5- DISCUSSION

Rabbits industry and production have been developed and expanded all over the world to fill the gap between available and required animal protein for human being. Great attention is directed to the diseases causing economic losses to this industry from time to time **(Finzi and Amici, 1991**).

Clostridial enteritis turns up as a big problem in various areas in Egypt and all over the world, therefore the present study was undertaken to through light on the incidence of ***Clostridial*** infections causing enteritis and sudden deaths in weaned rabbits and in their incidence the environment at different Egyptian governorates by using conventional and recent techniques for diagnosis. Also, we tried to induce experimental infection of weaned rabbit's using ***C. perfringens*** and ***C. difficile*** strains and finally we studied the susceptibility of both mentioned ***Clostridial*** strains to different chemotherapeutic agents *in-vitro*.

The observed clinical signs on the examined diseased weaned rabbits or shortly before death in the examined rabbits farms were severe bloat associated with offensive odour doughy brownish to bloody stained diarrhea that soil the region around anus and hind quarters, inability to walk, depression and ruffled fur. **Baskerville *et al.*, (1980)**, **Ivanics *et al.*, (1982), Nagi *et al.*, (1988)**, **Hunter *et al.*, (1992)** and **Mostafa (1992)** reported similar signs on naturally infected rabbits by ***Clostridial*** organisms.

Post-mortem examination of freshly dead rabbits in surveyed farms revealed that the small and large intestines had severe enteritis, typhlitis,

ballooning with thickened wall, different degrees of necrosis and hemorrhages of the mucousa, offensive odour doughy brownish or bloody stained contents mixed with gases with and the mesenteric blood vessels were engorged with blood which are the same as that recorded by **Prescott, (1977)**. The livers usually showed congestion, enlargement, sub-capsular hemorrhages, necrosis especially at the liver margins and friability while the gall bladder was distended. **Kunstyr *et al.*, (1975)** found similar liver's lesions in rabbits infected with ***C. perfringens***. The kidneys showed congestion and enlargement and the urinary bladders usually were filled with urine. Lesions detected in dead rabbits with different ***Clostridial*** infections by **Baskerville *et al.*, (1980), Nagi *et al.*, (1988), Abdel-Rahman *et al.*, (2006)** and **Shi XiShan *et al.*, (2008)** were resembling to our observed results.

The results of the histopathological examination of the liver of naturally infected dead weaned rabbits were fibrosis, newly formed bile ducts associated with diffuse kupffer cells proliferation, inflammatory cells infiltration in between the hepatocytes and severe congestion in the central vein while the surrounding hepatic parenchyma had brown pigmented material. **Prescott, (1977)** and **Heba, (2010)** observed similar histopathological alterations in dead rabbits due to ***Clostridial*** organisms. The small intestine showed necrosis involved the mucosal layer with desquamation of the lining epithelium while the underlying submucosa showed oedema, inflammatory cells infiltration and congested blood vessels and capillaries, while the large intestine revealed diffuse mucosal necrosis and ulceration all over the lining epithelium with inflammatory cells infiltration in the lamina propria. Nearly similar microscopical changes in the intestine of ***C. perfringens*** naturally infected dead rabbits were observed previously by **Badagliacca *et al.*, (2010)** and parallel

study in broiler chicken recorded same lesion by **Hofshagen and Stenwig (1992)**.

In this study, it is evident that the total number of the isolated ***Clostridial*** (single or mixed) spp. was 293 out of 367 samples that collected from apparently healthy, diseased and freshly dead weaned rabbits as well as feed and water samples from 19 rabbit's farm at different Egyptian governorates. The number (293) represents isolation rate of 79.9% which is higher than 37.6% **(McDonal and Duncan, 1975)**, 39.0% **(Szemeredi *et al.*, 1983)** and 35.2% **(Mostafa, 1992)**. This difference in the isolation rate between this study and the others may be related to the difference of the locality from which the organs were collected.

The results showed also that single infection with ***Clostridial*** organisms was 298 (78.7%), whereas mixed ***Clostridial*** spp. infection was 4 (1.08%).

