

Escherichia Coli as an Etiological Agent of Mucoïd Enteropathy in Rabbits.

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Abstract: Bacterial examination of two hundred and twenty – five specimens represented forty- five, either freshly dead or sacrificed examined rabbits suffered from mucoïd enteropathy syndrome was revealed 38 E.coli; 25 klebsiella spp. and 23 citrobacter spp. Isolates. Thirty- eight E.coli isolates were serologically identified into 13 serotyped and 25 unserotyped serotypes. The serotyped E. coli serotypes were identified as five O44-k74- and eight strains O158-k-serotypes. The most prevalent bacterial isolates were E.coli isolates. The pathogenicity of serotyped E.coli serotypes as a causative agent in induction of Mucoïd enteropathy in susceptible rabbits was performed. Clinical signs; postmortem lesions; morbidities and mortalities; reisolation trail and histopathological lesions of experimentally infected rabbits with E.coli serotypes were recorded.

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1. Introduction:

Bacterial causes of mucoïd enteropathy syndrome affecting rabbits remain one of the major causes of economic losses of rabbits. Screening of samples for bacterial causes of deaths revealed the isolation of Enterobacteriaceae organisms from about 73% of examined cases and *E. coli* was the more prevalent isolated organism (Saif-El din et al., 1994). *E. coli* is a normal component of rabbits digestive flora and it does not always exert direct pathogenic activity in rabbits. Stress factors may trigger overgrowth of *E.coli* along the intestinal tract as treatment with some antibiotics such as ampicillin (Milon, 1996 and Escoula, et al., 1981). Other pathogens as coccidiosis indirectly causes increase of cecal PH that can result in diarrhea and death (Peeters et al., 1984d and Wilber., 1999). Enteropathogenic *E. coli* (EPEC) is the only known class of *E. coli* in rabbits which induces acute intestinal pathology marked by inflammatory lesions of the gut where these *E. coli* are strictly located (Licois, 2004). This EPEC strain do not produce enterotoxins and are not invasive. They are able to attach the epithelial brush border of small and large intestine, followed by desquamation of enterocytes, villous atrophy and diarrhea (Cantey and Blake, 1977; Prescott, 1978 and Peeters et al., 1984a and 1988). The O serogrouping together with biotyping lead to a rapid identification of highly pathogenic strains that cause diarrhea in rabbits. The *E. coli* strains belonged to 12 different biotypes (Pisoni et al., 2004). This study was planned to investigate the role of *E.coli* in induction of mucoïd enteropathy syndrome experimentally in susceptible rabbits.

2. Materials and Methods:

A. Materials

A.1. Specimens:

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

A.2. Bacterial media:

A.2.1. Isolation media:

- Nutrient broth:
- Peptone water broth (CruickShank et al., 1975).
- Nutrient agar:
- MacConkey agar:
- Eosin Methylene Blue agar:
- Simmon's Citrate agar:
- Urea agar base (CruickShank et al., 1975):
- Semi-Solid agar medium

A.2.2. Biochemical identification media:

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

A.3.Reagents and solutions: Were prepared after (CruickShank et al., 1975; Koneman et al., 1992 and Baron et al., 1994)

1-1% tetramethyl-p-phenylenediaminedihydrochloride solution for oxidase test.

- 2- Hydrogen peroxide 3% (H₂O₂) freshly prepared for catalase test.
- 3- Urea solution 40% for urease test.
- 4- Kovac's reagent for indole test:
- 5- 0.02% methyl red solution for methyl red.
- 6- Solutions
 - a- 5.0% alpha-naphthol in absolute ethyl alcohol and
 - b- 40% potassium hydroxide for voges proskauer test.
- 7- Physiological saline.
- 8- Peptone water.
- 9- Phenol red for sugar fermentation test and urea utilization test which was used as 0.0012% for urea test and 0.0018% for sugar fermentation test (CruickShank et al., 1975).
- 10- Neutral buffered formalin 10% for fixation and preservation of the affected organs for histopathological examination.

A.4. Stains: Gram's stain (CruickShank et al., 1975).

A.5. Antibiotic:

2- Streptomycin sulphate used for preparation of *E. coli* resistant strain of inoculated for experimental infection.

A.6 *E. coli* antisera polyvalent and monovalent

Were kindly obtained from National Laboratory for Veterinary Quality Control on Poultry Production (NLQP); Animal Health Research Institute (AHRI); Dokki, Giza; Egypt .

