Closteridia as an Etiological Agent of Mucoid Enteropathy in Rabbits.

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Abstract: This study was carried out to investigate the anaerobic bacteria as a causative agent of Mucoid enteropathy in Rabbits. Thirty - three isolates of *Clostridia perfringens (C. perfringens*) were isolated mainly from caecum of 225 specimens of forty- five diseased and freshly dead rabbits. Genotyping of *C. perfringens type A* using Multiplex PCR revealed that alpha toxin genes are detected in 8 isolates. The pathogenicity of isolated clostridia in rabbits was carried out. The clinical signs, morbidities, mortalities and body weight gain were recorded for experimentally infected rabbits. Reisolation of *C. perfringens type A* from freshly dead and/or sacrificed experimentally infected rabbits has been done. Histopathological study of intestine of affected rabbits was carried out.

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1. Introduction:

During recent years, rabbits industry became well established in Egypt. Rabbit meat is used as a good source of animal protein, and some breeds are reared for fur production as well as for medical and biological purposes (Ragheb et al., 1999). Enteritis remain one of the major problems facing rabbitaries, causing high mortalities mainly after weaning which is of economic importance. Mucoid enteropathy in rabbits is a multifactorial disease associated with several factors, one main factor is changes in immature caecum at weaning (Lelkes, 1987). High carbohydrate and low fiber diet, these types of diet can cause production of bacterial toxins agents like C*lostridia perfringens, Clostridia defficile* and *Clostridia spiriforme* (Butt et al., 1994). Investigation were conducted on Mucoid enteropathy outbreaks in different rabbit farms in Egypt and Clostridia perfringens type A was mainly incriminated and could be isolated from caecum of rabbits which died suddenly after short illness with sever diarrhea. Clostridia organisms are widely distributed in nature and considered to be normal inhabitant in intestinal tract of man and animal. Under certain circumstances usually stress factors and disease conditions, these organisms may become active and produce toxins which are responsible for a variety of diseases in animals (Smith and Williams 1984). Also alpha toxins of *C. perfringens type A* could be detected in the caecal content of dead rabbits (Diab et al., 2003). Therefore, the aim of this study was to monitor the current bacterial agents responsible for enterotoxaemia in rabbits through isolation and identification of the prevalent clostridia anaerobic bacteria from field affected rabbits; typing and identification of C. *perfringens* toxins genes by multiplex PCR and finally histopathological studies of experimentally infected rabbits.

2. Materials and methods

A. Materials

A.1. Specimens:

Two hundreds fifty five specimens from liver, spleen, caeci and intestine were collected from forty five diseased and /or freshly dead rabbits of different breeds and from different localities at Sharkia and Dakahlia Governorates with an average 4-12 weeks- old. All rabbits were subjected to clinical and / or postmortem examination, specimens were subjected to bacterial isolation and identification.

A.2. Bacterial media:

A.2.1. Blood agar base

A.2.2. Cooked meat media

A.2.3. Neomycin sheep Blood agar medium (Willis., 1977):

A.2.4. Brain heart infusion broth

It was used for enrichment and preservation of pure isolates *C. perfringens* and for further identification to extract DNA for PCR.

A. 2.5. Peptone water broth

A.2.6. Production Medium (Roberts et al., 1970).

It was used for production of *C . perfringens* exotoxins

A.2.7. Gas generating kit and Gaspak anaerobic jar.

A.2.8. Enriched egg yolk agar medium (Cruickshank et al., 1975.

A.2.9. Semi-Solid agar medium

A.2.10. Gelatin Medium (Smith and Holdeman, 1968):

A.2.11. biochemical identification:

- Lactose, Glucose, Maltose and sucrose used for identification and differentiation of anaerobes with 0.0018% phenol red
- Christensen's urease agar slants
- Indol test media

A.3. Chemicals, reagents, stains and solutions:

The following chemicals and reagents were prepared according to (Cruickshank et al., 1975; Koneman et al., 1992 and Baron et al., 1994)

1- Hydrogen peroxide 3% ($H_2 O_2$) freshly prepared for catalase test.
2- Kovak's reagents for indole test.
3- 0.02% methyl red solution for methyl red.
4- Bromocresol purple for detection of lecithinase activity.
5- Phosphate Buffered saline (PBS).
6- Phenol red for sugar fermentation test as 0.0018% and urea utilization test as 0.0012%.
7- Natural buffered formalin 10% for fixation and preservation of the affected organs for histopathological examination.
8- Gram stains which was used to differentiate between the organisms into Gram-positive or Gram negative.