In regards to the recovery rate of ***Clostridial*** microorganisms at different Egyptian governorates, it was totally 41.03% (293 positive samples out of 714 ones).

On the basis on colonial morphology and biochemical tests, positive 293 ***Clostridial*** isolates were differentiated into six different spp. ***C. perfringens***, ***C. tertium***, ***C. sporogens***, ***C. bifermentans***, ***C. septicum*** and ***C. difficile***.

The prevalence rate of each of ***Clostridial*** spp. was determined in this study where ***C. perfringens*** was the most predominant one (26%). Nearly similar finding was reported by **Mostafa, (1992)** who isolated ***C. perfringens*** in percentages of 23%, while, higher incidences were

recorded by **Lee *et al.*, (1991)** 76.5 %, **Abdel-Rahman *et al.*, (2006)** 39.3% and **Heba, (2010)** 86%. **El-Rhaman and Atwa (2006)** recovered ***C. perfringens*** from intestine, liver and faecal samples in incidences of 30, 18 and 10%, respectively from apparently healthy rabbits and in incidences of 70, 60 and 36%, respectively from dead animals.

Most of weaned rabbit's enteritis cases usually associated with more than spp. of ***Clostridial*** organisms which possibly promote the invasiveness of other bacterial agents in various mixed infection **(Smith, 1975)**. In this concern, other ***Clostridial*** spp. were recovered from examined samples includes ***C. tertium***, ***C. sporogens***, ***C. bifermentans***, ***C. septicum*** and ***C. dfficile*** with incidences of 25.7, 14.2, 9.5, 3.9 and 2.5%, respectively.

The prevalence percentages of ***C. tertium*** and ***C. dfficile*** were 25.7 and 2.5%, respectively incomparable with 2.5 and 1.2% that recovered by **Hughes *et al.*, (1983)**. In addition, **Mostafa, (1992)** isolated only three and one positive cases for ***C. tertium*** and ***C. difficile***, respectively out of 358 diseased and apparently healthy rabbits, while **El-Rahman and Atwa (2006)** isolated ***C. difficile*** in percentage of 10% from dead rabbits with intestinal pathological lesions.

Reviewing available literatures, it is considered the first record as this study succeeded in reporting that the prevalence percentages of ***C. sporogens***, ***C. bifermentans*** and ***C. septicum*** in rabbits were 14.2, 9.5 and 3.9%, respectively, although **Peter *et al.*, (1986)** demonstrated that ***C. sporogens***, ***C. bifermentans*** and ***C. septicum*** are non pathogenic normal inhabitant pathogens in the rabbit's intestine.

The prevalence percentages of the isolated ***C. perfringens*** from feed and water samples were 15.78 and 2.63%, respectively.

Nevertheless, another study of **Heba, (2010)** revealed higher isolation percentage of ***C. perfringens*** as 58.3 and 50%, respectively from the previously mentioned environmental samples and these higher percentages in comparison with ours may be correlated to collection of water samples from manual not automatic drinkers that allow more water contamination by the rabbit's faecal matter.

Clostridial spp. other than ***C. perfringens*** were not isolated (0.00%) from either feed or water samples as they are normal non pathogenic inhabitant in the intestinal tracts of rabbits. **Peter *et al.*, (1986)** demonstrated that ***C. sporogens***, ***C. bifermentans*** and ***C. septicum*** are non pathogenic normal inhabitant pathogens in the rabbit's intestine.

