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BACTERIOLOGICAL STUDIES ON ANAEROBIC BACTERIA CAUSING HEPATIC AFFECTIONS IN AGED BUFFALOES WITH REFERENCE TO ITS PUBLIC HEALTH HAZARD

(With 5 Tables)

By

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**دراسات بكتريولوجية علي البكتريا اللاهوائية المسببة للإصابات الكبدية في
الجاموس المسن مع الإشارة إلي خطورتها علي الصحة العامة**

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الميكروبات اللاهوائية من الميكروبات الخطيرة التي تؤثر على صحة الإنسان وخاصة ميكروب الكلوستريديم بيرفيرنجينز ويزيد من خطورتها قدرتها على إفراز السموم لهذا أجري هذا البحث علي 67 عينات كبد من جاموس مسن عمره أكثر من 6 سنوات ذبح بمجازر أسوط. منها 44 عينة كبد كان بها آفات باثولوجية ظاهرية و 23 عينة سليمة ظاهرياً. كان الفحص البكتريولوجي ايجابياً من 48 عينة (71.64%) لعزل الميكروبات اللاهوائية منها في صورة عزلات مختلطة 41.7% أو عزلات منفردة 58.3%. كانت كل عينات الكبد المصابة بالديدان الكبدية ايجابية للعزل البكتيري. تم عزل ميكروب الكلوستريديم بيرفيرنجينز من 14 عينة كبد (29.2%) في صورة عزلات مختلطة (25% من عينات الكبد المصابة و 4.2% من عينات الكبد السليمة الايجابية للعزل البكتيري)، وقد أظهرت نتائج اختبار الضراوة لهذه العزلات أن ميكروب الكلوستريديم بيرفيرنجينز المفرز للسموم 64.3% والغير مفرز للسموم 35.7%. كما وجد أن أعلى نسب عزل كانت لميكروب البكتيرويدس والبيتوستربتوكوكس أنيرويس. بالإضافة أنه تم عزل ميكروب الفيوزوبكتريم نكرفورم من عيتين كبد بها خرايج بنسبة 4.2%. وبإجراء اختبار الحساسية لكل من هذه العترات المعزولة علي حدة ضد 12 من المضادات الحيوية المختلفة أسفرت النتائج عن حساسية جميع العترات المختبرة للأوفلوكساسين والسيبروفلوكساسين بنسبة 100% يليه كل من اوكسيتتراسيكلين والأرثروميسين بنسبة 94.1% و76.5% ،علي التوالي، كما أنها أظهرت مقاومة لكل من كولستين وأموكيسللين وأسيكتينومييسين وريفاميسين. ويمكن الخلاصة أن آفات كبد الجاموس المسن مصدر خطورة لوجود أنواع البكتريا اللاهوائية المختلفة فيها وبخاصة عندما تكون مصابة بالديدان الكبدية.

SUMMARY

Anaerobic bacteria are of great concern to the food industry, especially *Clostridium perfringens* that produce exotoxins. The present study was per-

formed on 67 liver samples from aged female buffaloes (over 6 years old) slaughtered in the slaughter houses in Assiut Governorate, Egypt. Forty-four liver samples showed gross hepatic lesions and twenty-three samples were apparently normal liver. Bacteriological examination of these liver samples revealed that 48 samples (71.64%) were positive for anaerobic bacterial isolation, in mixed infection 41.7% and in single infection 58.3%. All *Fasciola* infected livers were positive for anaerobic bacterial isolation. *Clostridium perfringens* was isolated in mixed form with incidence 29.2%, 14 out of 48 samples positive for anaerobic culture, (25% and 4.2% from liver samples showed hepatic affections and normal livers, respectively). The Pathogenicity of the isolated *Clostridium perfringens* in mice revealed that 64.3% and 35.7% were toxigenic and non-toxigenic, respectively. The most prevalent anaerobic isolates were *Bacteroides spp.* and *Peptostreptococcus anaerobius* either in single or mixed form. In addition *Fusobacterium necrophorum* was isolated from two liver abscesses in a single form (4.2%). Antimicrobial susceptibility testing of the most prevalent isolates, with 12 antibiotics, revealed their sensitivity 100% to Ofloxacin and Ciprofloxacin followed by Oxytetracycline (94.1%) and Erythromycin (76.5%). All tested bacterial isolates showed resistance to Colistin, Amoxicillin, Spectinomycin and Rifamycin. It can be concluded that livers of slaughtered aged female buffaloes considered as hazardous source of mixed anaerobic bacteria especially which are infected with *Fasciola*.