A.4. Antibiotics:

1- Neomycin sulphate used to obtain separate colonies of clostridium and prevention the growth of other anaerobes.
2- Streptomycin and Rivampicin for preparation of resistant strain of inoculated microorganisms (*C. perfringens type A*) for experimental infection.

A.5. Experimental rabbits:

Thirty - two native breed rabbits were obtained from private farm in Sharkia Governorate for experimental infection. Rabbits were fed on ration obtained from (El Marwa Company) containing: (protein 18.5%, fiber 15.5%, fat 3.4% and calories 26500). The ration contains Clazurel as anticoccidial drug and not contain antibiotics.

A.6. Material used for polymerase chain reaction

A.5.1 Materials, buffers and reagents used for multiplex PCR after (Yoo et al., 1997)

- Tris boric EDTA (TBE) 5x: Tris base-Boric acid-EDTA. It was used for DNA extraction and as buffer for visualization of PCR products in agarose.
- 10x PCR buffer (Gibco/ BRL, Grand Island, N.Y.).
- $MgCl_2$ (2.5 mM) applied biosystem PCR mix, USA)
- dNTPs (deoxy nucleotide triphosphate) 10mM) (Gibc6)
-Taq thermostable DNA polymerase (Biometra) (2U).
-Template DNA (*C.perfringens* alpha, beta and epsilon toxin genomes
-Reference strains of (alpha, beta and epsilon toxin genomes) were obtained from National Laboratory for Veterinary Quality Control (NLVQP), Animal Health Research Institute (AHRI), Dokki, Giza, Egypt.
- Oligonucleotide primers (100 pmol).

Primers for the four toxin genes (alpha, beta, epsilon and iota) of *C. perfringens* were selected after (Yoo et al., 1997) as shown in table (1).

Table (1) shows Primers for the four toxins genes of *C. perfringens* type

Primer name and direction	Nucleotide sequence	Size of amplified products (bP)
Cpa(alpha toxin genes Forward Reverse	5' GTT GAT AGC GCA GGA CAT GTT AAG3' 5' CAT GTA GTC ATC TGT TCC AGC ATCC3'	402
Cpb(beta toxin genes Forward Reverse	5' ACT ATA CAG ACA GAT CAT TCA ACC3' 5' TTA GGA GCA GTT AGA ACT ACA GAC 3'	236
Cpe(epsilon toxin genes Forward Reverse	5' ACT GCA ACT ACTACT CAT ACT GTG 3' 5' CTG GTG CCT TAA TAG AAA GAC TCC3'	541
Cpi (iota toxin genes Forward Reverse	5' GCG ATG AAA AGC CTA CAC CAC TAC3' 5' GGT ATA TCC TCC ACG CAT ATA GTC3'	317

A.5.2. Agarose gel electrophoresis buffers and reagents (Piattir et al., 2004)

a- Agarose gel 1.5%
b- Agarose 1.5gm
c- Electrophoresis buffer (TAE or TBE 100 ml)
d- TBE (Tris Boric EDTA).
Composed of 0.5 x (0.04 µl Tris-borate and 1 mM EDTA, pH 8.0
e- Ethidium bromide solution
Ethidium bromide powder 10 mg
Distilled water (D.W.) 1 ml

It mixed and put into melted agarose to reach to a final concentration of 1.0- o.5 mg /ml. It was used as a fluorescent dye to stain the DNA during examination by UV transilluminator.

f- Gel loading buffer
Bromo phenol blue 0.25%
Xylene cyanol 0.25%
Glycerol 30.0%

They are dissolved in sterile D.W. and covered with aluminum foil to be stored at room temperature. DNA ladder (100 bp, pharmacia, USA).

