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DIARRHEA SYNDROME CAUSED BY *CAMPYLOBACTER JEJUNI* IN CALVES

HALA, M. ISMAIL[1] AND SHEREEN S. MOUSTAFA[2]

[1] Pathology Unit, (Animal Health Research Institute, Mansoura Lab), Agriculture Research Center (ARC).
[2] Bacteriology Unit, (Animal Health Research Institute, Mansoura Lab), Agriculture Research Center (ARC).

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ABSTRACT

Campylobacter jejuni (*C.jejuni*) is the leading bacterial cause of food born diarrheal illness and enterocolitis worldwide in human and young animals. A total of 140 samples (100 fecal samples of calves and 40 raw milk samples of cow) were collected from Dakahlia governorate dairy farms for isolation and identification of *C.jejuni* by using cultural, biochemical, molecular methods and detection of the virulent *cad*F gene using PCR. Moreover, the isolated *C.jejuni* subjected for antimicrobial susceptibility test. Then, we develop an experimental infection of rabbits by inoculation with 1×10^9 colony-forming units (cfu) of completely identified *C.jejuni*. Rabbits divided into 3 groups: infected untreated (G1), infected treated with enrofloxacine (G2), control (G3) to study pathogenesis, fecal inflammatory response, histopthology, immunohistochemistry and ultrastructural changes post inoculation in the three groups. Results revealed that, from 140 fecal and raw milk samples only 41 isolates were bacteriologically and biochemically identified as *Campylobacter spp*. Seven isolated strains were positively amplified for *map*A gene specific to *C.jejuni* and carried the *cad*F virulence gene. *C.jejuni* isolates were resistant to amoxicillin and ampicillin and highly susceptible to norfloxacine and erythromycin. After oral infection with *C. jejuni* 90% of rabbits developed diarrhea with highly fecal inflammatory responses in G1, but mild in G2 (treated) and negative in G3 (control). Pronounced histopathologic changes were investigated in G1 during the acute phase (days 1 to 3) restricted on distal small intestine and colon including massive destruction of villi and loss of intestinal glands. The submucosa and muscularis mucosa showed the presence of edema with congested blood vessels, while hemorrhage was seen in the muscularis propria layer. The changes were mild and involved only the villi in treated group (G2), while abscent in control (G3). These results were confirmed by immunostaining, suggesting that *C.jejuni* is capable of invading deep intestinal tissues down to the submucosal layer in G1 while in G2 infection, the reaction was confined mainly to the villi, and was greatly reduced in the submucosa. Electron microscope showed all stages of invasion and associating damages from postinfection, colonizationa and villus damage. Thereby, the implementation of hyagenic practices during milking and proper handling of milk during calves feeding with regular monitoring of antibiogram profile are very crucial in preventing *C.jejuni* infection, colonization and intestinal damage and subsequently economic loss in dairy farm.

Keywords: C.jejuni; Calves; Diarrhea; PCR; cadF gene; Intestine; pathology.

Corresponding author: HALA, M. ISMAIL
E-mail address: ibrahimsabry.gravena@gmail.com
Present address: Pathology Unit, (Animal Health Research Institute, Mansoura Lab), Agriculture Research Center (ARC).

INTRODUCTION

Calf diarrhea is a multifactorial disease and a major problem in livestock production in Egypt and throughout the world (Ibrahim, 2007) which have serious financial and animal welfare implications in both dairy and beef sucker herds (Uhde *et al.,* 2008). It has been detected that 75% of early calf mortality in dairy herds is caused by acute diarrhea in the pre-weaning period, also still a major cause of economic loss to cattle producers worldwide (Bartels *et al.,* 2010). The aetiology of diarrhea is complex involving management, environmental, nutritional, physiological variations and variety of pathogens (Prescott *et al.,* 2008), but 80% of tested diarrheic calves indicated that infectious factor is still a major cause of calf diarrhea (Meir *et al.,* 2010). The majority of diarrheic cases were identified among 0 to 4 week old calves (Wudu *et al.,* 2008). Although *E. coli* and *Salmonella* are known to be the most common and economically important agents (Achá *et al.,* 2004), *Campylobacter spp,* principally *C.jejuni* is among the main causes of gastroenteritis in newly born calves worldwide. Moreover, it is important human pathogens that may cause outbreaks of food-borne diseases and thus are of high public health importance (Cho, 2012). Raw milk acts as the main source for *Campylobacter spp.* and primarily to be contaminated by bovine feces or direct contamination of milk as a consequence of mastitis (Modi *et al.,* 2015).

Campylobacter isolation and identification is considered the standard method for disease identification; however, it is laborious due to the complex nature of Campylobacter (Li *et al.,* 2014). Thus, molecular techniques, such as polymerase chain reaction (PCR) and sequencing, can permit the simple, fast and exact identification of *C. jejuni* and reveals its epidemiological characteristics (Miller *et al.,* 2010).

The disease seriousness relies upon the virulence of the strain and on the host's immune state (Younis *et al.,* 2018). *CadF* is one of the reference virulence genes that encodes proteins involved in the attack and attachment of *C. jejuni* (Elmali & Can, 2019), and this gene is a highly prevalent in *C. jejuni* isolates and have been proposed to play a role in enteritis and colonization in the luminal surface of the small bowel (Andrzejewska *et al.,* 2015).

Antimicrobial susceptibility test represents one of the most important an epidemiological tool and definition of the proper treatment of infections, consequently preventing or creating strategies that minimize the dissemination of resistant bacterial strains, mainly multiresistant ones especially *Campylobacter spp* (Shang *et al.,* 2016), which has a potentially serious impact on food safty in both animal and human health (Hagos *et al.,* 2021).

Despite extensive study, little is understood about the mechanism by which *C. jejuni* causes diarrheal disease, so various small animal models have been reported to study the process of enteroinvasiveness by *C. jejuni* (Heimesaat *et al.,* 2014). The gastroenteritis due to *Campylobacter* ranges from mild to severe diarrheal disease (often bloody diarrhea), other symptoms are cramping, abdominal pain and fever within 2-5 days after exposure to the organism, which typically lasting 1 week with Complications (Murray *et al.,* 2007), which attributed to virulence factors that could play a role of colonization, adherence, and invasion of epithelial cells in the animal and human being (Wilson *et al.,* 2010).

The aim of this study were to isolate and identify *C. jejuni* from naturally infected calves and raw milk, and to determine the antibiotic susceptibility pattern for perfect control and treatment. Moreover, using an experimental rabbit model to study histopathological, immunohistochemichal and ultrastructural changes, as well as to enable testing of new therapeutics to prevent and/or combat infection.

MATERIAL AND METHODS

1. Samples collection

Atotal of 140 samples including raw milk (40) and sterile cotton rectal swabs (100) were collected from different farms exhibited severe diarrhea in newly born calves at Dakahlia Governorate in the period from December 2019 to April 2020. All samples were collected in sterilized bottles and were immediately transported to the laboratory in an insulated ice box at 4C within 1-2 h from collection and processed immediately upon arrival for isolation.