Key word: Buffalo, anaerobic bacteria, liver.

INTRODUCTION

Liver is considered the most important organ for health production and reproduction. Liver infection is an important disease that affects all kinds of meat producing animals, this lead to great losses to livestock production and national income due to condemnation of great numbers of livers in slaughterhouses (Durham *et al.*, 2003; Sayed *et al.*, 2008). Livers of slaughtered aged female buffaloes were considered as hazardous source of mixed different bacterial species (Sayed *et al.*, 2008). Anaerobic microorganisms in single or mixed forms were identified and found to play a role in hepatic affection of buffaloes as *Fusobacterium necrophorum*; *Clostridium sordellii* and *Clostridium perfringens* (Zaki *et al.*, 2000). *Cl. perfringens* is an anaerobe responsible for a wide range of diseases in animals and humans (Niilo, 1986; Mohamed *et al.*, 2004).

Clostridia are widely distributed in nature and are found in soil as well as in fresh water and marine sediments throughout the world (Onderdonk and Allen, 1994).

The presence of the organism in soil has made it as an important soil pathogen, especially for those species that cause infections. The ecological factors usually play a very important role in the development of clostridial soil borne diseases (Siefert *et al.*, 1996). The clinical pictures of *Cl. perfringens* in animals were summarized into three forms; (A) intestinal diseases, (B) muscle diseases and (C) liver diseases. The potent exotoxins produced by the *Cl. perfringens* are the main way for its pathogenicity in animals and human (Mai and Ezz El-Deen 2003). Enterotoxaemia is a main syndrome caused by *Cl. perfringens* and characterized by high fatality rate, hemorrhagic enteritis and diarrhea (Manecta *et al.*, 2001)

Cl. perfringens remains a major cause of food borne illness world-wide and is continuing concern to the food service industry (Stringer *et al.*, 1980). ICMSF (International Commission on Microbiological Specification for Food, 1986) considered that *Cl. perfringens* gain access during processing of food service operations. Clostridia have been incriminated in many anaerobic infection, they produce various toxins that damage tissues of the nervous system. The most common clostridial infections are short-lived and relatively mild food poisoning, but may cause inflammation that sometimes destroys the wall of the large and small intestine, a condition called necrotizing enteritis (Kamakaraj *et al.*, 1998; Quinn, *et al.*, 2002). Among food borne pathogens, *Cl. perfringens* enterotoxin is an important virulence factor causing several illnesses in both humans and animals (Yanny and Shalaby, 2003). *Cl. perfringens* can grow at low temperature below 15 °C but the growth is slow. The growth rate is related to pH and medium as well as temperature, so that the *Cl. perfringens* grows slower in media at pH 5.8 than at pH 7.2. Germination of clostridia spores has been observed at 5 °C (Hobbs and Gilbert, 1969).

Cl. perfringens is a large gram positive, anaerobic rod that produces endo- spores. These endospores allow survival outside the body for long periods. They are highly resistant to many chemical and physical factors including desiccation and heat. The most heat resistant spores can survive at temperature of 100 °C for 60 min. and so remain viable after many types of cooking. *Cl. perfringens* is the type commonly implicated in food poisoning. In food poisoning a large dose of vegetative cells are ingested with the contaminated food. This large number of bacteria alters the normal intestinal flora multiply briefly and then sporulates in the intestine. During sporulation, the enterotoxin responsible for the signs of food poisoning is produced (Quinn *et al.*, 2002).