B. Methods

B.1 Clinical and postmortem examination:

Clinical examination of diseased rabbits was carried out for recording clinical signs. Faecal samples were examined microscopically for excluding coccidiosis. Post mortem examination of both freshly dead and sacrificed rabbits were carried out to recording the post mortem lesions. Specimens from liver, spleen, ceacum and intestine were collected in separate sterile container for bacteriological examination. In addition specimens of affected intestine from dead and sacrificed rabbits were collected in 10% buffered formalin for histopathological examination.

B.2 Bacterial isolation:

Specimens of liver, spleen, caeci, and intestine were cultivated on sterile freshly prepared cooked meat media in two tubes, one tube was heated in water bath at 80^{o}C for 15-20 minutes to kill any other vegetative form of the bacteria than clostridia spores, the other tube was left without heating then incubated anaerobically in anaerobic jar that contained catalyst and gas generator kits at 37^{o}C for 24-48 hours. A loopful from previously incubated tube was streaked into surface of 10% sheep blood agar with neomycin sulphate plate (100µg/ml). Then the plate was incubated anaerobically at 37^{o}C for 24-48 hours. Suspected colonies of Clostridia were picked up and examined for their morphological and cultural characters then subculture on brain heart infusion broth tubes for preservation. (Koneman et al., 1992 and Baron et al., 1994).

B.3. Bacterial identification.

B.3.1 Morphological identification

Bacterial smears were prepared from the suspected colonies and stained with Gram stain and examined microscopically for morphological characteristic. The colonial appearance was also studied to investigate their structure, surface edge and colour.

B.3.2. Biochemical identification.

Suspected colonies of isolated microorganisms were identified by a series of biochemical tests according to Smith and Holdeman (1968), Willis (1977) and Koneman et al., (1992).

- Catalase test using few drops of freshly prepared 3 % H_2O_2.
- Indol test using tubes with 2 % peptone water.
- Gelatinase – activity test using nutrient gelatin media.
- Methyl red test using glucose phosphate peptone.
- Lactose, glucose, sucrose and maltose fermentation test using 1% peptone broth.
- Lecithinase reactions test using egg yolk agar plate.

B.3.3. Molecular identification.

B.3.3.1. DNA extraction, crude cell lysate (Daube et al., 1994)

One to five pure colonies of C. *Perfringens* that showed double zone of haemolysis on blood agar were grown overnight in 10 volumes of brain heart infusion (BHI) supplemented with 1% (W/V) sodium thioglycolate under anaerobic conditions at 37^{o}C for 16-24hours. The cells were harvested by centrifugation (5000Xg, 15min, 4^{o}C). Take 1 ml of culture then were washed twice with 1 ml of phosphate buffer saline (PH 7.2) then pellet was suspended in 50 ml of TE buffer. The mixture was mixed by vortex apparatus, boiled for 10 min. for cell lyses, centrifuged at 13000xg for 2 min. and 5 ml of the supernatant was used as template.

B.3.3.2. PCR amplification and cycling protocol: (Yoo et al., 1997).

PCR was performed in thermocycler in a total reaction volume of 50 ml containing 5 ml of 10 x PCR buffer, 4 µl of $Mgcl_2$, 1 µl of dNTPs, 0.5 ml of each primers, 5 ml template DNA, 1 µl of Taq – polymerase and up to 50 µl of distilled water. The following program was used: initial denaturation at 94^{o}C for 5 min, followed by 30 cycles consisting of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min, and extension at 72^{o}C for 1 min.

B.3.3.3. Detection of PCR products.

Ten µl of amplified PCR products were mixed with gel loading buffer and electrophoresed in 1.5% agarose gel as shown in the following: The prepared agarose was melted by using microwave oven, let the solution cool down to about 60^{o}C at room temperature, then ethidium bromide was added by 0.5 µg/ml and mix thoroughly. It poured directly on gel casting try after proper installation of the desired comb at one side of the gel, about 5-10 mm from the end of the gel and after the gel was solidified at room temperature, carefully remove the comb. The holes that remain in the gel are the wells. Electrophoresis buffer (TBE or TAE buffers) that used for preparation of the gel was added into the tank to a level of 1-2 mm above the gel layer. Then the samples and a 100bp DNA ladder (market) were mixed with proper gel loading buffer then loaded in the wells, the cover of the tank was closed and the power supply was attached. A current of 60 -80 V for 1 hour was passed on the electrophoresis unit and bromophenol blue was allowed to run to run 2/3 of the gel length before terminating the run. Stopped the run and the gel was transferred to ultraviolet

transillumination to observe the specific amplicons, compared with the marker and photographed by digital camera.