2.Campylobacter Isolation

Samples were examined for the presence of *Campylobacter* spp. using selective enrichment and isolation protocol recommended by Roberts and Greenwood (2003). One ml. of the homogenized samples was aseptically inoculated into sterile screw capped tube, containing 9ml of Bolton broth (Oxoid Ltd, Basingstoke, Hampshire, England) containing 5% laked horse blood and Bolton broth selective supplement which incubated under appropriate microaerophilic conditions in anaerobic jar by using the Gas Pack System BBL (5% O2, 10% CO2 and 85% N2) at 37˚C for about 4 hours prior to increasing the temperature to 41.5˚C for the remainder of the 48 hours of the incubation time for resuscitation. Loopful of the incubated broth was plated onto modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, Oxoid) with CCDA selective supplement and the plates were incubated for 48 hours at 41.5˚C under appropriate microaerophilic conditions, suspected colonies were selected and isolated.

3.Campylobacter Bacteriological Identification

Presumptive colonies of *Campylobacter spp.* were subjected to standard biochemical tests (Foster *et al.,* 2004), including oxidase test, catalase production test, nitrate reduction test, hydrogen sulphide production using lead acetate paper, glycine tolerance test, NaCl 3.5% tolerance test, sensitivity to Nalidixic acid and Cephalothin and Hippurate hydrolysis test. Biochemically identified *C. jejuni* colonies were stored at -70 °C in nutrient broths with 15% glycerol until subjected to molecular PCR identification.

4.Molecular characterization of *C.jejuni*

4.1.DNA extraction:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations.

4.2.Polymerase chain reaction *(mapA gene)*

The amplification of the *mapA* gene for *C. jejuni* was carried out on 10 representative isolates that were biochemically confirmed utilizing the primers listed in Table 1. Amplification conditions were as follows: 6 minutes at 94 °C; 35 cycles of 50 seconds at 94 °C, 40 seconds at 57 °C, and 50 seconds at 72 °C; and a final extension of 3 minutes at 72 °C. The PCR products were analysed using 1.5% agarose gel electrophoresis ((Applichem, Germany, GmbH). Gelpilot 100 bp plus ladder (Qiagen, Gmbh, Germany) and Generuler 100 bp ladder (Fermentas, Thermo) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software (Shin & Lee, 2009).

4.3.Virulence gene characterization of *C. jejuni* isolates

The biochemically and molecular confirmed *C. jejuni* isolates were characterized for recognition of the *cadF* virulence gene by PCR (Konkel *et al.,* 1999) utilizing the primers listed in Table 1.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions of *C.jejuni*.

Target gene	Primers sequences	Amplified segmen (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
mapA	F (5`- CTA TTT TAT TTT TGA GTG CTT GTG) R (5 `-GCT TTA TTT GCC ATT TGT TTT TTA)	**589**	**94˚C 6 min.**	**94˚C 50 sec.**	**57˚C 40 sec.**	**72˚C 50 sec.**	**72˚C 3min.**	**Shin & Lee(2009)**
cadF	F (5`- TTG AAG GTA ATT TAG ATA TG) R (5 `-CTA ATA CCT AAA GTT GAA AC)	**400**	**94˚C 5 min.**	**94˚C 1 min.**	**45˚C 1 min**	**72˚C 1 min.**	**72˚C 5 min.**	**Konkel et al., (1999)**

5.Antimicrobial susceptibility test of *C.jejuni* isolate

All *C.jejuni* Confirmed isolates were screened for antimicrobial susceptibility using the stander agar disc diffusion technique as recommended by Clinical and Laboratory Standards Institutions (CLSI, 2014) for susceptibility to 8 different antibiotic disc: Amoxicillin (10µg), Ampicillin (10µg), Chloramphenicol (30µg), Erythromycin (15µg), Gentamycin (10µg), Norfloxacin (10µg), Sulfamethoxazole-trimethoprim (25µg), Streptomycin (10 µg). After 48h of microaerophilic incubation at 37˚C, the clear zones diameter for individual antimicrobial agents were measured and then translated into Sensitive (S) and Resistant (R) categories.

6. Experimental study

6.1. Experimental animal

Atotal of 60 infant rabbits at 10 days old and *C.jejuni* pathogen free, were obtained from Rabbit production unit, Faculty of Agriculture, Mansoura University. To ensure the absence of *Campylobacter* infection, rectal swabs were performed immediately after reception of rabbits and plated on sheep blood agar plates containing cefoperazone, vancomycin, and amphotericin B (CVA agar). Rabbits were housed in cages with pads in the bottom. Food and water were provided at libitum, and were allowed to acclimate for 2 days before experiment.

6.2. Experimental design and infection

Rabbits were divided into 3 groups, each group has 20 rabbits. Group1: infected untreated with orogastric inoculation with 1 × 10^9 colony-forming units (cfu) of *C. jejuni* that were isolated previously and fully identified from naturally infected calves and contaminated raw milk. Group 2: infected and treated with Norfloxacine within 12hr PI (1cm/kg). Group3: control group.

6.3. Clinical signs and postmortem:

Rabbits were observed daily for signs of diarrhea or death. Rectal swabs for bacterial re-isolation were obtained daily until the end of the 2-week observation period. Complete postmortem examinations were done on infected and control animals.

6.4.Fecal inflammatory markers: Were measured according to Nemelka *et al.* (2009), a stool sample is collected daily in a clean container provided by the laboratory. This sample should be uncontaminated by urine or water. Occult blood (Hemoccult, Beckman Coulter Kit) designed to evaluate

stool samples for hidden (occult) blood by measuring the heme (non-protein) part of hemoglobin from blood in the stool .Lactoferrin (Leuko-Test), is a glycoprotein present in activated neutrophils when the intestines are inflamed and are shed in stool. Gross blood was detected in stool by nacked eye.

6.5. Histopathological examination:

Three rabbits from each group were sacrificed each 12hr post infection until five days, then once each 24hr till the end of experiment. The intestines were removed immediately. Specimens were placed in formalin 10%. Representative sections were taken from each specimen and stained with hematoxylin and eosin (Bancroft *et al.*, 2013).

6.6. immunohistochemical staining for *C. jejuni*

Sections were permeabilized with 0.1% Triton X-100 for 15 min, treated with 3.3% H2O2 for 15 min, and washed. Samples were blocked for 30 min with 5% bovine serum albumin and incubated for 1 h with an in-house mouse polyclonal antiserum against *C. jejuni* or without serum as a control. Samples were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Sigma) for 1 h, developed with 3-amino-9-ethylcarbazole, counterstained with hematoxylin, and mounted with aqueous mounting medium (Shang *et al.,* 2016).

6.7. Ultra structural examination of intestine:

For electronmicroscopy, fragments of duodenum, ileum and colon were removed and fixed for 24 h at 4°C in callidine buffer containing glutaraldehyde 2.5%. Samples were then washed in buffer, fixed for 1 h in osmium tetroxide 1%, dehydrated, embedded in Epon (Merck), and cut with an ultramicrotome. Semi-thin sections were stained with toluidin blue; ultrathin sections were contrasted with uranyl acetate and lead citrate, before examination with a Philips EM300 electronmicroscope (Electron Microscopy Unit, Mansoura University).