The aim of this work was to investigate the prevalence of anaerobic bacteria in affected and normal livers of aged female buffaloes, the

antibiogram of the most prevalent these bacterial isolates, as well as pathogenicity of some isolated bacteria in experimental animals.

MATERIALS and METHODS

MATERIALS:

1- Samples:

A total 67 of liver samples (44 liver samples revealed gross hepatic lesions and 23 samples of normal liver) of aged female buffaloes (more than 6 years) were collected from slaughter houses in Assiut Governorate. For bacteriological examination, each sample was collected in a clean sterile plastic bag and transferred directly to the laboratory.

2- Culture media:

a- Cooked meat medium "Oxoid".

b- Blood agar medium with 7% sheep blood.

3- Media used for biochemical tests:

Sugar fermentation (glucose, maltose, sucrose and mannitol), gelatin medium, glucose phosphate broth medium, peptone water, triple sugar iron agar (T. S. I.), urea agar base and solid agar media.

4- Reagents, chemicals and stains used were:

Kovac's reagents, urea, methyl red, Andrade's indicator, Gram's stain, glucose 1%.

5- Experimental animals:

Swiss mice were inoculated through tail vein with 0.3ml of centrifuged supernatant of 48 h. culture of *Cl. perfringens*. The animals were kept under observation for 72 hours after inoculation (Carter, 1984; Buogo *et al.*, 1995).

6- Gas-pack anaerobic jar "Oxoid":

It was used for production of anaerobiosis by using disposable hydrogen-carbon dioxide bags with socket.

7- Antimicrobial sensitivity discs:

It was manufactured by Bioanalyse (Turkey) including; Amoxicillin (25µg); Ciprofloxacin (5µg); Cephalexin (30µg); Cefotaxime (3µg); Colistin (10µg); Erythromycin (15µg); Gentamycin (10µg) Oxytetracycline (30µg); Ofloxacin (5µg); Rifamycin (30µg); Spectinomycin (100µg) and Trimethoprim (5µg).

METHODS:

1- Isolation and identification of anaerobes:

The liver samples of aged female buffaloes were subjected to anaerobic bacteriological examination. Two grams of each liver sample were inoculated separately into two sterile cooked meat media tubes. Both inoculated media were incubated anaerobically at 37°C for 48 hours. One

of them was heated in water bath at 75°C for 15 minutes, while the other was left unheated. Subcultures from each of 48 hours cultures were streaked on blood agar plates and incubated anaerobically at 37°C for 24 hours. Only strict anaerobic isolates were examined and identified for microscopical appearance, culture characters, and motility then transferred to cooked meat medium for the other biochemical tests as described by Koneman *et al.* (1988).

2- Pathogenicity tests:

All strains that were identified biochemically as *Cl. perfringens* were evaluated for the presence of lethal toxin through inoculation into the mice models. Swiss mouse weighing about 50 g. body weights that kept for 15 days was considered as *Cl. perfringens* free mice and used for the pathogenicity test. *Cl. perfringens* strains were grown for about 48 hours in cooked meat medium (Oxoid). After centrifugation at 10000-x g for 10 min., 0.3 ml quantities of the supernatant of each strain was injected into the tail vein of two mice and two mice were inoculated with sterile saline as negative control according to Carter (1984) and Buogo *et al.* (1995).

3- Reisolation of the inoculated organisms was done as mentioned above.

4- Sensitivity test:

The most prevalent isolated strains were tested for sensitivity to different chemotherapeutic agents. One ml of 48 hours broth cultures was spread on the surface of blood agar. Antibiotic sensitivity discs were placed on the surface of seeded agar. Plates were incubated anaerobically at 37°C for 24 hours. The sensitivity was judged according to the diameter of clearance zone around the discs (Perelman *et al.*, 1991).