A.7. Experimental rabbits:

Forty- eight native breed rabbits aged either 4-6 ws-old or 8-10 ws-old, 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 containing diclazuril as anticoccidial agent and without any antibiotics as feed additive.

B- Methods

B.1-Clinical and postmortem examinations:

Clinical examination of diseased rabbits was carried out for recording clinical signs. Postmortem examination of both freshly dead and sacrificed rabbits was carried out to recording the postmortem lesions.

B.2. Parasitological examination

Faecal and intestinal content samples were collected from examined rabbits for recording or excluding presence of *Eimeria* spp. and helminthes. In addition, hepatic and bile smears were examined for investigation of hepatic coccidiosis.

B.3. Bacterial isolation

Specimens of caecum, liver, spleen, and intestine were collected aseptically and cultivated on nutrient broth tubes for enrichment of suspected microorganisms and incubated aerobically at 37°C

for 18-24 hours. A loopful from the previously incubated tubes were streaked onto the surface of MacConkey agar and incubated aerobically at 37°C for 24-48 hours. The suspected colonies were subcultured on eosin methylene blue agar then the suspected colonies were picked up and examined for their morphological and cultural characters then subcultured on slope agar 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's stain) and examined microscopically for morphological characteristics.

B.3.2. Biochemical identification

Suspected colonies were subjected to a series of biochemical tests according to (Smith and Holdeman, 1968; Willis, 1977 and Koneman et al., 1992) as follows:

- Indol test using tubes with 2% peptone water.
- Methyl red and Voges proskauer test using glucose phosphate.
- Citrate utilization test using Simmon's citrate agar.
- Urea utilization test using urea agar base.
- Motility test using soft agar media.

B.3.3. Serological identification of isolated *E. coli* isolates according to (Edwards and Ewing., 1972).

The suspected *E. coli* isolates were subjected to serological identification by slide agglutination test using polyvalent and monovalent *E. coli* antisera at National Laboratory for Veterinary Quality Control on Poultry Production (NLQP); Animal Health Research Institute (AHRI); Dokki; Giza; Egypt.

B.4.Preparation of resistant strains:

E. coli serogroups were treated with streptomycin for obtaining resistant serotypes. The organisms were cultured for 24 hours at 37°C in nine successive broth cultures containing increasing quantities of streptomycin, starting with 0.01 gm and ending by 1 gm streptomycin per liter broth, then subcultured on MacConkey's streptomycin plates; (MacConkey's agar containing 1 gm streptomycin per 1 liter). So *E. coli* were referred as streptomycin - resistant *E. coli*.

B.5. Titration of inoculums:

The inoculum of *E. coli* strain, used for challenge was prepared from 24 hours nutrient broth culture of *E. coli* strain. They were serially diluted in sterile PBS. The viable cell concentration of the inoculum was determined by colony count on MacConkey agar. The challenge dose was adjusted to 1.3×10^7 CFU in case of *E. coli* O44 and 2×10^8 CFU in case of *E. coli* O158 (Skrivanova and Marounek., 2007).

B.6. Experimental design:

To study the pathogenicity of isolated *E. coli* in rabbits. Forty - eight, native breed rabbits were divided into two main groups (1 and 2) according to age. First group (G1) containing 24 rabbits aged 4-6 weeks-old and second group (G2) containing 24 rabbits aged 8-10 weeks-old. Each group was subdivided into 3 subgroups (a, b, c), each subgroup containing 8 rabbits. Subgroup (1a) and (1b) rabbits were orally infected with *E. coli* serotype O44-k74 and O158-k- with a dose of 1.0 ml containing 1.3×10^7 CFU and 2×10^8 CFU respectively. Meanwhile, subgroup 1c rabbits were remained as negative control. Subgroup (2a) and (2b) rabbits were orally infected with *E. coli* serotype O44-k74 and O158-k- with dose of 1.0 ml containing 1.3×10^7 CFU and 2×10^8 CFU respectively. Meanwhile, subgroup 2c rabbits were remained as negative control. All experimental rabbits were observed for 2 weeks before experimental infection to be free from any infection. Clinical signs, gross lesions, morbidities, mortalities reisolation trail, and histopathological examination for all experimentally infected rabbits were recorded for 3 weeks post infection.