The total incidence rate of the isolated ***Clostridial*** spp. (***C. perfringens*** and other spp.) at different Egyptian governorates were 36.5, 36.5, 43.7, 42.5, 42.7, 43.2, 43.4 and 38.9% respectively in Port Said, Giza, Cairo, Beni Suef, Fayoum, El-Qaliubiya, Al-Sharkia and El-Menoufia. Other study of **Mostafa, (1992)** showed that, in Giza, the number of the isolated ***C. perfringens*** strains from 358 apparently healthy and diseases rabbits were 83 positive cases (35.2%). Also, **Heba, (2010)** recovered that the incidence of ***Clostridial*** infection in Giza were 86%. In El-Menoufia governorate, the incidence rates of 30, 18 and 10% from the intestines, livers and faecal samples, respectively was detected from 300 diseased and dead 4-12 weeks old rabbits **(El-Rahman and Atwa, 2006)**. Moreover, from El-Menia governorate, **Abdel-Rahman *et al.*, (2006)** demonstrated that the incidence rate of ***C. perfringens*** that isolated from 140 rectal swabs from apparently healthy, diarrhoeic and dead weaned rabbits was 39.30%.

Since ***C. perfringens*** represents as an intestinal commensal organism **(Petit *et al.*, 1999),** so we should differentiate between toxigenic and non toxigenic ***C. perfringens***. Toxigenic ***C. perfringens*** produces alpha, beta, epsilon and iota toxins according to types of the organism (A to E). The alpha toxin is phospholipase C and lecithinase that has been implicated in several diseases in rabbits due to its lethal and hemolytic effects **(Julian, 1998)**.

The toxigencity test in Swiss mice, Nagler's reaction, dermonecrotic reaction in Guinea pigs and serum neutralization test in Swiss mice revealed that out of 93 ***C. perfringens*** strains, there were 89 (95.69%) toxigenic and 4 (4.3%) non toxigenic strains. Partial agreements with our results were recorded in other studies of **Mostafa, (1992)**, **Abdel-Rahman *et al.*, (2006)** and **Heba, (2010)** who found that the percentage of ***C. perfringens*** toxigenic and non toxigenic strains were 68.3 and 31.7%, 81.82 and 18.18% and 75.4 and 24.56%, respectively.

Regarding the results of toxigencity, it was demonstrated that the percentages of the toxigenic single type ***C. perfringens*** strains were 16.12, 4.3, 16.12 and 4.35%, respectively for ***C. perfringens*** types A, B, D and E. Meanwhile, mixed ***C. perfringens*** types was (A and D), (A and E) and (B and D) were representing percentages of 34.4, 4.3 and 16.12%, respectively. Higher percentages were recorded by **Mostafa, (1992)** who isolated ***C. perfringens*** types A, B, D and E, respectively at 15.65, 4.45, 8.89 and 71.11%, while mixed types of ***C. perfringens*** (A and D) was isolated in percentage of 5%. Furthermore, **Heba, (2010)** isolated ***C. perfringens*** types A and D with percentages 47.3 and 20.0%, respectively.

The prevalence of single and mixed types as well as non-toxigenic ***C. perfringens*** at different Egyptian governorate in descending order was 30 (Giza), 19 (El-Qaliubiya), 10 (El-Sharkea), 9 (Cairo), 7 (Port-Said), 7 (Fayoum), 6 (Beni Suef) and 5 (El-Menoufia) governorate. Moreover, the prevalence of other spp. of ***Clostridia*** other than ***C. perfringens*** was the highest (44 in Giza) followed by (42 in El-Qaliubiya), (40 in Fayoum), (25 in El-Menoufia), (23 in Port-Said), (11 in Beni Suef), (10 in El-Sharkea) and (5 in Cairo).

Out of a total number 357 rabbit's samples, the total recovery of single ***Clostridial*** spp. was 283 strains representing (66.6%), mixed ***Clostridial*** spp. were 4 (1.1%), while the different types of ***C. perfringens*** was 51 (14.2%).

The PCR technique is used as a recent, rapid and accurate diagnostic tool for detection and typing of ***C. perfringens*** **(Uzal *et al.*, 1997)**.