RESULTS

Detailed obtained results are illustrated in Tables 1-5.

Table 1: Prevalence of anaerobic bacterial positive culture in different hepatic affections and normal livers (n =67).

Examined livers	Gross hepatic affections	No. of samples		Positive for anaerobic bacterial culture	
		No.	%	No.	%
Liver showing hepatic affections	1- Fascioliasis	12	17.91	12	17.91
	2- Hepatitis	23	34.32	16	23.88
	3- Necrosis	6	8.96	4	5.97
	4- Liver abscess	3	4.48	3	4.48
	Total	44	65.67	35	52.24
Normal livers		23	34.33	13	19.40
Total examined		67	100	48	71.64

Table 2: Anaerobic microorganisms isolated from liver samples positive for bacterial culture (n =48).

Hepatic lesions	Bacterial isolates					
	Single infection	No.	%	Mixed infection	No.	%
Fasciola	<i>Bacteroides</i> spp.	5	10.4	<i>Cl. perfringens</i> & <i>Bacteroides</i> spp.	1	2.1
	<i>Peptostreptococcus anaerobius</i>	4	8.3	<i>Cl. sordellii</i> & <i>Bacteroides</i> spp.	1	2.1
				<i>Cl. perfringens</i> & <i>Cl. sordellii</i> & <i>Bacteroides</i> spp.	1	2.1
Hepatitis	<i>Bacteroides</i> spp.	2	4.2	<i>Cl. perfringens</i> & <i>Bacteroides</i> spp.	2	4.2
	<i>Peptostreptococcus anaerobius</i>	6	12.5	<i>Cl. perfringens</i> & <i>Peptostreptococcus anaerobius</i>	4	8.3
				<i>Bacteroides</i> spp. & <i>Peptostreptococcus anaerobius</i>	2	4.2
Abscess	<i>F. necrophorum</i>	2	4.2	-	-	-
	<i>Peptostreptococcus anaerobius</i>	1	2.1			
Necrosis	-	-	-	<i>Cl. perfringens</i> & <i>Bacteroides</i> spp.	4	8.3
Normal liver Samples	<i>Bacteroides</i> spp.	7	14.6	<i>Bacteroides</i> spp. & <i>Peptostreptococcus anaerobius</i>	2	4.2
	<i>Peptostreptococcus anaerobius</i>	1	2.1			
				<i>Cl. sordellii</i> & <i>Bacteroides</i> spp.	1	2.1
				<i>Cl. perfringens</i> & <i>Cl. sordellii</i>	1	2.1
	Total	28	58.3		20	41.7

Table 3: Incidence of anaerobic bacteria isolates recovered from the examined liver samples (n =69).

Anaerobic bacterial isolates	No.	%
<i>Bacteroides</i> spp	29	42.02
<i>Peptostreptococcus anaerobius</i>	20	28.99
<i>Clostridium perfringens</i>	14	20.29
<i>Clostridium sordellii</i>	4	5.80
<i>Fusobacterium necrophorum</i>	2	2.90
Total	69	100

Table 4: Incidence of toxigenic and non-toxigenic *Clostridium perfringens* in the examined livers samples.

No. of examined liver samples	Positive for <i>Cl. perfringens</i>		Toxigenic <i>Cl. perfringens</i>		Non-toxigenic <i>Cl. perfringens</i>	
	No./67	%	No./14	%	No./14	%
67	14	20.9	9	64.3	5	35.7

Table 5: The percentage of in vitro antimicrobial susceptibility pattern of some frequent isolates tested against different antibiotics.