RESULTS

1.Campylobacter isolation and bacteriological identification

From the total of 140 collected samples, 41 campylobacter strains were isolated as following: 32 out of 100 (32%) calve fecal samples and 9 out of 40 (22.5%) raw milk samples from the same farms, by cultural methods. *Campylobacter. spp*. appear as grey color spreading colonies on blood agar media after incubation for 48hrs at 37c in microaerophilic condition (10% CO_2, 5% O_2 and 58% N_2). Gram staining examination showed pink color spiral rods were arranged as a single or in pairs under microscope (100x). All isolated strains of Campylobacter spp were then subjected to biochemical tests, which were positive hippurate hydrolysis, catalase test and indoxyl acetate hydrolysis.

2.Molecular Identification of *Campylobacter jejuni* isolates

Seven representative biochemically validated Campylobacter isolates were further molecularly identified through the amplification of *map A* gene specific to *C.jejuni*. All isolates recorded the specific product for *C.jejuni* (589 bp), as shown in fig.1.

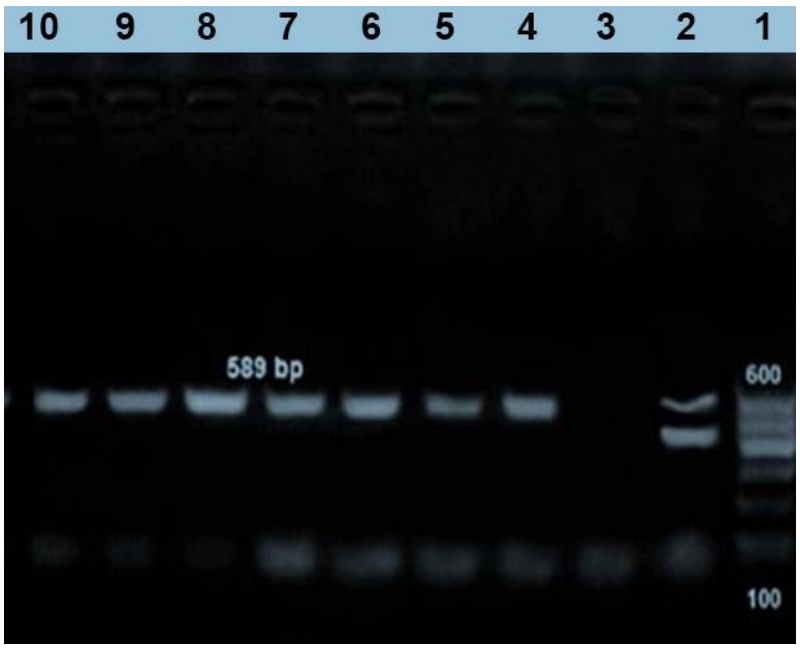

Figure 1: Amplification of the *mapA* gene of *C. jejuni* isolates. Lane 1: DNA ladder (100 bp.), lane 2: positive control, lane 3: negative control; lanes 4-10: positive *C. jejuni* isolates showing specific bands at 589 bp.

The virulence characterization of molecularly identified *C.jejuni* isolates revealed that eight (15.22%) carried the virulence cadF gene among 46 C. jejuni isolates and produced the expected product (400), figure 2.

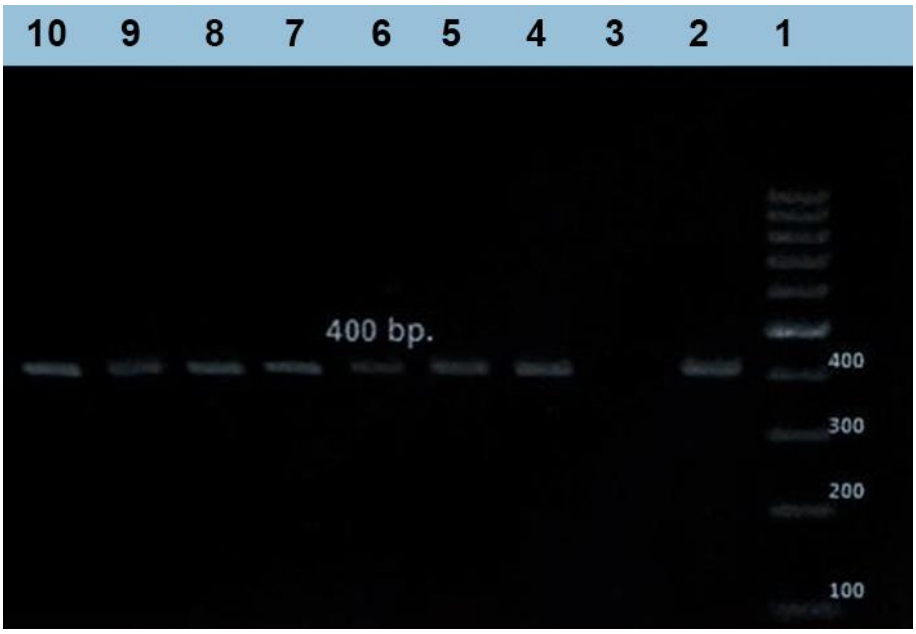

Figure 2: Agarose gel electrophoresis of *CadF* gene PCR products in *C. jejuni* isolates: Lane 1: DNA ladder (100 bp), lanes 2: positive control, Lane 3: negative control, Lanes 4 -10: positive *C. jejuni CadF* gene showing specific bands at 400 bp.

3.Antibiogram profile of isolated *C.jejuni*

The antimicrobial susceptibility test of molecularly identified *C.jejuni* isolates (7) were screened against 8 antibiotics. All strains were susceptible to Norfloxacine, Erythromycin, Sulfamethoxazole-Trimethoprime, and Gentamycin. However, isolates showed resistance to Ampicillin, and Amoxicillin, Table 2.

Table 2: Antimicrobial susceptibility pattern of *C.ejuni* isolates identified by disc diffusion method.

Type of antibiotics	Interpretations		
	Susceptability (%)	Intermediate (%)	Resistant (%)
Ampicillin (10µg)	0 (0%)	1 (14.3)	6 (85)
Amoxicillin (10µg)	1 (14.3)	2 (28.6)	4 (57.1)
S.Trimethoprim (25µg)	2 (28.6)	1(14.3)	4 (57.1)
Erythromycin (15µg)	5 (71.4)	1 (14.3)	1 (14.3)
Gentamycin (10µg)	4 (57.1)	1 (14.3)	2 (28.6)
Norfloxacin (10µg)	6 (85)	-	1 (14.3)
Chloramphenicol (30µg)	4 (57.1)	1 (14.3)	2 (28.6)
Streptomycin (10µg)	2 (28.6)	2 (28.6)	3 (42.9)

4.Experimental study:

4.1.Clinical signs after orogastric inoculation of *C. jejuni* into rabbits:

After oral challenge with 10^9 CFU of *C. jejuni*, all 20 (100%) infected untreated rabbits(G1) were infected, and exhibited diarrhea after 12hr post infection which characterized by release of loose, gelatinous unformed bloody stools followed by severe yellow diarrheal fluid. Duration of diarrhea varied between 1 to 4 days, and spontaneously remitted by day 6 after inoculation. None of the rabbits died but subsequently lost weight, off food and became very weak and lethargic. In contrast, in infected-treated group (Gr2), 4 of 20 (20%) of rabbits exhibited mild manifestation in the form of yellow watery diarrhea after 12hr post infection but did not develope to severe illness and completly recovered after 48hr. In control group (Gr3), appeared normal and did not exhibit any signs of illness, the change in their fecal consistency was not noted throughout the experiment.