Concerning the result of conventional PCR, it was revealed that ***C. perfringens*** types (A, B, D and E) were positive for Cp alpha toxin at (324bp). Moreover, the multiplex PCR showed that ***C. perfringens*** type (A) was positive for Cp alpha toxin at (324bp), ***C. perfringens*** type (B) was positive for Cp alpha toxin at (324bp), beta toxin at (196bp) and epsilon toxin at (655bp), ***C. perfringens*** type (D) was positive for Cp alpha toxin at (324bp) and epsilon toxin at (655 bp) and ***C. perfringens*** type (E) was positive for Cp alpha toxin at (324b) and iota toxin at (446bp). Parallel results were found by **Glenn Songer and Ralph Meer (1996)** and **Augustynowicz *et al.*, (2000**).

The results of *in-vitro* sensitivity of the most prevalent types of ***C. perfringens*** and ***C. difficile*** recovered from surveyed rabbit's Egyptian farms to different antibiotics showed that all types of ***C. perfringens*** as well as ***C. difficile*** were highly sensitive for Amoxicillin / Clavulanic acid and Ampicillin. Strains of ***C. perfringens*** types (E, A and E), (A and D) and (B and D) as well as ***C. difficile*** were also highly sensitive to Tylosin. For ***C. perfringens*** types (D, E, A and E), (A and D) and (B and D) and ***C. difficile***, Nalidixic Acid was highly effective. All strains of ***C. difficile*** were very sensitive to Gentamicin, Oxytetracycline, Penicillin G, Enrofloxacin, Doxycycline, Tetracycline and Chlorotetracycline. Also all types of ***C. perfringens*** were intermediately sensitive to Enrofloxacin, Doxycycline and Penicillin G, while ***C. difficile*** was intermediately sensitive to Metronidazole, Chlorotetracycline, Kanamycine and Sulphquinoxaline / Trimethoprim. On the other hand, all types of ***C. perfringens*** and ***C. difficile*** were resistant to Colistine, Erythromycin and Lincomycine. These results are nearly similar to those recorded by **Secasiu and Pastarnac, (1993)** who tested the *in-vitro* sensitivity of 78 strains of ***C. perfringens*** isolated from rabbits to some antimicrobial drugs and found that they were sensitive to Penicillin and Ampicillin, **Abdel-Rahman *et al.*, (2006)** who observed that ***C. perfringens*** isolated from diarrheic rabbits was sensitive to Ampicillin and resistant to Gentamycin, **Agnoletti *et al.*, (2010)** who detected that rabbit's ***C. perfringens*** isolates were sensitive to Tylosin and Oxytetracyclin, and **Heba, (2010)** who demonstrated that ***Clostridial*** isolates from diarrheic rabbits were highly sensitive to Penicillin G and Tylosin. On the other hand, our results disagree with **Mostafa, (1992)** who reported sensitivity of ***Clostridial*** spp. Colistin sulfate and Sulphquinoxaline/ Trimethoprim and resistance to Penicillin G and Tertracycline, **Abdel-Rahman *et al.*,**

(2006) who found that ***C. perfringens*** isolates were resistant to Gentamicin and **Heba, (2010)** who recorded on resistance of ***Clostridial*** strains to Nalidixic acid, Gentamicin, Ampicillin, Oxytertracycline and Doxycycline.

The results showed that ***C. perfringens*** types (A, B, D and E) and ***C. difficile*** were the most prevalent recovered strains in this study, so, our experimental study focused on detecting of the role of these five ***Clostridial*** strains in induction of infection in newly weaned rabbits.

In regard to the experimental infection in weaned rabbits, the highest mortality rate was observed within 24 hrs. post-challenge in ***C. perfringens*** infected rabbits with type (B) (S/C), type (A) (S/C), type (D) (S/C), type (E) (S/C), type (A) (oral), type (D) (oral) and type (E) (oral) at percentages of 80, 60, 60, 40, 20, 20 and 20%, respectively. The findings recorded by **Mostafa, (1992)** are totally agrees with ours as he induced experimental infection of weaned rabbits with ***C. perfringens*** types (A, D and E) through both S/C and oral routes and recorded mortalities in rates of 40 and 30%, respectively in type (A) by S/C and oral routes, while it reached 50.0% in type (E) through both oral and S/C routes, also mortalities reached 70 and 60 % in type (D) through both oral and S/C routes, respectively. Added to that, **McDonel and Duncan, (1977)** and **Matthes, (1981)** are virtually agree with this work as they noticed mortality rates between 30-50% among experimentally infected rabbits with ***C. perfringens*** type (A). Opposite results were observed by **Abdel-Rahman *et al.*, (2006)** who recorded mortality rates of 75 and 37.5 %, respectively in orally challenged rabbits with ***C. perfringens*** types E and A. The variation in mortality rates percentages in infected rabbits among the different studies may be referred