Isolated microorganisms	No. of the tested isolates	Amoxicillin (25µg)	Ciprofloxacin (5µg)	Cephalexin (30µg)	Cefotaxime (3µg)	Colistin (10µg)	Erythromycin (15µg)	Gentamycin (10µg)	Oxytetracycline (30µg)	Ofloxacin (5µg)	Rifamycin (30µg)	Spectinomycin (100µg)	Trimethoprim (5µg)
		Number and percentage of sensitive strains											
<i>Cl. perfringens</i>	12	3/12 (25%)	12/12 (100%)	12/12 (100)	3/12 (25%)	0/12 (0%)	6/12 (50%)	3/12 (25%)	12/12 (100%)	12/12 (100%)	0/12 (0%)	3/12 (25%)	3/12 (25%)
<i>Bacteroides</i> spp.	12	0/12 (0%)	12/12 (100%)	4/12 (33%)	4/12 (33%)	0/12 (0%)	12/12 (100%)	8/12 (67%)	12/12 (100%)	12/12 (100%)	4/12 (33%)	0/12 (0%)	4/12 (33%)
<i>Peptostreptococcus anaerobius</i>	10	0/10 (0%)	10/10 (100%)	6/10 (60%)	0/10 (0%)	0/10 (0%)	8/10 (80%)	6/10 (60%)	8/10 (80%)	10/10 (100%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
Total	34	3/34 (8.8%)	34/34 (100%)	22/34 (64.7%)	7/34 (20.6%)	0/34 (0%)	26/34 (76.5%)	17/34 (50%)	32/34 (94.1%)	34/34 (100%)	6/34 (17.6%)	3/34 (8.8%)	7/34 (20.6%)

DISCUSSION

Intestine and liver are considered to be the main target organs of anaerobic infection; intestine, because of its good vehicle for growth of the pathogenic bacteria and the production of its toxins. However the liver via the portal circulation is considered the core of the body metabolism and reflects the condition of the animal body in health and disease (El-Shahawy and Abd El-Gaied, 2006). *Cl. perfringens* is a known pathogen that causes enterotoxaemia in human and domestic animals, also is known to be a soil saprophytic and widespread in nature (sewage, faces, water and feeds) (Van Baelen and Devriese, 1987).

In the present study, bacteriological examination of aged female buffalo's liver samples revealed 71.64% were positive to anaerobic bacterial isolation (52.24 from hepatic affections and 19.40 from normal liver examined) (Table 1). It was thought that those bacterial isolates were found to play a role in the detected hepatic affections, since Zaki *et al.* (2000) found 77.8% of the examined liver samples of buffaloes were positive for obligatory anaerobic bacterial isolation. Failure for bacteriological isolation in 9 affected liver's buffalo samples, in the present study, may be due to other causes incriminated as viruses, molds or parasites. So, the etiology of liver infection is complex and multifactor which are either non-infectious or microbial determinates including bacteria, viruses and fungi (Blood and Radostitis, 1989).

Fasciola spp. play an important role in aiding microbial invasion to the infected animals either by transportation as a result of migration of immature liver flukes or depression of hepatic tissue resistance to be a good media for bacterial growth (Mousa, 1993; Osman, 2005; Sayed *et al.*, 2008). All liver samples of aged female buffaloes infected with *Fasciola* (n=12) in the present work were positive for anaerobic isolation (Table 1). Moreover, parasitic infestation of liver may lower the resistance of the hepatic tissues and give chance for bacterial agents to attack as secondary invaders and produced their pathological lesions (Ahmed 1991; Mousa 1993).

As shown in Table 2, the incidence of mixed and single bacterial isolates was 41.7% and 58.3%, respectively. The single and mixed infection of anaerobic isolates from buffalo's liver showing hepatic lesions recorded by Zaki *et al.* (2000) were 42.9% and 57.1%.

In the present work as shown in Table 2, *Cl. perfringens* was isolated in mixed form in an incidence of 29.2% (14 out of 48 liver samples were positive for anaerobic culture). High incidence (68.8%) was obtained by Zaki, *et al.* (2000), which may be attributed to age of the slaughtered

female buffaloes, change in hygienic measure, stress factors and immune status of the infected animal.