B.4. Preparation of resistant strains.
C. Perfringens type A. was treated with streptomycin and Rifampicin for obtaining resistant serotype. It was subcultured for 24-48 hours anaerobically in 5 successive brain heart infusion broth containing increased concentration of streptomycin and Rifampicin (0.01gm,1 µg to 1 gm, 50µg) respectively, then subcultured on blood agar containing 1 gm streptomycin and 50 mg Rifampicin per litter.

B.5. Titration of inoculum:
Clostridia strain was prepared by selection one colony from blood agar to be cultivated on brain heart infusion broth and incubated anaerobically for 48hours. Serial dilution of microorganism in sterile PBS. The viable cell concentration of the inoculum was determined by colony count on blood agar. The infected dose was adjusted to $1x10^9$CFU.

B.6. Experimental design:
To study the pathogenicity of *Clostridia perfringens type A* in rabbits. Thirty- two native breed rabbits were divided into two main groups according to age. First group (G1) containing 16 rabbits aged 4-6weeks. Second group(G2) containing 16 rabbits aged 8-10weeks. Each group of rabbits were subdivided into two subgroups (1a, 1b) and (2a, 2b) each subgroup containing 8 rabbits. Subgroup (1a) rabbits were infected orally with C. *Perfringens* type C in a dose of 1.0 ml containing $1x10^9$CFU. Meanwhile, subgroup (1b) was remained as a negative control. Subgroup (2a) rabbits were infected orally with C. *Perfringens* type A in a dose of 1.0 ml containing $1x10^9$CFU. Meanwhile, subgroup (2b) was remained as a negative control. All experimental rabbits were observed for 2 weeks before experimental infection to be examined to exclude coccidia, *E coli* and clostridium infection. Clinical signs, morbidities, mortalities of all experimentally infected rabbits were recorded for 3 weeks post infection, an histopathalogical examination was carried out.

B.7. Histopathological examination:
Histopathalogical examination was carried out according to Schauer et al., 1998). The necropsy was performed and samples were collected from the affected intestine and fixed in 10 % buffered neutral formalin solution. Five - micron thick paraffin sections were prepared, stained with hematoxyline and eosin and then examined microscopically for histopathological finding.

3. Results
3.1 Clinical findings
Clinical examination of collected diseased rabbits was shown that watery brownish and/or yellowish diarrhea, or white mucoid discharges staining the hair around the anal, and the hind quarters, belly swollen, impacted ceacum and off food. In late stages of the disease, examined rabbits showed dehydration, emaciation and deaths among all ages especially young rabbits 3-12 weeks age.

3.2. Post mortem findings
Post mortem examination of both freshly dead and scarified rabbits revealed congestion of the liver; heart and kidneys; caecum filled with watery to mucoid contents and gases; distention of the small intestine with watery fluid contents; congested mesenteric blood vessels, in addition, some examined rabbits revealed ceacum containing pasty feted odor contents and colon filled with mucoid material instead of hard pellets.

3.3. Bacterial isolation.
Specimens from liver, spleen, intestine and ceacum were collected from rabbits involved in enteric problems and cultivated on cooked meat media for enrichment of clostridia species and incubated an aerobically at 37^oC for 18-24 hours. Aloopful from previously enriched culture were streaked on neomycin sulphate sheep blood agar and incubated anaerobically at 37^oC for 24-48h. Colonies were rounded, raised, smooth, opaque glistening, showed double zones of haemolysis and 2-4mm in diameter.