III-2-7- Histopathological examination:

Histological examination of both naturally and experimentally infected rabbits were carried out according to (Schauer et al., 1998). Specimens 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 examined rabbits was showed clinical signs in the form of watery brownish and/or yellowish diarrhea, or white mucoid discharges staining perinum, and hind quarters fur, swollen belly, impacted caecum and off food. Later on, , examined diseased rabbits showed dehydration, emaciation and deaths among all ages especially young aged rabbits 3-12 weeks old.

3.2. Post mortem findings

Post mortem examination of both freshly dead and scarified rabbits revealed gross lesions in the form of 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 caecum

containing pasty feted odor contents and Colon filled with mucoid material instead of hard pellets.

3.2. Parasitological findings.

Faecal and intestinal content samples were collected from examined rabbits for recording or excluding presence of *Eimeria* spp. and helminthes. In addition, hepatic and bile smears were examined for investigation of hepatic coccidiosis.

3.3. Bacterial isolation.

Bacterial isolation of examined specimens revealed 66 *E. coli*; 23 *Citrobacter* spp and 25 *Klebsiella* spp. in an incidence percentage of 25.9%; 9.0% and 9.8% respectively. *E. coli* isolates were isolated from examined duodenum, jejunum, ileum, caecum, liver and spleen in an isolation percentage of 4.8% (11/225); 6.6% (15/225); 4% (9/225); 7.5% (17/225); 4.4%(10/225) and 1.7% (4/225) respectively. *Citrobacter* spp isolates were isolated from examined duodenum, jejunum, ileum, caecum, liver and spleen in an isolation percentage of 1.7% (4/225); 2.2% (5/225); 2.2%(5/225); 1.7(4/225); 1.3%(3/225); 0.8%(2/225) respectively. Meanwhile, *Klebsiella* spp isolates were isolated from examined duodenum, jejunum, ileum, caecum, liver and spleen in an isolation percentage of 1.7(4/225); 2.6%(6/225); 2.6%(6/225); 2.6%(6/225); 0.8%(2/225); 0.4%(1/225) respectively.

3.4. Bacterial identification.

3.4.1 Morphological identification

Smears from colonies were stained with Gram's stain, for microscopical examination. *E. coli* isolates appeared to be Gram negative bacilli or coccobacilli, 1-2 mm in diameter, convex and appeared bright pink on MacConkey agar, and blue green metallic sheen on eosin methylene blue (EMB) agar., Fig (3).

3.4.2. Biochemical identification:

Indol test was positive with *E. coli* and *Klebsiella* and negative with *Citrobacter*. Methyl red test was positive with *E. coli* and *Citrobacter* and negative with *Klebsiella*. Citrate test was positive (blue media) with *Citrobacter* and negative with *E. coli* (green media).

3.4.2. Serological identification of *E. coli* isolates.

Thirty -eight out of 66 *E. coli* isolates recovered from examined specimens were serologically identified into 25 untyped serotypes and 13 typed serotypes Table (4). The serotyped *E. coli* serotypes were identified as five O44-k74- and eight strains O158-k-serotypes with an incidence percentage of 38.46% and 61.5% respectively.

Table (1) Shows : Prevalence of isolates from examined rabbits according to site of isolation.

	<i>E.coli</i>	<i>Citrobacter</i>	<i>klebsiella</i>	<i>Coccidia</i>	<i>Nematode</i>
Duodenum	11	4	4	-----	-----
Jejunum	15	5	6	-----	-----
Ileum	9	5	6	-----	-----
Ceacum	17	4	6	19	27
Liver	10	3	2	2	-----
Spleen	4	2	1	-----	-----
Total isolate no.	66	23	25	21	27
Total number	255	255	255	45	45
percentage	25.88 %	9.0%	9.8%	46.66%	60%

225 = number of specimens and 45 = number of rabbits

Table (2)Shows: Incidence of bacterial isolates according to age:

Age	No	<i>E.coli</i>		<i>Klebsiella</i>		<i>Citrobacter</i>	
		no	%	no	%	no	%
1M	8	6	75%	2	25%	1	12.5%
2M	27	17	63%	9	33.3%	5	18.5%
3M	10	2	20%	3	30%	2	20%
Total	45	25		14		8	

Percentages of *Klebsiella* and *Citrobacter* at 2-3 months old rabbits were 32.4%, and 18.9%, respectively, but *E. coli* at 1-2 months old rabbits was 65.7%.

