2. REVIEW OF LITERATURE

2.1. Historical account about nomenclature of *Clostridium perfringens*:

Welch and Nutall (1892) described anaerobic gram positive bacillus organism under the name of ***Bacillus aerogens capsulatus*.**

Frankel (1893) suggested the name of ***Bacillus phlegmonis emphysematosa*** for anaerobic gram positive bacillus organism isolated from gas gangrene cases.

Veillon and Zuber (1898) described the anaerobic gram positive bacillus organism as ***Bacillus perfringens***.

Migula (1900) suggested the name of ***Bacillus welchii*** for the anaerobic gram positive bacillus organism.

Wildson (1931) firstly named ***Clostridium welchii*** as ***C. perfringens***. The name "perfringens" is derived from the Latin name, meaning "very fringed". ***C. perfringens*** is not simple organism; there are many types which could be differentiated by their toxins and their antigenic structure.

2.2. Incidence of *Clostridial* spp. enteric infections:

Prescott (1977) isolated for the first time ***Bacillus piliformis*** organism from young rabbits with severe diarrhea and 50% mortality rate in United Kingdom.

Borriello *et al.*, (1985) implicated enterotoxigenic strains of ***C. perfringens*** in some cases of antibiotic-associated diarrhea in rabbits.

Harris and Portas (1985) isolated ***C. spiroforme*** strains from newly arrived batches of rabbits with profuse scouring and sudden death with mortality rate of 20%.

Scharmann and Wolff (1985) isolated ***Clostridium Welchii*** from weaning rabbits with diarrhea and mortality rate 27-50%.

Peeters *et al.*, (1986) examined commercial rabbits showed clinical signs of enteritis-complex for the presence of ***C. spiroforme*** and its iota-like toxin. The bacterium was detected by Gram's stain in 149 caecal samples (52.4%), the iota-like toxin was in 29 tested strains (7.4%) and 26 were toxigenic that originated from 24 of 29 rabbitries. In 13.4% of the samples, ***C. spiroforme*** was present as the only known disease agent.

Lee *et al.*, (1991) carried out a quantitative and qualitative analysis of faecal samples for ***Clostridial*** infection in laboratory mice, rats, hamsters and rabbits using selective as well as nonselective culture techniques. About 364 ***Clostridial*** isolates were identified from mice, rabbits, rats and hamsters as percentage of 79.4, 76.5, 25.4 and 28.6%, respectively. The most common 16 identified ***Clostridial*** spp. were: ***C. coccoides***, ***C. cocleatum*** and ***C. innocuum*** from mice; ***C. cocleatum, C. cochlearium*** and ***C. irregularis*** from rats; ***C. cocleatum*** and ***C. irregularis*** from hamsters and ***C. aminovalericum*** and ***C. irregularis*** from rabbits. Unidentified ***Clostridium*** spp. were isolated from each tested animal spp. and were tentatively classified into 26 groups. None of those groups was found in more than one animal spp. in that study.

Mostafa (1992) examined 358 rabbits representing 208 diseased and 150 apparently healthy ones in Giza to isolate different ***Clostridial spp***.

Bacteriological examination revealed that ***Clostridial*** organisms positive cases were 87 where 83, 3 and one cases were positive for ***C. perfringens***, ***C. tertium*** and ***C. difficile***, respectively.

Barral *et al.*, (2000) isolated ***C. perfringens*** from cases of rabbit's enteritis that were associated with isolation of ***Coli*** forms, especially serotype (O2).

Abdel-Rahman *et al.*, (2006) collected 140 rectal swabs; 20 from apparently healthy, 80 from diarrhoeic and 40 from dead and sacrificed diarrhoeic rabbits aged 6-8 weeks-old from El-Minia and Assiut governorates. The incidence of ***C. perfringens*** and ***C. spiroforme*** were 39.30% (n=55) and 7.14% (n=10), respectively. There was variation in the prevalence of ***Clostridium*** spp. among the rabbits, being 15% in apparently healthy (n=3), 40% in diarrhoeic rabbits (n=32) and 75% in dead and scarified diarrhoeic ones (n=30). The incidence of ***C. perfringens*** toxigenic type was 81.82% (n=45), while it was 18.18% (n=10) in non-toxigenic one. Among the toxigenic types, ***C. perfringens*** type E was the most predominant (71.11%), followed by types A, D and B with incidence 15.56, 8.89 and 4.45%, respectively. The pathogenicity test of the isolates revealed high mortality of rabbits infected with ***C. perfringens*** type E (75%) and type A (37.5%), while ***C. spiroforme*** reached 62.5%. All dead experimentally infected rabbits showed profuse watery diarrhoea and died within few first days after onset.