Table (2) Shows Prevalance of bacterial and non bacterial isolates among examined rabbits.

number	Total no. of C. perfringens	Ceacal Coccidiosis	Hepatic coccidiosis	Round nematodes
45	33	19	2	27
Percentages	73.33%	42.2%	4.4%	60%
Site	Ceacum	Ceacum	Liver	Ceacum

Table (3) bacterial isolates according to different ages:

Age	No of rabbits	Clostridium		
		No	Percentage	
1M	8	4	50%	
2M	27	20		74%
3M	10	9	78,4%	90%
Total	45	33		

C .perfringens, at 2-3 months old rabbits were 78.4%

3.4. Bacterial identification.

3.4.1 Morphological identification

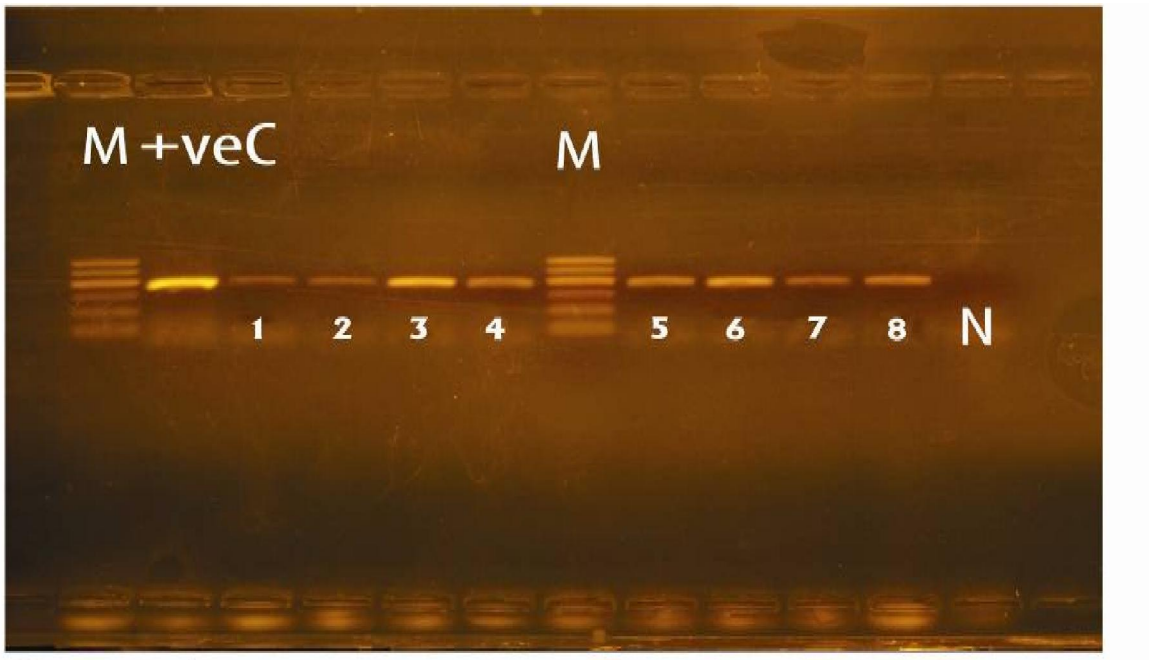
Smears from the colonies were stained with Gram's stain for microscopical examination. Clostridium isolates were Gram positive, short bacilli, straight with parallel sides and rounded ends. Some strains kept in refrigeration for 4-5 months showed central oval non bulging spore.

Biochemical identification

Clostridium isolates were positive with Gelatinase activity, Lecithinase reaction, Sugar fermentation test (Lactose, Glucose, Sucrose, Maltose), Methyle red test and Indol test and negative with Catalase test.

3.4.2- Molecular identification by Polymerase chain reaction:

PCR used for identifying and typing of toxin genes of *C. perfringens* that were isolated from diseased or freshly dead rabbits. C. perfringens *type A* identified by presence of alpha toxin genes and gave at 402bp fragment that were shown in eight strains as in picture no. A.



Picture no. A

Lane "M": 100 bP DNA ladder (Marker).
Lane "C": Positive (+ ve) control of *C. perfringens type "A".*
Lane "1, 2, 3, 4, 5, 6, 7, 8" were only alpha toxin genes producing $_{C}$*. perfringens type "A"* field isolates.
Lane "N" Negative (- ve) control.