4.2.Fecal inflammatory markers

Lactoferrin, gross blood and occult blood were measured in feces to investigate the degree of inflammatory response to *C. jejuni* infection in each group as showed in table (3).

Table 3: Fecal inflammatory response of *C. jejuni* experimental infected rabbits.

Groups	Days	Fecal inflammatory marker [no. positive/no. tested (%)]		
		Gross blood	Occult blood	Lactoferrin
Infected untreated	1	12/20 (60%)	9/20 (45%)	10/20 (50%)
	2	10/20 (50%)	8/20 (40%)	9/20 (60%)
	3	7/20 (35%)	7/20 (35%)	8/20 (40%)
Infected treated	1	3/20 (15%)	3/20 (15%)	5/20 (25%)
	2	0/20	1/20 (5%)	2/20 (10%)
	3	0/20	0/20	0/20
control	Any day	All fecal inflammatory markers were negative (0/20)		

All markers were negative on days 6 and 7 in the collected sample stool.

All infected untreated rabbits that developed diarrhea (100%) showed gross blood in their stool, this value gradually declined to 35% by day 3, but no gross blood was present beyond day 5. Whereas the infected treated group revealed gross blood in (15%) of animals and disappeared in day 2 of experiment. In control group, all fecal inflammatory markers were negative during the study period. lactoferrin was more sensitive compared with gross and occult blood in detecting inflammation and antimicrobial susceptibility.

4.3.Gross examination:

The intestine of most infected untreated rabbits (G1) revealed acute enterocolitis indicated by bloody content, hyperemia, petechial hemorrhage and swollen. Small intestine, ceca and proximal colon were distended and fluid-filled (Fig3. A), compared with infected treated rabbits (G2) which exhibit mild gross lesions and control group that show normal intestine.

Figure (3): Gross findings in rabbits inoculated with *C. jejuni* isolated from naturally infected calves and cow raw milk. **A**. The infected untreated rabbits (G1): Showing congested, swollen, distended and fluid-filled intestine. **B.** infected treated rabbits (G2): Showing mild focal congestion and not distended with fluids. **C**. Control group (G3): Showing normal intestine.

4.4.Histopathology:

The abnormalities of histological analysis of infected rabbits were recorded mainly in the distal small intestine as well as in the colon**.** Lesions in infected untreated rabbits revealed characteristic progressive changes of the intestinal epithelial morphology. At 12 hr PI, intestinal villi appeared mostly normal with intact lining epithelium (Fig4.A). by 24 hr, heterophils were observed in the lamina propria of villi, with small clusters of bacteria were attached and associated with erosions in the epithelial surface (Fig4.B). However most of the lining epithelial layer remained intact except little debris was observed in the lumen. Untill that point there was no evident of extensive disruption and disintegration of villus epithelium. By 36hr PI, we assessed gradual histopathological changes: the erosions were more pronounced, causing cavities in the epithelial surface and resulting in loss of the actin ring that encircles and protect the villus, epithelial cells became extruding and unprotected which enhance epithelial hyperplasia, at that time luminal depris as well as heterophils and bacteria became more abundant in the lumen and lamina propria (Fig4,C). But by 48hr till 72hr PI there was widespread damaged, degenerated, and sloughed intestinal mucosa, marked congestion of capillaries in the villi, severe epithelial hyperplasia leading to ulceration, serosal hemorrhage and diffuse polymorphonuclear neutrophilic infiltration (PMN) at the crypt epithelium. Loss goblet cell and crypt, smucosal ulceration, submucosal congestion and edema were extensive. Moreover, the inflammation extended from the submucosa to the muscle layer (Fig4.D). By the day 5 PI, the severity of intestinal lesions decreased and the damaged epithelial cells from the villus is balanced by proliferation in the crypts (Fig4.E) by day 7 PI, villus epithelial coverage was nearly normal and absence of any significant changes (Fig4.F).

The infected treated group (Fig 5), approximately 20% of rabbits developed clinical symptoms of *C. jejuni*. By 24hr PI, the intestinal villi and epithelial coverage were normal with normal mucosa, submucosa and muscularis (Fig5.A). By 36hr PI, exhibited rather mild histopathological changes such as focal damage of epithelial lining villi associated with small clusters of bacteria and heterophilic aggregation in the lamina properia (Fig5.B). By 48hr PI, congestion, edema in lamina properia, single to mild scattered cell infiltrates in mucosa and lamina propria were recorded, but the lesions did not extend to submucosa and muscularis, normal goblet cells and crypts (Fig5.C). By day 3PI recovery phase occurred and characterized by regeneration of villus epithelium with absence of congestion and edema (Fig5.D). By day 4PI, complete regeneration of lamina properia of villi with marked increase of villus length and width (Fig5.E). By day 5PI, intestine showed conical shaped villi

lining with simple columnar epithelium, with marked increase of villus width and length, normal cryptal glands and goblet cells were seen (Fig5.F).

The comparative histopathological evaluation between infected, infected treated and control groups were illustrated in table (4).

Table 4: Comparision of histopathological findings in all experimental groups:

Histopathological changes	Experimental groups		
	Infected untreated	Infected treated	control
Mucosal ulceration	extensive-diffuse	mild-focal	-
mucosal congestion	prominent	minimal	-
submucosal congestion	prominent	-	-
submucosal, edema	prominent	-	-
Inflammation progress	Mucosa/submucosa/muscle	Mucosa	-
Muscle edema	prominent	-	-
Bacterial Invasion (immunohisto)	Up to muscle layer	mucosa	-