Table (3)Shows: Prevalence of non bacterial isolates.

No.	Total No. of examined rabbits	Caecal Coccidiosis	Hepatic Coccidiosis	Round Nematodes
		19	2	27
percentage	45	42.2%	4.4%	60%
site		ceacum	liver	ceacum



Fig (1) shows: Colonies of *E.coli* appeared green metallic sheen on Eosin Methylene Blue (EMB) agar.

Fig (2) shows: Biochemical tests of *E. coli*, urease, citrate, methylene red, and indole test.

Table (4) Shows: serological identification of isolated *E.coli*.

Total No. of isolated strains of <i>E.coli</i>	Total isolates of <i>E.coli</i>	No. of untyped strains	No. of serotyped strains	Typed <i>E.coli</i> O. serogroup	
				O158-k-	O44-k74
66	38	25	13	8	5
percentage				61.5%	38.46%

Table (5) Shows: percentage of different *E.coli* O serogroup according to location of examined rabbits.

Location	Typed strain of <i>E.coli</i>	O serogroup	No. of typed strains	Percentage
Dakhliya Gov.	7/38	O158-k-	5	38.5%
		O44-k74-	2	15.3%
Sharkia Gov.	6/38	O158-k-	1	7.7%
		O44-k74-	5	38.5

3.5. Clinical findings of experimentally infected rabbits with *E.coli*.

The clinical findings of experimentally infected rabbits with *E.coli* O44-k74- were more severe than infected rabbits with *E.coli* O 158-k-serogroup. The clinical findings were decrease feed intake; anorexia; ruffled fur; swelling abdomen; watery brownish to yellowish diarrhea, sometimes, faeces admixed with mucus material.

3.6. Post-mortem findings of experimentally infected rabbits with *E.coli*.

Post mortem examination of both freshly dead and/or sacrificed experimentally infected rabbits revealed gross lesions in the form of

engorgement of subcutaneous blood vessels, congestion of liver, spleen, heart; fully distended stomach with fluids and gases, distension of small intestine and caecum with watery to mucoid contents and gases and catarrhal typhilitis.

3.6.3. Reisolation trial of *E. coli* from experimentally infected rabbits.

reisolation *E.coli* was performed on MacConkey medium containing 1.0 gm/liter streptomycin in percentage of 90% in case of *E.coli* O44-k74 and 80% in case of *E.coli* O 158-k-

Table (6) Morbidities and mortalities percentages of experimentally infected rabbits.

Group number	Inoculums	Age	Morbidities		Mortality	
			NO	Percentage	N NO x̄	Percentage
1a	<i>E.coli</i> O44	6-8 wks	4/8	50%	1/8	12.5%
1b	<i>E.coli</i> O158	6-8 wks	3/8	37.5%	1/8	12.5%
		-ve	2/8	25%	2/0/8	0%
2a	<i>E.coli</i> O44	8-10 wks	3/8	37.5%	0/1/8	12.5%
2b	<i>E.coli</i> O158	8-10 wks	3/8	37.5%	0/0/8	0%
2c	-ve	8-10 wks	2/8	25%	0/0/8	0%

C.6.4 . Histopathological results of experimentally infected rabbits.

The small intestine of rabbits inoculated with *E.coli* O44-k74 and O158-k-, mainly duodenal

mucosa revealed partial loss of the intestinal villi, which resulted in villous atrophy with hyperplasia of intestinal crypts and intestinal leukocytic infiltration, mainly round cells in lamina propria and submucosa

Fig.(3). Moreover, submucosal edema with mononuclear leukocytes and necrosis of intestinal glands, beside partial desquamation of intestinal epithelium could be seen. Other rabbits showed complete absence of intestinal villi, and replaced by mucus epithelial sheets and intense inflammatory cells, mainly lymphocytes Fig.(4). The jejunum contained little mucus and portions of sloughed intestinal villi inside its lumen Fig. (5). The large intestine mucosa of rabbits inoculated with *E.coli* showed hyperplasia and metaplasia to goblet cells in the surface epithelium and intestinal glands beside mucosal and submucosal round cell infiltration Fig (6). On the other hand the intestinal coats of non infected rabbits revealed normal intestinal coats Fig. (7).