3.6 Clinical finding of rabbits experimentally infected with *C. perfringens type A.*

The most clinical findings in experimentally infected rabbits with *C. perferingens type A.* were depression, ruffled fur anorexia, slight swollen belly, watery brownish to yellowish diarrhea, decrease feed intake and sometimes, faeces admixed with mucus material. The mortalities of experimentally infected rabbits begin 5 days post infection with an incidence percentage 0f 25% . The most sever symptoms were observed in experimentally rabbits aged 8-10 weeks – old.

3.6.2. Post-mortem findings of rabbits experimentally infected with *C. perfringens type A.*

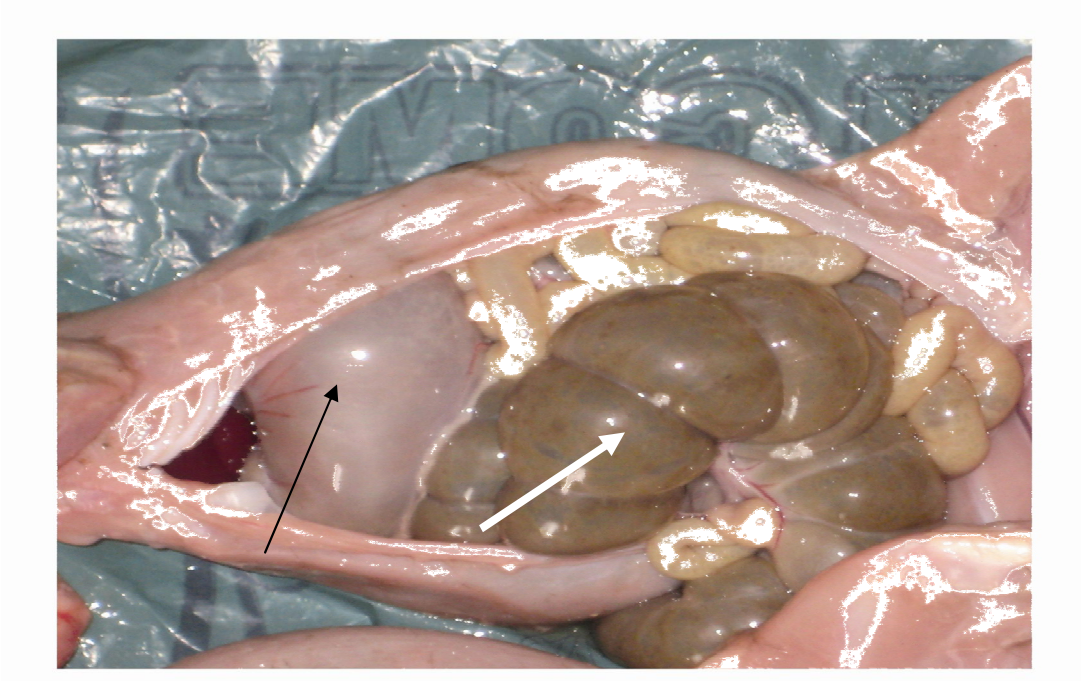
The main gross lesions were congestion of liver, spleen, heart and engorgement of subcutaneous blood vessels. fully distended stomach with fluids and gases, also small intestine and ceacum was distended with watery to mucoid contents and gases. Catarrhal enteritis in ceacum in some cases. Fig. (1&2).

3.6.3. Reisolation trial of *C. perfringens type A* from experimentally infected rabbits.

Reisolation of *C. perfringens type A* was performed in cocked meat medium containing 1.0 gm/liter streptomycin.

Table (4) Shows result of morbidity and mortality percentage of in experimentally infected rabbits.

Group number	Inoculums	Age	Morbidity		M Mortality	
			NO	Percentage	NO×	Percentage
1a	*C. perfringens type A*{	wks4-6	86/	75.%	2/8	25 25%
1b	-ve	wks4-6	3 3/8	37. 37.5%	0/8	0%
2a	*C. perfringens type A*	8-10 wks	6/8	75.%	2/ 2	25 25%
2b	-ve	8-10 wks	/83	37 37.5%	0/ 0	0%



Fig, (1): Caecum of rabbit experimentally infected orally with *C. perfringens type A* at 4-6 weeks –old showing ceacum filled with watery mucoid material and gases, distention in the jejunum and ileum with watery fluid content and congestion of liver. (sacrificed experimental case 7d after infection).