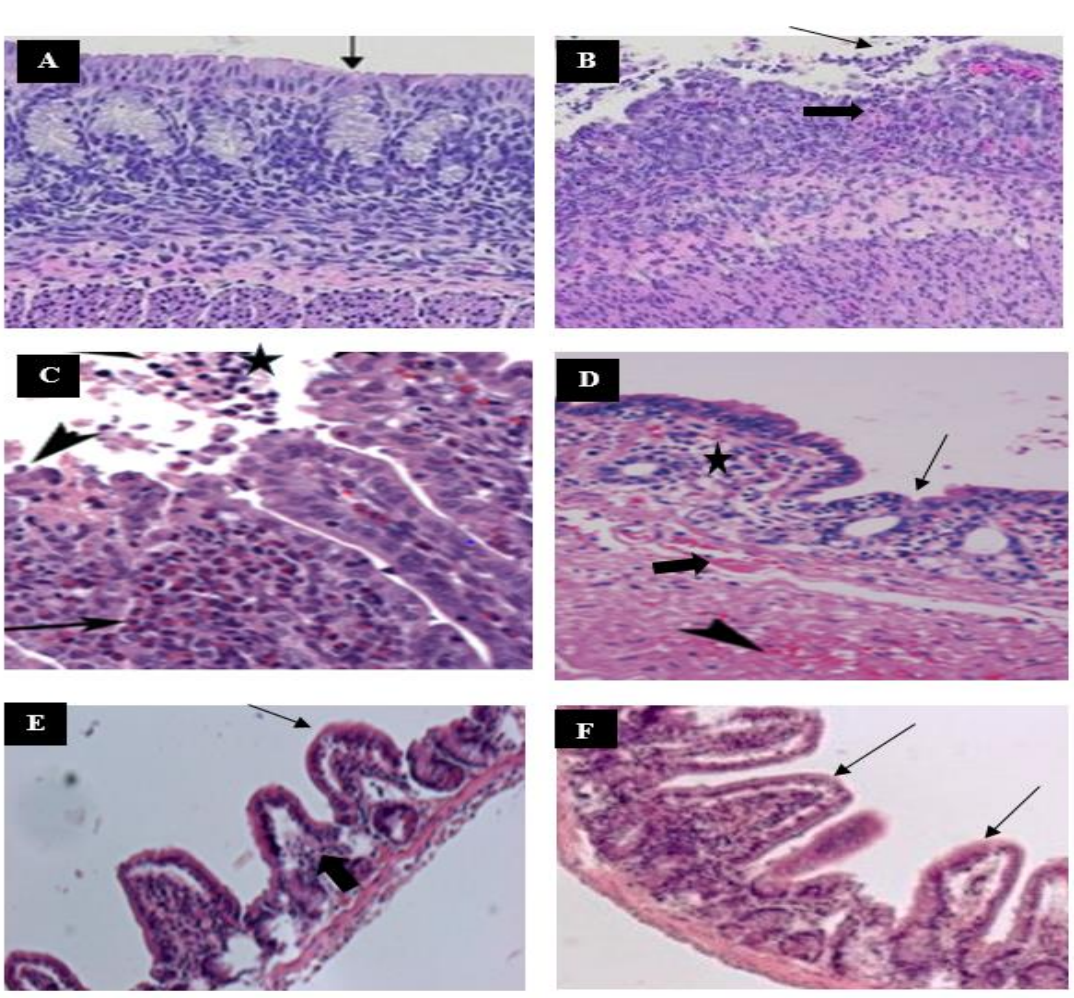

Figure 4: Histopathological changes in the distal small intestine of rabbits infected with *C.jejuni* isolated from naturally infected calves and raw milk. A: by 12hr PI, intestinal surface appear normal and smooth with intact villi and lining epithelium, normal mucosa and submucosa (arrow), H&E, x20. **B**: by 24hr PI, erosion of epithelial surface associated with bacterial clusters (thin arrow) and mild heterophilic aggregation in the lamina propria (thick arrow), H&E, $_{x20}$. **C:** by 36hr PI, sloughed or desquamated villus epithelium associated with heterophilic aggregation (star), attached bacteria (arrow head), heterophilic aggregation in the lamina properia (thin arrow), H&E, $_{x40.}$ **D:** by 48hr PI, degeneration and sloughing of villus epithelium (thin arrow). Congestion of blood vessels and infiltration with PMN in the lamina propria (star), submucosal congestion and edema (thick arrow), Congestion and edema in the muscularis externae (arrow head), H&E, x40. **E:** by the day 5 PI, regeneration of the damaged villus epithelium (thin arrow), by proliferation in the crypts (thick arrow). **F:** by day 7 epithelial coverage appear nearly normal with marked increase in length and width of villi (arrow), H&E, x_{100}.

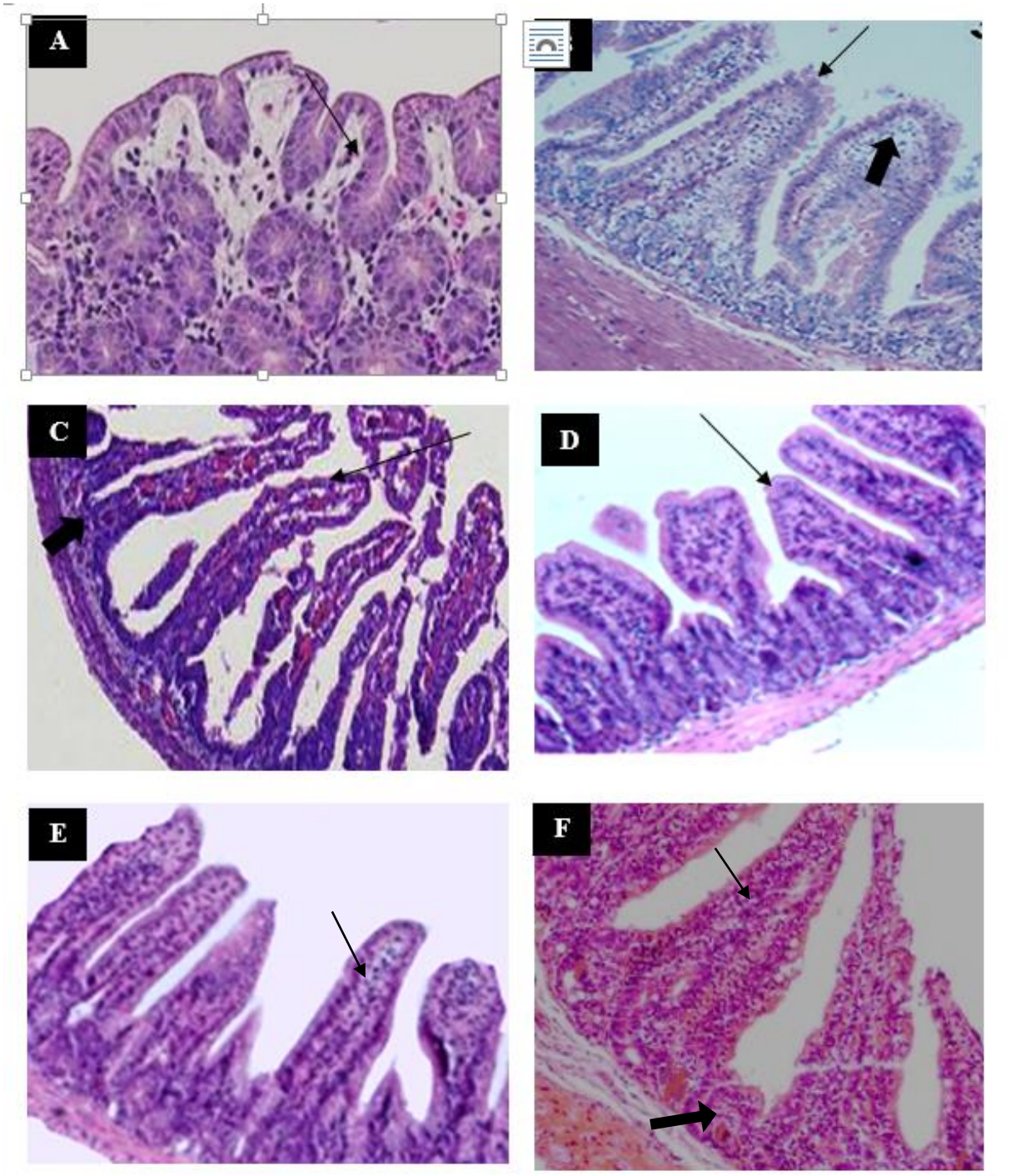

Figure 5: Histopathological changes in the distal small intestine of rabbits infected with *C.jejuni* isolated from naturally infected calves and raw milk and treated with Norfloxacine. A: by 12hr PI, normal intestinal villi with intact epithelial coverage (arrow) H&E, x10. **B:** by 24hr PI, focal damage of epithelial lining villi (thin arrow), associated with small clusters of bacteria and heterophilic aggregation in the lamina properia (thick arrow) H&E, x40. **C:** by 48hr PI, mild congestion, edema and single scattered cell infiltrates in lamina propria (thin arrow), normal submucosa and muscularis, normal goblet cells and crypts (thick arrow) H&E, x40. **D:** by day 3PI, regeneration of villus epithelium and absence of congestion and edema (arrow) H&E, x40. **E:** by day4, normal lamina properia of villi with marked increase in length (arrow), H&E, x40. **F:** by day 5PI, showing conical shaped villi lining with simple columnar epithelium (thin arrow), with marked increase of villus width and length, normal cryptal glands and goblet cells (thick arrow) H&E, x40.