to the route of inoculation and the dose of the inoculated viable ***Clostridial*** bacterial cells.

Blank control non infected group exhibited no mortalities along the course of the disease.

No clinical symptoms were also seen in control blank non infected rabbits.

All ***C. perfringens*** challenged groups (A, B, D and E) through both oral and S/C routes showed general signs of depression, off food, in-ability to walk and ruffled fur. Oral infection of rabbits with ***C. perfringens*** type (A) revealed severe brownish offensive odour diarrhea accompanied with bloat, while S/C infection with ***C. perfringens*** type (A) showed severe watery dark brownish and bloody diarrhea with offensive odour and the urine became purulent with offensive odour. Rabbits infected with ***C. perfringens*** type (B) orally suffered from light bloat without diarrhea, whereas subcutaneously infected ones showed bloody urine and severe degree of bloat without diarrhea. Subcutaneous infection of rabbits with ***C. perfringens*** type (D) induced bloody urine and bloat. Infected rabbits either orally or subcutaneously with ***C. perfringens*** type (E) revealed light brownish watery diarrhea. These results are almost equal to that detected by **Nagi *et al.*, (1988), Hunter *et al.*, (1992)**, **Mostafa, (1992)** and **Jorge *et al.*, (2008)** who inoculated weaned rabbits with various types of ***C. perfringens***.

The observed bloody diarrhea with offensive odour in group of rabbits infected S/C with ***C. perfringens*** type (A) were confirmed by the histopathological findings of Small intestine that show necrosis in mucosal layer and large intestine also show necrosis of the mucosa with congestion in blood vessels.

The observed purulent cystitis in group of rabbits infected S/C with ***C. perfringens*** type (A), was subjected for bacteriological examination which resulted in isolation of ***E. coli***. and confirmed by the histopathological findings of kidney that show coagulative necrosis in the tubular lining epithelium of the cortex. That finding could be attributed to the stress of toxic effect of ***C. perfringens*** type (A) which leads to flourishing of some bacteria as ***E. coli***, similar observation was recorded by **Barral *et al.*, (2000)** who isolated ***C. perfringens*** from cases of rabbit's enteritis that were associated with isolation of ***Coli*** forms, especially serotype (O2).

The observed bloody urine in group of rabbits infected S/C with ***C. perfringens*** type (B) were confirmed by the histopathological findings of the kidney that revealed degeneration in the tubular lining epithelium.

The observed bloody urine in group of rabbits infected S/C with ***C. perfringens*** type (D) were confirmed by the histopathological findings of the kidney that revealed focal haemorrhage in between the degenerated tubules at the renal cortex.

No clinical signs were seen in rabbits challenged with ***C. difficile*** after S/C infection except signs of light brownish diarrhea was detected in orally infected animals at the 3rd day post infection. **Prescott, (1977)** and **Keel and Songer, (2006)** referred to the role of ***C. difficile*** in induction of enteritis in rabbits.

Post-mortem findings in livers of orally infected rabbits with ***C. perfringens*** types (A, B and D) were congestion, friability, sub-capsular hemorrhage and necrosis at margins and the gall bladder was distended with bile as these reported by **Mostafa, (1992)**. The kidneys were congested with focal necrosis. The spleen was highly congested. The stomach impacted with offensive odour content. The small intestine

showed ballooning, containing un-digested feed particles with congestion and peticheal hemorrhage on mucosa and mesenteric blood vessels were engorged with blood. Identical intestinal lesions were observed by **Prescott, (1977)**. The large intestine was impacted with greenish brown doughy content with offensive odour.