Fig, (2) showing intestine of rabbit experimentally infected orally with *C. perfringens type A* at 4-6 weeks – old with presences of desquamated epithelium. (sacrificed 7 days after infection

3.6.4. Histopathological results of experimentally infected rabbits.

Intestinal lumen of rabbit inoculated with *C. peferingens type A* had excess mucus casts mixed with a few leukocytes with villous destruction and sever inflammatory reaction, mainly macrophages and lymphocytes in both mucosa and submucosa Fig. (3). Other rabbits that infected with *C. peferingens type A* revealed proliferation of intestinal glands, submucosal edema and leukocytic infiltration with regeneration attempts in the surface epithelium Fig (4). On the other hand the intestinal coats of non infected rabbits revealed normal intestinal coats Fig. (5).

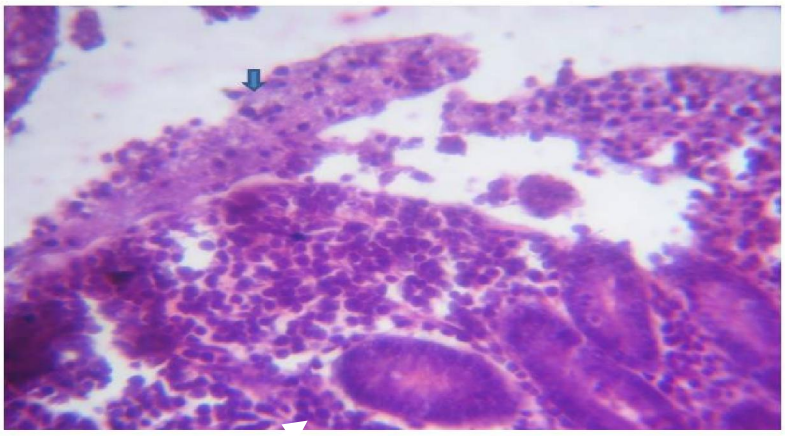


Fig. (3). Small intestine of rabbit infected with *C.perferingens type A,* showing mucus casts with a few leukocytes (arrow) inside intestinal lumen. H&E x 1200.

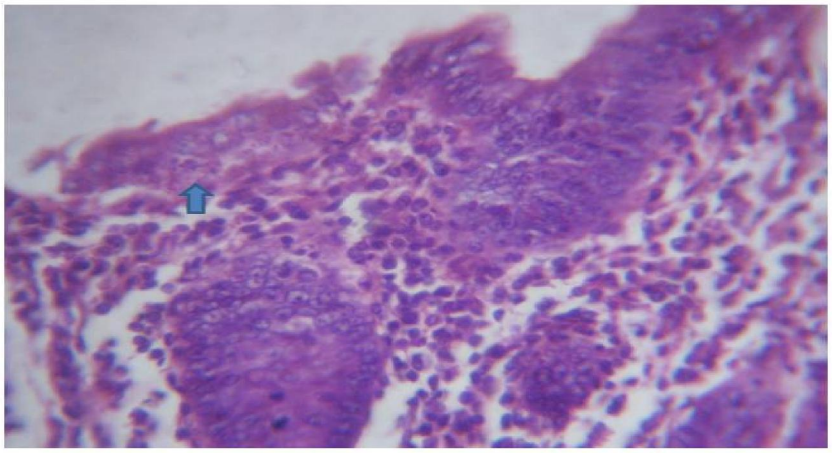


Fig. (4). Large intestine of rabbit infected with *C.perferingens type A* showing proliferation of surface and glandular epithelium. H&E x 1200 .

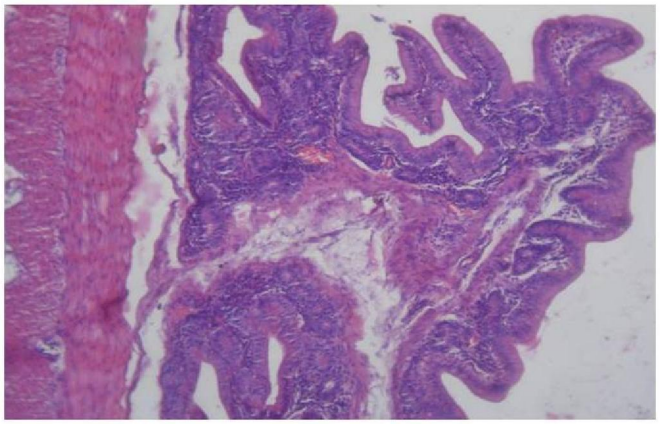


Fig. (5). Small intestine of rabbit showing normal intestinal coats H&E x 300.