El-Rahman and Atwa (2006) collected from rabbit's farms in El-Menoufiea governorate 300 specimens including intestine, liver and faeces from 150 apparently healthy and 150 dead rabbits with intestinal pathological lesions at age of 4-12 weeks. ***C. perfringens*** was the most

prevalent organism that recovered from apparently healthy rabbits, with incidences of 30, 18 and 10% from intestine, liver and faecal samples, respectively. ***C. difficile*** was respectively isolated from 6, 4 and 4% from the intestine, liver and faeces, respectively. ***C. perfringens*** was also the most prevalent type from dead rabbits with incidences of 70% from the intestine, 60% from the liver and 36% from faeces followed by ***C. difficile*** at 10, 10 and 8% from the intestine, liver and faeces, respectively. Typing of ***C. perfringens*** isolates revealed that type A was the most prevalent followed by type D with respective incidences of 40 and 20 in the intestine, 33.3 and 11.1 in the liver and 40 and 20% in the faeces in apparently healthy rabbits. The incidences of type A and type D in dead rabbits were 42.9 and 25.7 in the intestine, 36.6 and 23.3 in the liver and 38.9 and 22.2% in the faeces, respectively.

Marlier *et al.*, (2006) succeeded in isolation of ***C. perfringens*** with its toxins and non-enterotoxigenic *Escherichia coli* ***(E. coli)*** from intestinal contents of dead rabbits suffered from Epizootic Rabbit Enteropathy (ERE) disease complex. Also, they succeeded in reproduction of the disease experimentally. High (30–80%) mortality rate of rabbits that aged 6–14 weeks was recorded in experimentally infected animals.

Yao ChunYang and Zhuang JinQiu (2007) detected that ***C. welchii*** type A was the major pathogen infecting rabbits in China. Also, few cases involved ***C. welchii*** type E were detected.

Bano *et al.*, (2008) investigated the prevalence of ***C. difficile*** and its toxins in 132 Italian rabbit farms. The organism was isolated from the content of the small intestine and impacted caecum of 317 diseased rabbits and 80 apparently healthy ones with variable ages.

Jiang XiangYu (2008) identified ***C. welchii*** organisms in rabbits aged 8 weeks, in China.

Romero *et al.* (2009 a) considered ERE as a major disease affecting intensive European rabbits farms. The concentration of ***C. perfringens*** in digestive contents of young rabbits had been shown to be highly correlated with average diarrhea and mortality in the fattening period. To evaluated the effect of ERE on production performance in young rabbits, the mean of ***C. perfringens*** enumeration in soft faeces was the most important. It was observed that counts were significantly higher around ten days after weaning, irrespective to the age of weaning. Furthermore, it was showed that high counts of ***C. perfringens*** in ERE diseased rabbits were associated with reduced body weight at slaughter.

Romero *et al.*, (2009 b) carried out two experiments to show the effect of ***C. perfringens*** on rabbit's performance and they detected that ***C. perfringens*** proliferation in the caecum helped in the development of ERE, causing impaired growth and increasing weight variability in weaned rabbits.

Romero *et al.*, (2009 c) investigated the effect of dietary fiber content at weaning age on ***C. perfringens*** proliferation in the caecum. The results of that study indicated that high counts of ***C. perfringens*** in the caecum caused ERE which was the main cause of fattening rabbits mortalities.

Heba (2010) examined 28 apparently healthy rabbits, 32 diseased rabbits, 24 water samples and 48 pelleted feed samples for the presence of ***C. perfringens*** at Giza governorate. The researcher found 114 samples were positive for ***C. perfringens*** out of 132 ones where the positive samples typed

as 62 ***C. perfringens*** type A, 24 *C.* ***perfringens*** type D and 28 non-toxigenic ***C. perfringens*** strains.

Huybens *et al.*, (2011) found that Epizootic rabbit enteropathy (ERE) emerged and spread in Europe within the last 13 years causing major economical loss in weaned rabbits.

2.3. Predisposing factors for infection with *Clostridial* organisms:

Boyd *et al.*, (1948) recognized that the most prevalent and important predisposing factor which help in the growth of ***C. perfringens*** was coccidial pathogen which cause intestinal damage.

Brooks (1979) demonstrated that the incidence of rabbit's enteric disorders was greatly increased by feeding finely ground dehydrated Lucerne pellets. In addition, un-restricted feeding of young rabbits resulted in over eating and production of excessive food mass in the stomach which was poorly penetrated by the antimicrobial gastric acids. That condition forms a suitable environment for colonization, multiplication and toxin production by ***C. perfringens*** type E during the critical transition of the relatively sparse gastrointestinal flora of suckling rabbit. Also, the incidence of diarrhea and mortality were increased by changing stomach pH from one to two.