Similar lesions were seen in animals subcutaneously inoculated with ***C. perfringens*** types (A, B, D and E) except presence of subcutaneous gelatinous exudates in types (A, B and D).

The characteristic lesions in rabbits infected with ***C. perfringens*** types (B and D) subcutaneously were that presence of bloody urine in the urinary bladder, the small intestine was severely ballooned, congested containing yellow to green undigested feed particles mixed with gases and the large intestine was filled with greenish watery content mixed with gases with offensive odour and its mucosa was congested in some parts.

Type (E) ***C. perfringens*** orally challenged animals showed very characteristic liver lesions as there were shrinkage, firmness, adhesion between liver loops, caseous perihepatitis with adhesion between the liver and gall bladder, also there was circumscribed white raised necrotic foci scattered on mucosal surface of large intestine in both orally and S/C challenged animals with ***C. perfringens*** Type (E). Nearly similar findings had been reported by **Mostafa, (1992)**.

All dead and living rabbits during the observation period after challenge exhibited positive results of re-isolation of different types of C**.** ***perfringens*** and ***C. difficile*** as they were present in all of the examined animals.

The histopathological finding of collected tissue specimens of control group revealed that there was normal histological structure of the liver, kidney, small intestine and large intestine. While the common histopathological findings of dead rabbits during experimental period were as follow: liver tissue showed sever dilatation was noticed in the central veins and sinusoids, associated with mononuclear leucocytes inflammatory cells infiltration in between the hepatocytes, similar findings were recorded by **Prescott, (1977)**. Kidney tissue showed degenerative change in the lining epithelial cells of the tubules at the renal cortex, identical intestinal lesions were observed by **Heba, (2010)**. Small intestine showed different degrees of necrosis in the mucosal layer associated with oedema and hypertrophy of the muscular layer with Focal inflammatory cells infiltration and congestion of blood vessels in the lamina propria, similar histopathological alterations were also observed previously in broiler chickens by **Hofshagen and Stenwig, (1992)**. Large intestine showed different degrees of necrosis in the mucosal layer with congestion in the underlying of muscular layer and congestion in blood vessels, similar findings were observed in broiler chicken by **Hofshagen and Stenwig (1992) and** similar findings were observed in rabbits by **Badagliacca *et al.*, (2010)**.

The statistical analysis of the cumulative feed consumption of ***C. perfringens*** and ***C. difficile*** experimentally infected rabbits during observation period was not significant ($P \leq 0.05$) when compared with control group.

The cumulative weight gain of ***C. perfringens*** and ***C. difficile*** challenged rabbits revealed that all orally infected groups were significantly ($P \leq 0.05$) lower than control group except the group of animals challenged with ***C. difficile*** orally.

The results of cumulative FCR of ***C. perfringens*** and ***C. difficile*** challenged rabbits showed that all orally infected groups were significantly ($P \leq 0.05$) higher than control group except the group of animals challenged orally with ***C. perfringens*** type (D) and ***C. difficile***.

The feed conversion rates of experimentally infected rabbits with most types of ***C. perfringens*** was significantly ($P \leq 0.05$) higher (bad) than that of the control group and this result agreed with that recorded by **Romero *et al.*, (2009 a and b)** who detected that ***C. perfringens*** proliferation helped in the development of epizootic rabbit enteropathy, causing impaired growth and increasing weight variability at weaned rabbits.

The observed cumulative weight gain and feed conversion rate showed that ***C. perfringens*** **type** (A) group was significantly ($P \leq 0.05$) the lowest in weight gain but the highest in feed conversion rate. This result agrees with that recorded by **Genigeorgis *et al.*, (1973)** who found that ***C. perfringens*** **type (A)** was badly affecting the performance parameters of infected rabbits as a result from production of enterotoxins that destruct the intestinal mucosa inducing poor absorbability of the nutrients and also causing impairment in the liver function that subsequently affect on the protein synthesis.