4.5.Immunohistochemistry:

The intestinal tissue sections from all groups were immunostained with *C. jejuni* antibody. By 24hr PI, In the infected rabbits (G1& G2), *C. jejuni* antigen were detected through the lumen of the small and large intestines as well as between enterocytes, or intestinal absorptive cells, are simple columnar epithelial cells which line the inner surface of the small and large intestines (fig.6A). But, by 48hr PI, the infected untreated group (G1), immunoperoxidase labeling was intense in the mucosal region and this intense labeling extended into the submucosa and muscularis as well as the paracellular junction and at the basolateral surface of the epithelium. (Fig. 6B). However, by 48hr PI in the infected treated group (G2), immunostained sections showed immunoperoxidase labeling in the mucosa only and not extended to ather layers of intestine (fig.6C). The tissue sections of negative control group showed no immunoperoxidase labeling was seen (fig.6D).

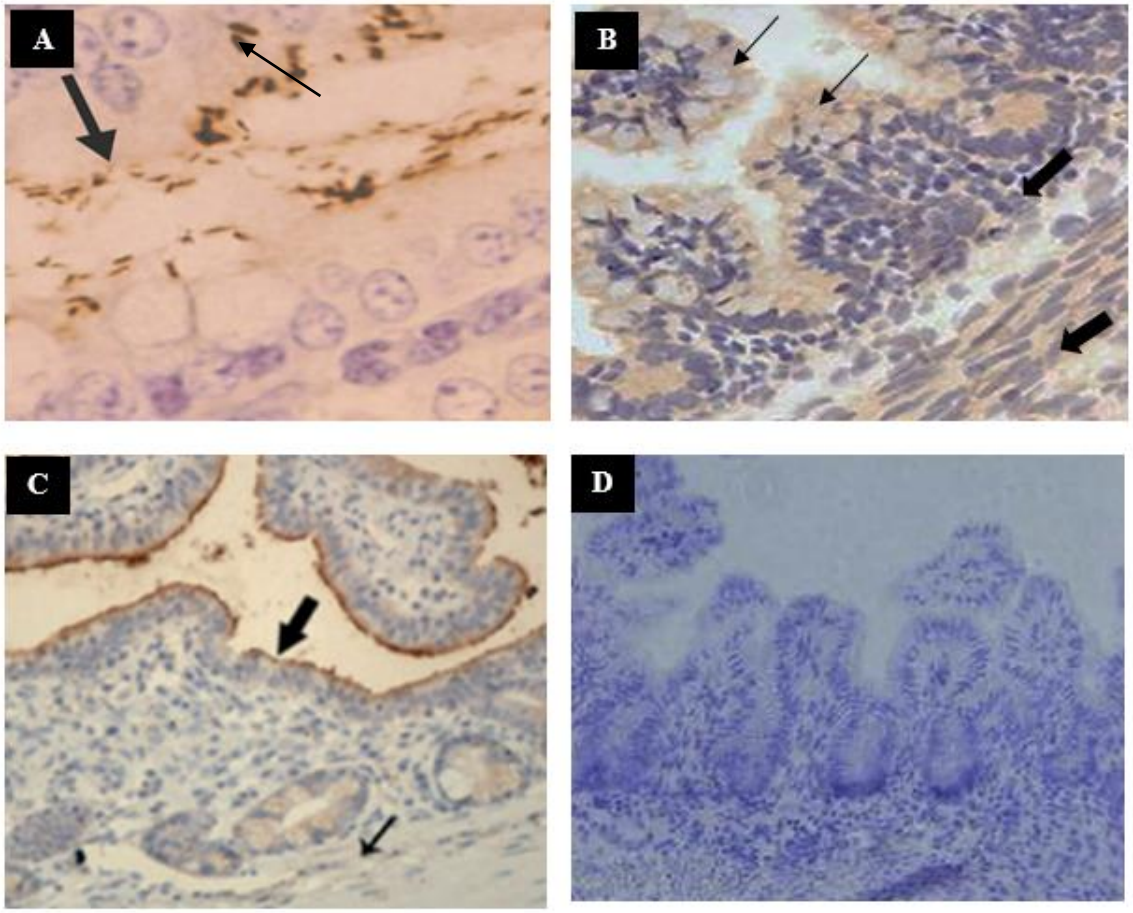

Figure 6: Immunohistopathological staining of rabbit intestinal tissue sections infected with *C.jejuni* isolated from naturally infected calves and raw milk: A: by 24hr PI, in the infected rabbits (G1&G2) showing presence of *C.jejuni* through the luminal surface of intestine (thick arrow) as well as between enterocytes which lining the inner surface of villi (thin arrow)**.** X40**. B:** by 48hr PI, in the infected untreated rabbits showing diffuse brown immunoperoxidase reaction in the cell surface and mucosa (thin arrow) x40. The reaction extended to the submucosa and muscularis (thick arrow). **C:** by 48hr PI in the infected treated treated rabbits showed a significant immunoperoxidase reaction on the cell surface (thick arrow), and also labeling within the mucosa (thin arrow), but not extended to submucosa and muscularis. **D:** no positive brown staining reaction are seen in negative control rabbits.

3.4.6. Ultrastructural changes in intestinal epithelial cells induced by *C.jejuni* in rabbits:

Transmission electron microscopy (EM) was used to further study how *C. jejuni* interacts with host epithelial cells of small intestine post infection. Transmission EM showed that *C. jejuni* did not induce any structural changes in the epithelium during the first 12 h post infection. Moreover *C. jejuni* was not observed by EM in the lumen, tight junctions, desmosomes, or villus brush border, which remained intact in all experimental groups (Fig. 7A). By 24hr PI, numerous clusters of bacteria were observed in the lumen and closely attached to the villus tips causing partial destruction of the brush border while the adjacent epithelium without bacteria appeared normal (fig.7B). By 36hr PI, intraepithelial lymphocytic infiltration were stimulated, cellular swelling and edema were also observed with cavities at the sight of attachment for invasion (Fig. 7C). By 48hr PI, the cellular membrane next to bacteria was invaginated, followed by diffuse disorganization of the epithelium architecture, and degeneration in the untreated group (fig.7D). By 60hr PI and after invasion, bacteria were surrounded by enterocyte vacules in which most of them lysed in the treated group. While in the untreated group retained their shape and size (fig.7E). By 72hr PI, the bacterial invasion fllowed by severe intracellular lymphocytic infiltration and cell swelling in the untreated group (fig.7F).