4. Discussion:

Rabbit farming enterprises in Egypt have experienced serious losses in weaned animals due to gastrointestinal problems. Investigations had confirmed that these animals suffered from Mucoid enteropathy caused by *C. perfringens type A* Diab et al., (2003). In the present study, isolation and characterization of some anaerobic pathogens causing Mucoid enteropathy in rabbits was carried out. Mucoid enteropathy syndrome (MES) or Epizootic rabbit enteropathy (ERE) have emerged and disseminated in different farms causing great

economic losses during the past years all over the world including Egypt. Enteric diseases are responsible for high morbidity characterized by growth depression, poor food conversion rate or mortality specially in young rabbits_Licois., (2004). In this study samples from liver, spleen, caeci and intestine were collected from rabbits either showing signs of diarrhea or freshly dead rabbits suffering diarrhea from different localities at sharkia and Dakhalia governorates. The isolated anaerobes were clostridia and identified from intestinal samples were 73.33% . The high incidence of clostridia infection at 2-3 month - old rabbits (78.4%) these means that the bacterial isolates were more common at the weaning age rather than suckling age. This results agreed with Mcpherson., (2000) who recorded that enterotoxaemia disease most commonly seen in weaning rabbits. Moreover, Patton et al. (1978) and Carman and Borriello (1984) recorded that the predisposition of rabbits to anaerobic infections is increased by stress factors, bacterial infection, parasitic infection, dietetic disorder and excessive antibiotic administration. The biochemical finding suggested that all isolates of Clostridia recovered from intestinal samples of examined rabbits were *Clostridia perfringens*. This result was confirmed by multiplex PCR. This result agreed with Ali et al., (1994) who isolated *C. Perfringens* (81%) from total of 120 rabbit samples and types toxigenic strains (22 strains) into type A, D and E. The type A was the most predominant one (16 strains). In addition Cocchi et al., (2008) showed that *C. Perfringens type A* was most commonly recorded type from ceacum of diseased rabbits and represented by 99.33 % which agree with our results. PCR was used for identification and confirmation of the causative agents, PCR is very sensitive and specific technique for detection of genes encoding alpha, beta, epsilon and iota exotoxins of *C. perfringens* (Nillo., 1980: Titball et al., 1989: Daube et al., 1994 and Yoo et al., 1997). In the present study, multiplex PCR was very sensitive test for genotyping of *C. perfringens* isolates. The recorded results revealed that the tested strains were identified and typed as eight *C. perfringens* according to presence of alpha toxin gene and give a

Characteristic band at 402 bp. This result was agreed with results obtained by Yoo et al., (1997) who developed multiplex PCR assay for determination the toxin genes of *C. perfringens* that give similar characteristic band. Experimentally infected rabbits, aged 6-8 weeks and 8-10 weeks-old, with *C. perfringens type A* showed depression, ruffled fur, anorexia slight swollen belly, watery brownish to yellowish diarrhea staining hind quarter decrease feed intake and sometimes, faeces admixed with mucus material. Mortality reach was the same, as it reach to 25. The main gross lesions were congestion of liver, spleen, heart and fully distended stomach with fluid and gasses, catarrhal enteritis, ceacum and colon were distended with watery mucoid contents and gases. Histopathological findings revealed that the intestine of rabbits infected with *C. perfringens type A* had excess mucous, casts mixed with a few leucocytes with villous destruction and sever inflammatory reaction, mainly macrophages and lymphocytes in both mucosa and submucosa. Intestine of other rabbits infected with *C. perfringens type A* revealed proliferation of intestinal glands, submucosal odema and leucocytic infiltration with regeneration attempts in the surface epithelium. Similar findings were recorded by Percy et al., (1993) and Wilber., (1999).

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