Mogollon *et al.*, (1981) suggested that aflatoxin B1 could be considered a predisposing factor for proliferation of ***C. perfringens*** organisms in 40 days old rabbits.

Scharmann and Wolff (1985) found that when a ration contained 20% crude protein and 10% fibers, the frequency of diarrhea caused by ***Clostridial*** organisms decreased considerably.

Peeters *et al.*, (1986) suggested that ***C. spiroforme***-mediated diarrhea was favored by maldigestion and initiated by infectious agents and/or nutritional factors.

Branton *et al.*, (1987) reported that the high energy diets or those rich in rye, wheat and barley and diets rich in fish meal may also play a role in aggravation of ***Clostridial*** infection.

Ficken and Wages (1997) suggested that the understanding the pathogenesis of ***Clostridial*** enteritis is very difficult due to its complexity and because of several predisposing factors such as mechanical irritation of the gut and sudden gut micro-flora changes appear to contribute to this syndrome.

Mehmet Haligur *et al.*, (2009) stated that Mucoid Enteropathy (ME) or ERE was a common cause of death in commercial or laboratory rabbits. The incidence of the disease has been associated with several factors, including a high-carbohydrate/low-fiber diet and obesity. Feeding on those types of diets may predispose animals to intestinal proliferation of bacteria such as enteropathogenic ***E. coli***, ***C. perfringens, C. difficile and C. spiriforme***.

2.4. *Clostridial* spp. and their toxins:

Smith (1955) defined ***C. perfringens*** as anaerobic, Gram positive rod, spore-former with central or sub terminal spores, encapsulated and non-motile. Organisms fermented glucose, maltose, lactose, sucrose and starch. Six types of the organism are defined as: A, B, C, D, E and F and are differentiated on the basis of toxin production. All types of ***C. perfringens*** produce alpha toxin, hemolycin and lethal lecithinase.

Wilson and Miles (1975) characterized ***C. perfringens*** morphology as Gram positive bacilli, about 3-7 μm in length and 0.04 - 1.2 μm in thickness. The vegetative bacilli are straight or curved with parallel sides and rounded or somewhat with trunicated edges.

Rahman (1978) characterized ***C. perfringens*** morphology as strongly Gram positive rods, atrichous, non-motile and straight with parallel sides and blunt ends that occur singly or in pairs. Spores of the organism are rarely seen and when present they are large, oval and central or sub terminal and distend the cell.

Joklik *et al.*, (1980) observed that ***C. perfringens*** strains produce at least 12 different soluble exotoxins proteins. Alpha, beta, epsilon and iota are the main lethal exotoxins. Alpha toxin is the most important toxin and produced by all types of the organism.

Cato *et al.*, (1986) typed ***C. perfringens*** into five types (A, B, C, D and E) according to the production of the four major toxins (Alpha, beta, epsilon and iota). Alpha toxin is produced by all strains and involved in the disease pathogenesis.

Banwart (1989) found that the spores of some ***Clostridia*** spp. are very heat resistant and may survive under the heat treatment. If the surviving spores germinate, the vegetative cell grows and spoilage of the food will occur.

Awad *et al.*, (1995) mentioned that one of the most important lethal and dermonecrotic toxins produced by ***C. perfringens*** is phospholipase C (PLC) which is commonly known as alpha toxin. It is produced in varying amounts by all types of ***C. perfringens*** and is considered as a primary virulence factor involved in ***Clostridial*** necrosis.

Merck (1998) described ***C. perfringens*** as an anaerobic Gram positive bacterial pathogen. This organism is widely spread in soil and gastrointestinal tract of animals and characterized by its ability to produce potent exotoxins which are responsible for specific enterotoxaemia in rabbits.

Petit *et al.*, (1999) stated that ***C. perfringens*** produced variety of exotoxins. Four of these toxins are alpha, beta, epsilon and iota, which considered as the major toxins and are used to group the bacteria into five types (A, B, C, D and E). Alpha toxin is produced by all strains and is involved in the disease pathogenesis.

Titball *et al.*, (1999) reported that alpha toxin produced by ***C. perfringens*** is a single polypeptide with a molecular weight about 43000 Dalton (Da).

Brynestad and Granum (2002) characterized ***Clostridia*** spp. morphologically as large Gram positive rods mostly motile, spore-former, fermentative and catalase negative. They are widely distributed in soil and as normal commensals in many animal spp. and man.