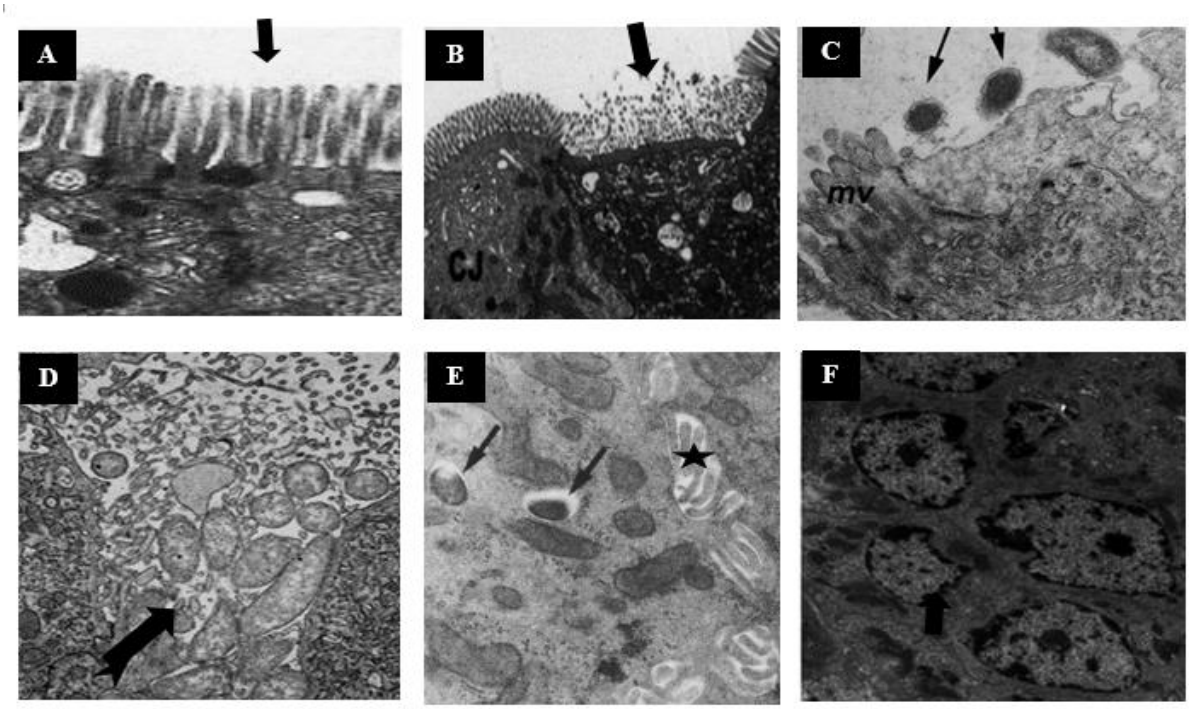

Figure 7: Transmission electron microscopy of the distal small intestine of rabbits inoculated with *C. jejuni*. A: by 12hr PI, *C. jejuni* are not observed in lumen and brush border remain intact with tight junction between villi (arrow), X54,000. **B**: by 24hr PI, clusters bacteria attached to the epithelial cell surface and associated with loss of microvilli at the site of attachment (arrow), while the adjacent epithelium without bacteria appeared normal and intact (thick arrow), X35,000. **C**. By 36hr PI, C. jejuni closely attached to epithelial surface causing cavities for invasion (arrow), X 54,000 .**D:** by 48hr PI, C.jejuni accumulated in the cavities of invasion below the normal level of adjacent intact epithelium (arrow), X 54,000. **E:** by 60hr PI, intracellular *C.jejuni* apparently free in the cytoplasm were surrounded by enterocyte vacules (arrow). mitochondria (star). X 35,000. **F:** by 72hr PI, bacterial invasion Followed bycell swelling, edema and severe inflammatory infiltrate of intraepithelial lymphocytes, (arrow), X 65,000.

DISCUSSION

The main goal of this work was to determine the virulence of *C. jejuni* and the post infection pathogenesis as well as antimicrobial resistance as part of the characterization of this strain for future treatment planning and efficacy studies.

C. jejuni is generally considered commensals of livestock and did not normally cause clinical disease in adult animals, but now has emerged as one of the most common causes of serious foodborn zoonotic diseases worldwide for both human and animal and is responsible for gastroenteritis in young livestock in many industrial countries (Kaakoush *et al.*, 2015). In the current study, Cultural examination, staining characteristics, biochemical tests and finally PCR were performed for the characterization of the *Campylobacter spp*. and the colony characteristics were exhibited grey color which was supported by Kabir *et al.* (2015) and Mehedul *et al.* (2018). The routine isolation and identification of *Campylobacter* spp. in laboratories were conducted on the basis of cultural and biochemical methods which was supported by Jamshidi *et al.* (2008). Hippurate hydrolysis test was used for discriminating between *C. jejuni* and *C. coli* which was also used by several researchers (Kabir *et al.*, 2014 and Shiramaru *et al.*, 2012). The current study recorded 32 (32%) and 9 (22.5%) *Campylobacter* spp. from 100 fecal and 40 raw milk samples respectively during the study period**.** The PCR is adefinitive, reliable, easy method and is required to facilitate rapid identification for *C.jejuni* (El-Kholy *et al.*, 2016). In the current study, seven *C.jejuni* isolates that were biochemically identified were further molecularly characterized by *mapA* gene amplification specific to *C.jejuni*, all the isolates demonstrated positive specific product (589bp) for *C.jejuni* which is supported by Ghoneim *et al.* (2020). With respection to the virulence properties of *C.jejuni*, all the seven isolates carried virulence campylobacter adhesion fibronectin (the *cad*F) and generated the expected product (400bp), which agree with Elsayed *et al.* (2019) who reported that *cad*F is the most virulent gene campylobacter adhesion.

Antimicrobial susceptibility test represents one of the most important tasks of the clinical microbiology laboratory especially in veterinary medicine, because of the interrelation between resistance found in strains of animal origin and humans. Also it can be used as an epidemiological tool and for the definition of the proper treatment of infections, consequently preventing or creating strategies that minimize the dissemination of resistant bacterial strains, mainly multiresistant ones of *C. jejuni* (Hagos *et al.*, 2021) which is emerged by WHO as aproblem of puplic health importance (Heredia and Garcia, 2018).

In the current study, 7 *C.jejuni* isolates were investigated for their antimicrobial sensitivity pattern. The percentage of ampicillin and amoxicillin resistant *C.jejuni* isolates were 85% and 57%, respectively, and highest susceptibility percent with norfloxacine and erythromycin 85% and 71.4% respectively even with multidrug resistance isolates. This was in agreement with Faris (2015) who reported 97.2% and 83.3% for ampicillin and amoxicillin, respectively. Moreover, Macrolides and Fluoroquinolones are usually the drug of choice for treatment *C.jejuni* isolates.