Cl. perfringens was recovered in mixed form with *Bacteroides* spp. and *Peptostreptococcus anaerobius*; this indicated that these microorganisms played a role in initiating hepatic affections in buffaloes (Tydon *et al.*, 1980; Itman *et al.*, 1989). Moreover, *Fasciola* infection plays an important role in stimulation of Clostridium infection especially *Cl. perfringens* due to the changes which were attributed to toxic environment created by the organisms in liver tissues (Samad and Haque, 1987).

Clostridial species occur in a quiescent state in a considerable number of apparently normal livers and spleen of cattle and camel (Carter, 1984; Osman and Nour, 2002), in the present study 19.40% of the normal buffalo's liver were positive for anaerobic bacterial isolation (Table 1), and *Cl. perfringens* was isolated from two normal livers of aged female buffaloes with a percentage 4.2% (Table 2). *Cl. perfringens* is one of the food poisoning microorganisms associated with enteric contamination (Silliker *et al.*, 1980); also it was incriminated in food poisoning outbreaks due to liver consumption (Bryan, 1980).

Identification of the anaerobic pathogens in the present study cleared the isolation of *Bacteroides* spp.; *Peptostreptococcus anaerobius*; *Cl. perfringens* (42.02%, 28.99% and 20.29%, respectively); *Cl. sordellii* and *F. necrophorum* (5.8% and 2.9%) as shown in Table 3. These were the most common anaerobic bacteria isolated from buffalo's liver by Itman *et al.* (1989); El-Sayed *et al.* (1991) and Zaki *et al.* (2000).

Fusobacterium necrophorum was isolated in single form from two hepatic abscesses (4.2%) (Table 3), this is similar to that obtained by Makhareta (1988); Ciftci *et al.* (1993); Abd El-Fattah, *et al.* (1995); El-Shahawy and Abd El-Gaied (2006), while Lichtenberg *et al.* (2005) detected it in 47%. *Fusobacterium necrophorum* is considered to be one of the most common cause of hepatic abscesses (Blood and Radostitis, 1989).

From Table 4, it seems clear that the incidence of toxigenic *Cl. perfringens* was higher than non-toxigenic one. This result seemed to agree with the observation of Menazie (2000); Zaki *et al.* (2000). The virulence of the *Cl. perfringens* is associated with production of several toxins (exotoxin and enterotoxin), among them four are known as the major lethal toxins (α ; β ; E and I). Intravenous injection of these toxins to mice is highly lethal (Collier *et al.*, 1998; Petit *et al.*, 1999).

The present antimicrobial susceptibility testing (Table 5) showed that all tested isolates were highly sensitive to Ofloxacin and Ciprofloxacin (100%) then Oxytetracycline (94.1%), while the all tested strains were resistant Colistine, Spectinomycin and Amoxicillin. Non- spore forming

anaerobes were susceptible to level of Oxytetracycline, but Gentamycin was ineffective (Prescott and Chirino-Trejo, 1984). *Cl. perfringens* was highly sensitive to Ciprofloxacin, but it was resistant to Colistine (Abd El-Gwad and Abd El-Kader, 2001).

Cl. Perfringens infections have a variety of origins including human and animal faeces, abattoirs, sewage and flies. Certain types of the organisms produce spores that resist boiling for four or more hours. If the spores are present as contaminants on raw meat they may resist boiling or stewing, and on slow cooling the spores will germinate into rapidly multiplying bacterial cells and produce large amounts of toxin (Gracey and Collins, 1992).

It can be concluded that livers of slaughtered aged female buffaloes are considered as hazardous source of mixed anaerobic bacteria especially that livers infected with *Fasciola* spp. In addition, *Cl. perfringens* in the normal livers may constitute a public health hazard if consumed by human being.

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