Uzal and McClane. (2011) found that ***C. perfringens*** type C causes necrotizing enteritis in humans and several other animal species. Type C isolates must produce at least beta toxin (CPB) and alpha toxin (CPA) and most strains produce several other toxins as perfringolysin O (PFO).

2.5. Diagnosis of *Clostridial* infections:

2.5.1. Clinical signs:

Niilo (1971) inoculated rabbits intravenously with a cell extract of an enteropathogenic strain of ***C. perfringens*** type A. The rabbits showed

excessive salivation, frequent defecation, tranquility and dyspnea followed by death.

Harbola *et al.*, (1975) intravenously inoculated white Swiss mice with intestinal filtrates for the detection of ***C. perfringens*** toxins. Mice died shortly after inoculation.

Prescott (1977) detected that ***Bacillus piliformis*** could induce an acute disease in young rabbits characterized by severe diarrhea and 50% mortality rate.

Baskerville *et al.*, (1980) recorded an outbreak of enteritis caused by ***C. perfringens*** type E in laboratory rabbits that manifested by profuse watery diarrhea and death within few hours. They experimentally infected mice and rabbits with caecal samples contained enterotoxins of ***C. perfringens***. Nervous signs and deaths occurred within 7 - 24 hrs. post infection were developed. Toxin neutralization test with specific antisera suggested that the enterotoxaemia was caused by ***C. perfringens*** type E.

Ivanics *et al.*, (1982) observed symptoms of copious bloody – stained diarrhea, salivation, weakness and death within 48 hours post infection with ***Bacillus piliformis*** in 5 out of 110 hares aged more than one year.

Hughes *et al.*, (1983) found ***C. difficile*** toxin (200 micro liter crude extract) in rabbit's ileum *in-vitro* and they described how ***C. difficile*** toxin could induce diarrhea.

Abd El-Rahman (1985) concluded that intradermal injections of albino Guinea pigs with supernatant cultures of different ***C. perfringens*** strains were useful for typing of isolates.

Nagi *et al.*, (1988) noticed that enterotoxaemia caused by ***C. perfringens*** in rabbits was a toxaemic disease lead to sudden death after short illness and characterized by bloody tinged diarrhea.

Hunter *et al.*, (1992) found that epsilon toxin which produced by type D and B ***C. perfringens*** was responsible for enterotoxaemia, diarrhea and deaths of rabbits.

Markovic *et al.*, (1992) studied the effect of ***C. perfringens*** enterotoxins in Guinea pigs and mice through intraperitoneal inoculation of partially purified enterotoxins from spores of ***C. perfringens*** type B strain. The results of inoculation were erythema in Guinea pigs and necrotic changes in the intestine of mice.

Tripathi *et al.*, (1992) inoculated growing culture toxin or washed cell of ***C. perfringens*** type C into washed jejunal loops of 10 Guinea pigs. The produced necrotic enteritis was characterized mostly by excessive fluid exudation in the lumen and congestion.

El-Naenaeey and El-Fetouh (1993) inoculated cell extract and culture filtrate of various strains of ***Clostridial*** spp. in the ileal loops of rabbits. Infected rabbits showed an intestinal response as accumulation of exudates with distension.

Rahman *et al.*, (1999) injected crude ***C. perfringens*** toxins intradermally and intraperitoneally in Guinea pigs at various doses. Intradermal injection had a dermonecrotic effect at the injection site, while intraperitoneal injection induced typical clinical and pathological changes. The researchers concluded that Guinea pig was an excellent model for studying clinical and pathological effects of these toxins.

Fernandez-Miyakawa *et al.*, (2007) reported that *in-vitro* toxin production was an important tool not only for diagnostic purposes, but also for the study of pathogenesis of ***C. perfringens*** infections.

Jorge *et al.*, (2008) found that ***C. perfringens*** types B (CPB) and C produce beta-toxin, causing fatal intestinal disease of humans or livestock. It was demonstrated that CPB was necessary for type C to cause bloody necrotic enteritis in a rabbit's ileal loop model.

2.5.2. Post-mortem lesions

Kunstyr *et al.*, (1975) examined three rabbits that died suddenly after a short period of diarrhea. They found purulent hepatitis with small necrotic foci on the liver and enlarged hemorrhagic caecum with purulent typhlitis. ***C. perfringens*** type A was isolated in pure culture from the liver and caecum of all rabbits.