In the current study, we developed a simple rabbits model of campylobacteriosis induced intestinal pathology and diarrhea. This experimental model enabled us to define several key features of the pathogenesis. Rabbits developed diarrhea within 48 h after oral inoculation of live *C. jejuni* contained mucus and gross blood. The clinical signs and pathological lesions produced in the rabbits like those observed in humans and other large-animal models. *C. jejuni* multiplied in the distal small intestine and cecum of rabbits with large

populations of *C. jejuni*, indicating its possible role in the pathogenesis of campylobacteriosis (Shang *et al.,* 2016). Although diarrhea resolved spontaneously by day 4 post infection, the untreated rabbits may excrete bacteria asymptomatically for as long as 2 wk, these results supported by Black *et al.* (1988); Wassenaar and Blaser (1999). In the current study, the inflammatory nature of the diarrhea was further confirmed by the presence of lactoferrin and occult blood in stools. Compared with occult blood, lactoferrin was more sensitive in detecting inflammation that agreed with Stintzi (2005). Fecal inflammatory markers were highly positive in G1(infected untreated), whereas were mild in G2 (treated), and negative in G3(control). These results agreed with Cook *et al.* (2020) who reported that fecal biomarkers result reflects the underlying GIT inflammatory conditions, moreover it can be used as amonitor response to intestinal therapy.

In the current study, infected untreated rabbits showed marked disruption of the villous epithelial surface in the small intestine, damage of the brush border was noted in areas adjacent to bacteria that appeared to be bound to the epithelial cells, all appear to contribute to villus disruption and the breakdown of epithelial barrier function, this may be attributed to effacement of microvilli, re-distribution of cytoskeletal and tight junction proteins, and extrusion of epithelial cells in the small intestine as recorded by Nemelka *et al.* (2009). Our histopathological finding revealed that microbial activities occurring mainly at the intestinal level and more significant than extraintestinal invasion as no histologic changes were seen in livers, kidneys, lungs, or mesenteric lymph nodes, that agreed with Shang *et al.* (2016) who reported that peritoneal histopathologic evaluation revealed no significant changes in infected animals at anytime during the study.

In the current study, based on immunohistochemical staining, we suggested that *C.jejuni* is capable of invading intestinal tissues. In the infected untreated rabbits, stained bacterial cells were observed in deep tissues as well as the paracellular junction of the epithelium and extended to muscularis compared to treated group which observed in lamina properia and mucosae only, similarly with Nemelka *et al.* (2009) who reported the immunohistochemical stained *C. jejuni* (brown stain) can be seen in deep tissues of infected rabbit.

In the current study, for further understanding how *C. jejuni* interacts with host epithelial cells, we visualized the epithelium in the small intestine of infected rabbits by electron microscopy (EM), which revealed that adherence would be the first step of invasiveness as *C. jejuni* did not induce any structural changes in the epithelium during the first 12 h after infection and *C. jejuni* was not observed in the lumen, tight junctions which remained intact. However, clusters of attached bacteria were observed 24 h postinfection, particularly near the villus tips. Partial destruction of the brush border, infiltration of intraepithelial lymphocytes, and intercellular swelling were observed at 63hr PI. the epithelium was disorganized, and necrotic with severe infiltration of intraepithelial lymphocytes was observed at 72 hr PI, this results agree with Shang *et al.* (2016).

CONCLUSION

The implementation of hyagenic practices during milking and proper handling of milk during calves feeding, regular monitoring of antibiogram profile are very crucial in preventing *C.jejuni* infection, colonization and intestinal damage and subsequently economic loss in dairy farm. suggesting that experimental models may be useful to study the mechanisms and the pathogenesis of *C. jejuni*-induced intestinal disease.

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متلازمة الاسهال التي تسببه الكمبيلوبكتر جيجوناى في العجول

هالة محمد اسماعيل ، شيرين سامى مصطفى

Email: ibrahimsabry.gravena@gmail.com Assiut University web-site: www.aun.edu.eg

أجريت هذه الدراسة بسبب ظهور حالات حقلية من الاسهال في العجول لمرحلة ما قبل الفطام في بعض مزارع الدقهلية والتي لم تستجيب للعلاج ببعض المضادات الحيوية مما أدى زيادة عدد الحالات وأطالة فترة العلاج وبالتالي خسائر اقتصادية كبيرة.

تم تجميع ١٠٠ مسحة شرجية وعينات اسهال من العجول المصابة بالإضافة الى ٤٠ عينة من اللبن الخام من نفس المزارع والتي يتغذى عليها العجول. تم عزل الميكروب وتصنيفة بيوكيميائيا بالطرق المرجعية ثم استخدام تفاعل انزيم البلمرة المتعدد للكشف عن جينات الضراوة بالميكروبات المعزولة واجراء اختبار الحساسية لتحديد المضادات الحيوية المناسبة للعلاج.

ولدراسة التغيرات الباثولوجية المصاحبة لهذا المرض قمنا بعمل عدوى اصطناعية على الارانب حيث قسمت الى ثلاث مجموعات: مجموعة مصابة ولم تعالج، ومجموعة مصابة وتم علاجها ومجموعة ضابطة سلبية وبعد مرور ١٢ ساعة على العدوى يتم اخذ عينات من انسجة الأمعاء كل ١٢ ساعة لمدة خمس أيام وتوضع هذه العينات في الفورمالين لعمل دراسات هستوباثولوجية ومناعية باثولوجية والكشف بالميكروسكوب الالكترونى لرصد مراحل التغيرات من بداية العدوى وحتى توقف الاسهال في المجموعات الثلاثة.

وأوضحت النتائج أنه من ١٤٠ عينة من براز العجول واللبن الخام الذى يتغذى علية العجول المريضة، تم التعرف على ٤١ عزلة من الناحيتين الجرثومية. تم تضخيم سبع سلالات معزولة بشكل إيجابي لجين *mapA* الخاص بـ *C.Jejuni* وحملت جين الضراوة *cadF*. جين.

كما أوضحت اختبار الحساسية لهذة المعزولات انها تقاوم الأموكسيسيلين والأمبيسيلين وقابلة للتأثر بشدة بالنورفلوكساسين والإريثروميسين.

وأوضحت العدوى الاصطناعية للأرانب بهذة السلالة عن طريق الفم اصابة ٩٠٪ من الأرانب بالإسهال. تم فحص التغيرات النسيجية المرضية الواضحة في (المجموعة الأولى) خلال المرحلة الحادة (الأيام ١ إلى ٣) في الجزء الأخير للامعاء الدقيقة والقولون بما في ذلك تدمير الزغابات وفقدان الغدد المعوية. ، بينما شوهد نزيف في طبقة البروبريا العضلية. كانت التغييرات خفيفة وتضمنت فقط الزغابات في المجموعة المعالجة (المجموعة الثانية) ، بينما غابت التغيرات الباثولوجية في المجموعة الضابطة(المجموعة الثالثة). تم تأكيد هذه النتائج عن طريق التلوين المناعي. مما يشير إلى أن بكتيريا *C.jejuni* قادرة على غزو أنسجة الأمعاء العميقة وصولًا إلى الطبقة تحت المخاطية في المجموعة الاولى بينما في عدوى المجموعة الثانية كان التفاعل محصورًا بشكل أساسي في الزغب ، وانخفض بشكل كبير في الطبقة تحت المخاطية. كما أظهر المجهر الإلكتروني جميع مراحل الغزو والأضرار المرتبطة به من العدوى ، والاستعمار ، وتلف الزغابات ، وغزو الخلايا الظهارية. وبالتالي ، فإن تنفيذ الممارسات الصحية أثناء الحلب والتعامل السليم مع الحليب أثناء تغذية العجول ، والرصد المنتظم لملف المضادات الحيوية أمر بالغ الأهمية في منع هذه عدوى ، والاستعمار ، والأضرار المعوية ، وبالتالي الخسائر الاقتصادية في المزارع الحلابة.