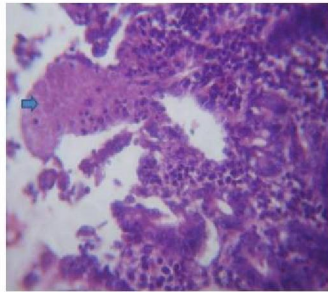


Fig. (3). Small intestine of rabbits injected with *E.coli*O44-k74 showing partial loss of duodenal intestinal villi, (arrow) hyperplasia of intestinal crypts and intense leukocytic infiltration. H&E x 1200.

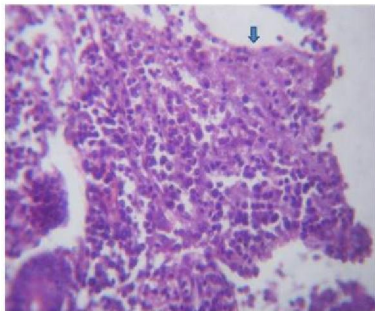


Fig.(4). Small intestine of rabbit injected with *E.coli*O44-k74 showing complete loss of intestinal villi and replaced by mucus, (arrow) mixed with leukocytes and epithelial sheets. H&E x 1200.

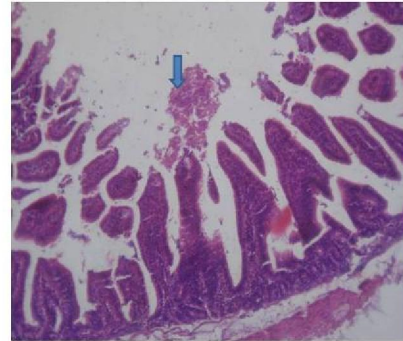


Fig.(5). Small intestine of rabbits injected with *E.coli*O158-k- showing little mucus within the intestinal lumen (arrow). H&E x 300.4. **Discussion:**

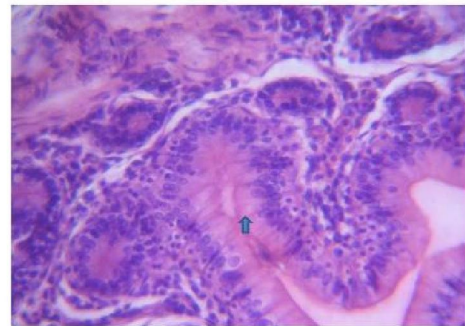


Fig. (6). Large intestine of rabbit injected with *E.coli*O158-k- showing metaplasia to goblet cells in surface and glandular epithelium H&E x 1200

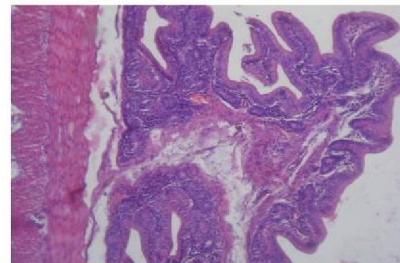


Fig. (7). Small intestine of rabbit showing normal intestinal coats H&E x

4. Discussion

Mucoid enteropathy syndrome (MES) or Epizootic Rabbit Enteropathy (ERE) is still nowadays one of the main severe intestinal disorders in rabbit farms all over the world including Egypt. This enteric disease causes the mortalities of fattening rabbits up to more than 60%. (Coudert et al., 1997). In addition, Marlier et al., (2006) mentioned that there are two main digestive syndromes that cause mortality in industrial fattening rabbit farms Colibacillosis and Epizootic Rabbit Enteropathy (ERE), these digestive diseases are rarely caused by one single pathogen.