Prescott (1977) described in details the post-mortem lesions caused by ***C. difficile*** causing enteritis, diarrhea and mortalities of young rabbits. The caeca contained watery brown fluids and gases with necrotic mucosa. The caecal wall was spongy edematous and occasionally contained serosal hemorrhages. The peritoneum contained bloody tinged serous exudate. The liver was enlarged and congested but rarely necrotic. Chronic cases mortalities were observed in 6-8 weeks old rabbits that consists of 50 commercial does and 200 laboratory rabbits. Dead animals showed distention of the caecum and colon with fluid or pasty contents and edematous wall.

Baskerville *et al.*, (1980) found distended, inflamed and necrotic caeca on postmortem examination of rabbits died from enteritis that caused by ***C. perfringens*** type E.

Ivanics *et al.*, (1982) described pathological changes as inflammation of the intestine and focal necrosis of the liver in rabbits infected with ***Bacillus piliformis*** organisms.

Nagi *et al.*, (1988) detected that the postmortem findings of enterotoxaemia caused by ***Clostridium*** spp. in rabbits were mainly congestion of small intestine and caecum and foul-smelling watery brown fluid contents with large amount of gases. In some cases, there were milliary necrosis in heart and liver.

Abdel-Rahman *et al.*, (2006) reported the lesions of ***C. perfringenes*** in dead rabbits with diarrhea. There were varying degrees of inflammation and ulcerative lesions on the mucosal surface of the caecum, colon and ileum while the internal organs were congested. Necrotic foci were observed in the liver of some rabbits.

Shi XiShan *et al.*, (2008) recorded that dead 100 rabbits from ***C. welchii*** infection showed abscessation of gastric mucosa, haemorrhages on the mucosa of large intestine, swollen liver and congested lungs.

Heba (2010) described the pathological lesions caused by ***C. perfringens*** in experimentally infected dead rabbits. The liver was congested with grayish red necrotic foci, while in few cases it was friable. The kidney was enlarged and congested and the intestine was severely congested.

2.5.3. Histopathological findings:

Prescott (1977) examined microscopically the intestine of rabbits died from ***Bacillus piliformis*** infection. There were extensive necrosis of the surface epithelium (with large numbers of 10 X 0.8 μ bacilli) that attached to the necrotic tissue in the muscle layers in addition to massive neutrophilia in the lamina propria. Miliary necrotic foci were scattered through the liver

with swelling of hepatocytes and kuffer cells, and occasional bacteria were present in the hepatocytes fringing the necrotic areas.

Hofshagen and Stenwig (1992) found intestinal necrosis caused by ***C. perfringens*** type A exotoxins in broiler chickens.

Campos *et al.*, (2004) inoculated culture suspensions of ***C. perfringens*** type A CPB-2 carrier strains or its filtrates into rabbit's ligated ileal loops and observed no histolopathological changes.

Badagliacca *et al.*, (2010) found that the microscopical pathological lesions in rabbits associated with ***C. perfringens*** were predominantly formed of non-inflammatory enteropathies. In other spp., ***C. perfringens*** was not only associated with congestive haemorrhagic enteropathy, but also with fatal traumatic lesions and degenerative diseases.

Heba (2010) described the microscopical changes in organs of rabbits experimentally infected with ***C. perfringens***. The liver showed different degrees of hepatic cell necrosis and nuclear pyknosis, the kidneys revealed marked interstitial haemorrhage within renal cortex and metastatic calcification with glomerular tuft and the spleen showed numerous numbers of haemosidrin laden macrophages.

Warren *et al.*, (2010) found that ***C. difficile*** toxin-induced enteritis is characterized by exuberant intestinal tissue inflammation, epithelial disruption and diarrhea. Adenosine, through its action on the adenosine A2A receptor, prevents neutrophillic adhesion and oxidative burst and inhibits inflammatory cytokine production. Alanyl-glutamine enhances intestinal mucosal repair and decreases apoptosis of enterocytes.

2.6. Isolation, identification and typing of *Clostridial* organisms:

Willis and Hobbs (1959) used Nagler's reaction as a serological test for typing of ***C. perfringens*** into A, B, C, D and E.

Horadniceanu and Sasarman (1964) used enriched agar medium containing 10-20% sheep blood and 2% glucose plus 250µg/ml neomycin sulphate and then incubate it at 46-47˚C for the isolation of ***C. perfringens***.

Cruickshank *et al.*, (1975) used cooked meat medium for growing ***Clostridial*** organisms and preservation of stock cultures, toxin production medium for ***Clostridial*** toxins production, neomycin sulphate sheep blood agar for isolation and purification of ***Clostridium*** spp., semi-solid medium to detect bacterial motality, egg yolk agar medium to detect lecithinase activity and Gram staining for routinely identification of the organism. They differentiated ***Clostridial*** isolates biochemically through some tests as gelatin liquefaction, lecithinase activity, urease, indole, sugar fermentation, meat digestion and motility tests.

