sensitivity test of the identified *C. perfringens type A* was performed against different antimicrobials and garlic oil (table, 4) revealed that the organism was sensitive to Ciprofloxacin (5µg), Amoxicillin/Clavulaniacid (10µg), Erythromycin (15 µg), Flumequine (30µg), Gentamycin (10µg), Chlormphenicol (30µg), Vancomycin (µg); Nitrofurantoin (300µg), intermediately sensitive to tetracycline (30µg), Enerofloxacin (5µg), and resistant to Amoxicillin (10µg), Neomycin (30µg) Streptomycin (10µg), Trimethoprim/ sulfamethoxazole (25µg) and garlic oil. The susceptibility to antimicrobials was nearly similar to that reported by Mafruza *et al.* (2012) and the resistance to garlic oil was in agreement with Banerjee and Sarkar (2003) who recorded that *C. perfringens* was not sensitive to garlic slice. The studies revealed that the antibacterial effect of garlic depending on the organism, growth medium and garlic preparation used (Al-Delaimy and Ali, 1970). The time between maceration and filtration of garlic should not be more than 4 hours as Saleem and Al-Delaimy (1982) found that the maximum inhibitory activity of the extract was held for 4 hrs. before filtration keeping in mind its possible volatility so the filtrate must be used for inhibition studies with in an hour of filtration.

Escherichia coli is an enteric Gram-negative, rod-shaped, flagellated, motile, oxidase negative, facultative anaerobe and is classified under the family Enterobacteriaceae (Buxton and Fraser, 1977), it produces epticaemia and diarrhoea in a wide range of hosts including lambs (Paul *et al.*, 2010).

In the present study pathogenic *E. coli* represented as 6%, 25% in diarrhoeic and apparently healthy sheep respectively and 31.5% in all faecal swabs examined (table, 2). Similar results were reported by Nibal (2012), higher results were recorded by Sharif *et al.* (2005); Ibrahim *et al.* (2010) and Purkayastha *et al.* (2010).

Pathogenic *Ps. aeruginosa* could be isolated from diarrhoeic and apparently healthy sheep with in a percentage of 10% and 9.1% respectively and 9.3% from all examined faecal samples (table, 2). The

obtained results were higher than that reported by Arsalan *et al.* (2009).

Staphylococcus aureus coagulase positive could be isolated with in a percentage of 20% from diarrhoeic sheep, 9.1% from apparently healthy sheep and 11.1% from all faecal swabs examined (table, 2). Higher results were recorded by Nibal (2012) and lower results were reported by Sharif *et al.* (2005).

From the previously mentioned results, we can concluded that sheep can be infected with different enteropathogenic bacteria that leading to high economic losses in sheep farms so some recommendations must be followed to avoid the infection with such pathogens including; Proper hygienic measures in the sheep farms, Vaccination of dams at last stage of parturition against *Clostridium perfringens* type A for control of enterotoxaemia in sheep, feeding animals with balanced ration. In addition to establishment of more researches dealing with using of other preparations of garlic as the results in this study are not absolute because of studying of the oil preparation only; also, *in vivo* trials for administration of garlic preparations may have more satisfactory results than *in vitro* ones.

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عزل وتصنيف بعض البكتيريا المعوية الممرضة في الأغنام مع مرجعية خاصة للكوليستروديم برفرينجنز ومدى حساسيتها للمضادات الحيوية المختلفة وزيت الثوم

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تتعرض الأغنام للإصابة بالعديد من البكتيريا المعوية والتى تتسبب في الكثير من الخسائر الاقتصادية لذلك أجريت هذه الدراسة لمعرفة بعض البكتيريا المعوية الممرضة التى تصيب الأغنام للتوصل لطرق الوقاية منها وذلك عن طريق الفحص البكتيريولوجى لعدد 54 مسحة برازية تم تجميعها من أغنام مختلفة الأعمار مصابة بالإسهال (10) وأخرى سليمة ظاهريا (44) من أماكن مختلفة بمحافظة الإسكندرية والذى أسفر عن عزل بكتيريا الايشريشيا كولاى، المكور العنقودي الذهبي ، السيدوموناس ارجينوزا بنسب 31.5، 11.1، 9.3% على التوالي كما تم عزل بكتيريا الكوليستروديم برفرينجنز بنسبة 44.5% والتى تم تصنيفها باختبار تفاعل البلمرة المتسلسل والذى أوضح أن 25% منها كان من النوع أ ، كما وقد تم أجراء اختبار الحساسية للكوليستروديم برفرينجنز نوع أ وتعيين مدى حساسيتها للمضادات الحيوية المختلفة وزيت الثوم والذى أوضح أنها مقاومة لبعض المضادات الحيوية المستخدمة في الدراسة وزيت الثوم. وقد تمت مناقشة النتائج والتوصيات تفصليا.