This study aimed for isolation and characterization of enterobacteriaceae from rabbits suffering from Mucoid enteropathy syndrome and the role of more prevalent *E. coli* serotypes in induction of the disease in susceptible rabbits. Specimens from liver, spleen, duodenum, jejunum, ileum and caecum were collected from rabbits either showed signs of diarrhea or freshly dead rabbits suffering from diarrhea from different localities in Egypt specially at Sharkia and Dakahlia governorates,. The parasitic agents recovered from forty –five examined rabbits in this study were Nematodes (60%), caecal coccidiosis (42.2%) and hepatic coccidiosis (4.4%). Meanwhile, the isolated enterobacteriaceae were *E. coli*, 55.5% (25/45), *Klebsiella* 31.11% (14/45) and *Citrobacter* 17.8% (8/45). The isolated bacteria were biochemically identified as *E. coli* 66, *klebsilla* 25 and *citrobacter* 23. This study revealed that the high incidence of *E. coli* infection was at 1-2 month- old rabbits (65,7%) while *Klebsilla* and *Citrobacter* were at 2-3 month-old rabbits at percentage of (32,4%) and (18.9%) respectively. These result means that the bacterial isolates were more common at the early weaning period rather than suckling period. This may be attributed to stress at weaning and change of diet leading to increase caecal pH. This result was agreed with Pillien et al., (1996) and Penteado et al., (2002) who recorded that enteric disease of rabbits due to enteropathogenic *E. coli* strains affect rabbits at weaning age and Saif-Eldin et al.,(1994) who recorded that the percentage of rabbits suffering from intestinal disorders and diarrhea associated with *E. coli* infection in young age were in early post-weaning period was 54%. Thirteen strains out of thirty – eight of isolated *E. coli* were serotyped in this study while the remaining twenty –five *E. coli* isolates were unserotyped. The serotyped strains of *E. coli* from examined rabbits were five strains *E. coli* O44-k74 in (5/13) and eight strains *E. coli* O158-k- (8/13) with an incidence percentage of 38.5% and 61.5% respectively. These results were agreed with El-Bakry., (2009) who identified *E. coli* O44 as the most prevalent (44%) in rabbits suffered from diarrhea at Sharkia governorate, Egypt.

Experimental infection of apparently healthy rabbits aged either 6-8 or 8-10 weeks –old with *E. coli* O44-k74 and *E. coli* O158-k- in a dose of 1.3×10^7 CFU and 2×10^8 CFU respectively. Infected rabbits showed watery brownish to yellowish diarrhea, decrease feed intake, abdominal bloating and caecal impaction detected on palpation, anorexia, ruffled fur, sometimes, faeces admixed with mucus material, that can be mostly observed as early as the 3rd day post-infection, the presence of mucus under the cages were not constant and can be observed intermittently, the peak of the disease was developed 5 to 7 days post- infection.

The mortality rate was 12.5% in both infected age groups with *E. coli* O44-k74 while rabbits infected with *E. coli* O158-k- at 4-6 weeks-old was 12.5% and zero in other age groups. These results were agreed with Peeters et al., (1988) and Blanco et al., (1996) who recorded that highly pathogenic strains of *E. coli* required small numbers of cells to cause high mortality rate while strains belonging to other serotypes showed moderate or mild pathogenicity and required higher doses to cause clinical signs. Greenham, (1962) and Katoch et al., (1993) recorded that enteropathogenic *E. coli* constitute one of the main infectious agents in diarrhoeic rabbits and are responsible for 10-60% of the losses.

The most pathognomonic lesions in both naturally and experimentally infected rabbits were distension of the stomach and duodenum by fluid and gaseous content, empty of distal colon, congestion of liver, spleen, heart and engorgement of subcutaneous blood vessels, distension of small intestine and ceacum with watery to mucoid contents and gases catarrhal typhilitis during the first 5-7 days.

Histopathological findings of the small intestine of rabbits infected with *E. coli* O44-k74 and O158-k-, were mainly duodenal mucosa revealed partial loss of the intestinal villi, which result in villous atrophy with hyperplasia of intestinal crypts and intestinal leukocytic infiltration, mainly round cells in lamina propria and submucosa. Moreover, submucosal edema with intense mononuclear leukocytes and necrosis of intestinal glands, beside partial desquamation of intestinal epithelium could be seen. In addition, other small intestine of examined rabbits, revealed complete absence of intestinal villi, and replaced by mucus epithelial sheets and intense inflammatory cells, mainly lymphocytes. The jejunoileal part contained little mucus and portions of sloughed intestinal villi inside its lumen. Furthermore, large intestine mucosa of rabbits inoculated with *E. coli* showed hyperplasia and metaplasia to goblet cells in the surface epithelium and intestinal glands beside mucosal and submucosal

round cell infiltration. Similar findings were recorded by Cantey and Balke, 1977; Peeter et al., 1984 c and d; Licois et al., 2000 and Licois et al., 2005. Finally, it could be concluded that pathogenic E.coli serotypes were a common cause of mucoid enteropathy of rabbits and stress factors either concurrent infections or hygienic disorders exaggerated their influence in inducing the diseases. The severity of the disease was correlated with the age of affected rabbits.

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