Stern and Batty (1975) applied dermonecrotic test in albino Guinea pig as a method for typing of ***C. perfringens*** isolates.

Willis (1977) mentioned that ***Clostridial*** spp. could be recovered anaerobically after overnight cultivation of examined samples onto blood agar plates at 37˚C.

Ivanics *et al.*, (1982) detected ***Bacillus piliformis*** in the affected tissues by Levaditi's silver impregnation technique and electron microscopically in liver's cell vacuoles. However, the ***Bacillus piliformis*** was not isolated by culturing or chick-embryos inoculation.

Haghour and Murad (1984) diagnosed ***Clostridial*** enterotoxaemia in rabbits on the basis of direct smears from the intestinal contents and culturing onto Robertson's cooked meat and blood agar media. ***Clostridial*** isolates were identified biologically by mice inoculation and serologically by passive mouse protection tests.

Mead (1985) found that trypose sulphite cycloserine (TSC) medium with or without addition of egg yolk could be used for isolation of ***C. perfringens***. Added to that, this medium was more effective than other selective media for recovering both vegetative and spores forms of the organism that damaged by exposure to cold storage conditions or certain sub-lethal heat treatment.

Downes *et al.*, (1986) used three types of selective media to determine which one would allow the optimal recovery of anaerobic organisms. Those media were kanamycin agar (KA), neomycin agar (NA) and nalidixic acid-tween 80 (NAT) agar. The major difference among media was in the recovery of anaerobic Gram positive cocci which accounted for 40 % of the total isolates on NAT, 25 % on NA and only 11 % on KA. ***Clostridium*** spp. were isolated on both NA and KA.

Peter *et al.*, (1986) found that ***C. perfringens*** is oxidase and catalase negative.

Niilo (1988) described the hemolytic patterns for presumptive identification of ***C. perfringens*** type C. Blood agar with trypticase soya agar base and 5% heparinized sheep blood was coated with 2 parallel bands of type A (at 1:10 dilution) and type C (undiluted) ***C. perfringens*** antisera then inoculated with ***C. perfringens*** strains at right angles to the antiserum bands

and incubated for 24 hrs. Classical ***C. perfringens*** type C produced a characteristic arrow shaped hemolysis adjacent to both sides of type A antiserum band and that hemolysis was inhibited by type C antiserum band.

Abd El-Salam and El-Sanousi (1992) designed a scheme for identification of ***C. perfringens*** and ***C. perfringens*** like organisms. The scheme was based on the presence or absence of lecithinase enzyme synergic hemolysis with *Streptococcus* group B toxin, inhibition with appropriate antisera and reaction in the gelatin nitrate motility (GNM) test with the fermentation of some sugars.

Koneman *et al.*, (1992) formulated lombard-dowell agar medium for growth of obligatory anaerobic bacteria and applied milk digestion test using lombard-dowell agar medium to identify different ***Clostridium*** spp. depending on casein hydrolysis property.

Geppert and Eisgruber (1995) found that phosphate reagent was successful for demonstration of acid phosphatase of ***C. perfringens*** only. With that reagent, enzyme activity was shown in 95.2% of all investigated ***C. perfringens*** strains.

Han *et al.*, (1997) identified 51 field isolates of ***C. perfringens*** from the intestinal contents of rabbits with diarrhea using biochemical tests. The characteristics of the isolates were positive fermentation of glucose, lactose, sucrose and maltose, hydrolysis of gelatin and production of lecithinase and positive reverse Christie Atkins and Munch-Peterson (CAMP) test.

Kadra *et al.*, (1999) identified 90 strains of ***C. perfringens*** by classical methods. Identification of different ***C. perfringens*** was developed using seroneutrilization test in mice.

Labbe (2000) detected that ***C. perfringens*** colonies usually show a double zone of hemolysis on blood agar plates with a clear inner beta-toxin zone and a hazy outer zone caused by alpha toxin production. The organism can grow between 15 and 50º C with an optimum of 45º C for most strains. The generation time (GT) for most strains (temperature between 33 and 49º C) is below 20 min, although GT of 8 min has been also reported.

Vaikosen and Muller (2001) observed colonial growth pattern of typical culture of ***C. perfringens*** which appeared as flat with double zone of hemolysis and olive colouration. Also, they applied certain biochemical tests for identification of ***C. perfringens*** and found that most isolates reduce nitrate to nitrite, give a dark violet coloration after addition of acid phosphatase and test positive for charbohydrate fermentation tests (lactose, maltose and sucrose), while mannitol sugar fermentation is negative.

Jong (2002) demonestrated that ***C. perfringens*** could be sporulated well in Duncan and Strong (DS) medium which could be optimized by adding theophylline. Pepton-bile theophylline (PBT) (with or without starch) was the most suitable medium that yielded higher spore numbers.

Quinn *et al.*, (2002) demonstrated that dermonecrotic test in albino Guinea pig is used for typing of ***C. perfringens*** toxin.

Fernandez-Miyakawa *et al.*, (2007) used 3 different conventional culture media; commercial cooked meat (CMM), brain heart infusion (BHI) and tryptone glucose yeast (TGY) medium for isolation of ***C. perfringens*** type A.

2.7. Molecular identification:

Glenn Songer and Ralph Meer (1996) developed polymerase chain reaction (PCR) assays for detection of genes for á toxin (*cpa*), â toxin (*cpb*), å toxin (*etx*), é toxin (*i*A), and enterotoxin (*cpe*) in ***C. perfringens*** infected rabbits, allowing classification of the organism into genotypes A (positive for *cpa*), B (positive for *cpa*, *cpb*, and *etx*), C (positive for *cpa* and *cpb*), D (positive *cpa* and *etx*) or E (positive for *cpa* and *i*A). The genotype of strians of known toxigenic phenotype (*n* = 131) was identical in 99.2% of cases. Field isolates (*n* = 616) were assigned to genotype A (*n* = 570, 92.7%), genotype B (*n* = 1, 0.1%), genotype C (*n* = 28, 4.5%), genotype D (*n* = 13, 2.1%) and genotype E (*n* = 4, 0.6%). About 8% (*n* = 50) of the total strains were PCR-positive for *cpe*, as genotype A strains was predominant among these. Those results suggested that PCR genotyping may be an acceptable substitute for *in-vivo* typing of ***C. perfringens*** in establishing enteric disease etiology.

Uzal *et al.*, (1997) applied PCR for detection of the genes encoding major toxins of ***C. perfringens*** in rabbits. This method has been highlighted as a rapid and accurate method for detection of low copy numbers of genes. Also, the sensitivity and specificity of this method are confirmed by amplification of specific target DNA under a unique condition. This method is more accurate and faster than serum neutralization test.

Campos *et al.*, (2002) observed that B-2 toxin produced by ***C. perfringens*** strains might be an important virulence factor to ***C. perfringens*** type A. In that work, rabbit's ileal loops were inoculated with filtrates or bacterial culture suspensions of *cpb*-2 positive and negative ***C. perfringens*** strains, as defined by PCR and isolated from intestinal contents of diarrhoeic and healthy piglets, respectively. There was no difference between using of

filtrate or a bacterial culture suspension on the fluid accumulation for each of the used strain. However, a significant difference was observed between using inoculum from *cpb*-2-positive or *cpb*-2-negative. Also, the first kind of inoculum produced more fluid accumulation than the second one.

Campos *et al.*, (2004) verified the pathogenicity of ***C. perfringens*** type A strains carrying the *cpb*-2 gene that coded for the beta-2 toxin production. The rabbit ligated ileal loop model was used. Culture suspensions of ***C. perfringens*** type-A *cpb*-2 carrier strains or their filtrates were inoculated. The dilatation of loops was observed in 19 out of 24 loops received inocula from *cpb*-2 positive strains and in only one out of 12 loops inoculated with *cpb*-2 negative inocula that constituted a significant difference. Insignificant difference in the dilatation of loops was observed in comparing with the use of filtrates or bacterial suspensions. In the loops treated with suspensions of *cpb*-2 positive strain cultures, severe lesions were observed than in those inoculated with filtrates of the same cultures. In the loops inoculated with culture suspensions or filtrates from a *cpb*-2 negative strain and in those treated with sterile Brain Heart Infusion (BHI) broth, no histological changes were observed. The ***C. perfringens*** biotype A strains carrying the *cpb*-2 gene showed its virulence when inoculated into the rabbit's ileum.

Chai *et al.*, (2007) investigated the presence and frequency of ***C. perfringens*** among apparently healthy farm animals in China. About 748 faecal samples were collected from 9 pigs, 4 sheep, 7 cattle and 5 rabbit's farms. ***C. perfringens*** was isolated from 124 samples (16.6%). The PCR was used to classify the strains according to toxin production through detection of genes encoding those toxins. All isolates were identified as ***C. perfringens*** toxin type A. There was a relationship between the incidence of

isolation of type A ***C. perfringens*** and the occurrence of enterotoxaemia in rabbits.

Drigo *et al.*, (2008) succeeded in detection of 80 field strains of ***C. spiroforme*** and their binary toxin coding genes in rabbits using PCR.

Osman *et al.*, (2008) studied the protein profile characters of ***C. perfringens*** type A strains isolated from chickens, rabbits and large animals and found that there were common protein bands between different isolates at molecular weights of 190, 128, 76 and 56 kilo Dalton.

Sayeed *et al.*, (2008) isolated ***C. perfringens*** type C which is the causative agent of enteritis necroticans in humans and enterotoxaemia in domestic animals. Using PCR was found to produce beta toxin (CPB), alpha toxin (CPA) and perfringolysin O (PFO) during log-phase growth.

Sting (2009) found that ***C. perfringens*** alpha toxin and the genes encoding beta2 and epsilon toxin could be frequently detected by means of phenotypical and PCR examinations of diseased rabbits.

Badagliacca *et al.*, (2010) used a multiplex PCR protocol to identify ***C. perfringens*** *cpa*, *cpb1*, *cpetx*, *cpi* genes and a duplex PCR to identify the *cpc* and *cpb2* genes encoding for alpha , beta 1, epsilon , tau , enterotoxin and beta 2 toxins in rabbits. The *cpa* gene alone or in association with the *cpb2* gene were detected in all examined DNA samples. The *cpa* gene together with *cpb2* gene were detected in seven out of 19 rabbit's ***C. perfringens*** strains (36.8%) and in nine isolates out of 41 necropsies (21.9%). The *cpa* gene was found in 63.2% of rabbit's strains and 76.9% of strains from other animal spp.

Pritt *et al.*, (2010) observed that 20 seropositive ***C. piliforme*** in rabbits were negative for the organism using PCR.

2.8. *In vitro* antibiotic sensitivity test:

Secasiu and Pastarnac (1993) tested 435 strains of ***C. perfringens*** strains (292 from mink, 65 from arctic foxes and 78 from rabbits) for the *in-vitro* sensitivity to some antimicrobial drugs. The greatest antibiotic sensitivity was found with Rifampicin, Pristinamycin, Penicillin, Erythromycin, Ampicillin, Chloramphenicol, Tetracycline, Furazolidone and Lincomycin, while relative sensitivity was found with Streptomycin, Neomycin, Colimycin, Kanamycin and Novobiocin.

Abdel-Rahman *et al.*, (2006) observed that the *in-vitro* sensitivity test of ***C. perfringens*** isolated from diarrheic rabbits was sensitivity to Ampicillin and Norfloxacin, but resistance to Streptomycin and Gentamicin.

El-Rahman and Atwa (2006) found that ***Clostridial*** isolates from diarrheic rabbits were sensitive to Enrofloxacin, Spectinomycin and Flumequine *in-vitro*.

Agnoletti *et al.*, (2007) evaluated the minimal inhibitory concentrations (MICs) of Zinc bacitracin in 123 ***C. perfringens*** strains isolated from rabbits in Italian fattening units. The agar dilution method was performed in Brucella agar supplemented with sheep blood, haemin and vitamin K. Most strains (94.3%) had low MIC values (≤0.5 μg/ml) and few strains (4%) were inhibited by a concentration of 1 μg/ml. Two isolates (1.6%) had a MIC value of 16μg/ml. The MIC required to inhibit 90% of organisms was 0.5 μg/ml and the presence of only two strains with MIC=16

μg/ml revealed the susceptibility of rabbits ***C. perfringens*** strains to Zinc bacitracin and of the absence of acquired resistance.

Agnoletti *et al.*, (2010) evaluated the antimicrobial susceptibility of Valnemulin, Tiamulin, Tylosin, Tilmicosin, Spiramicin, Zinc bacitracin and Oxytetracycline for 30 field strains of ***C. perfringens*** which was the causative agent of enterotoxaemia in rabbits. Not only Valnemulin displayed the highest *in-vitro* activity, but also Tiamulin and Zinc bacitracin showed good results with no resistant strains against those antimicrobials. Among Macrolides, 13% of strains showed resistance to Tylosin and Tilmicosin, while all strains were intermediately or completely resistant to Spiramicin. The Oxytetracycline displayed a bimodal MICs distribution and <7% of strains were susceptible.

Heba (2010) found that ***Clostridial*** strains from diarrheic rabbits were highly sensitive to Penicillin G, Clindamycin, Lincomycin and Tylosin, while they were resistant to Cephaloridine, Ampicillin, Chloramphenicol, Gentamicin, Doxycycline, Erythromycin, Streptomycine, Nalidexic aid and Oxytetracycline *in-vitro*.