3 *Egypt. J. Vet. Sci.* **Vol. 44**, pp. 37- 47 (2013)

Enteric Diarrheal Disease in Sheep Specially Campylobacter Infection

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A TOTAL of 75 faecal samples were collected from 30 apparently normal and 45 diarrheic sheep. The bacteriological examination revealed isolation of *C. jejuni* and *C. coli* from 9 (12%) and 3 (4%) of examined sheep respectively. The prevalence of *C. jejuni* and *C. coli were* higher in the diarrheic sheep (15.6%) and (4.4%) than the apparently healthy (6.66%) and (3.33%) respectively. Campylobacter isolates were identified biochemically and serologically and using pathogenicity test two antigens cytotoxin and whole cell antigens associated with campylobacter. Campylobacter whole cell and cytotoxins were identified for their immunogenic activity by neutralization test and ELISA. Campylobacter isolates were also tested for susceptibility against 9 antibiotics.

Keywords: Campylobacter, Enteric diarrhea, Cytotoxin, Neutralization, ELISA, Antibiotic sensitivity.

Diarrhea is the most common symptom of illness in young sheep and calves. Scours can be caused by many organisms and more than one causative agent can be present in one animal. Although Salmonella, *E.coli*, and viruses such as rota virus are the most common cause of scours in young animals but protozoa such as cryptosporidia and coccidia and other bacteria such as campylobacter can also cause diarrhea (Radostitis *et al.,* 2000).

Campylobacter is a Gram-negative slender, curved, and motile rod. It is a microaerophilic organism, which has a reduced level of oxygen. It is fragile and sensitive to several environmental stresses as 21% oxygen, drying, heating, disinfectants, and acidic conditions).Because of its microaerophilic characters, the organism requires 3 to 5% oxygen and 2 to 10% carbon dioxide for optimal growth conditions (Betty *et al.*,1998) and (Tongkorn, 2010). Campylobacter species are recognized as one of the major cause of diarrhea in domestic animals and human throughout the world (Butzler and Oosterom, 1991, Siemer *et al.*, 2005). Moreover, epidemiological data have provided strong evidence that animals and food products of animal origin are the main sources for human infection (Shakespeare, 2002 and Hartmut *et al.,* 2003).

Several hypotheses have been proposed, based on clinical observations including suggestions that the organism is invasive, can produce cytotoxins and enterotoxins (Prasad *et al*., 1996 and Lee *et al.,* 2000). Invasiveness is compatible with the occurrence of bloody diarrhea, often associated with endoscopic evidence of colitis or bacteraemia (Fernandez & Trabulsi, 1995 and Coote *et al.,* 2007). One of the mechanism by which *Campylobacter* species show its pathogenicity might be the production of toxins. Some of these toxins have a similarly to those of Vibrio cholera (Goossens *et al.,* 1990 and Schulze *et al.,* 1998). Both *C. jejuni* and *C. coli* produce a heat labile enterotoxin (McCardell *et al.,* 1986). However 70% of *C. jejuni* and *C. coli* strains produce a cytotoxin. (Johnson and Lior, 1988 and Lee *et al.,* 2000).

Some Campylobacter strains produce cytotoxic response in tissue culture system as Vero cell lines and the pathogenic significance of these cytotoxins has not been well evaluated (Muna, 2009). Increasing antimicrobial resistance in campylobacter is a recognized problem, (Sáenz *et al.,* 2000 and Jensen and Aarestrup, 2001). The increasing uses of antibiotics in treatment of animal diseases especially enteric diseases may create more resistant strains of campylobacter in human and animal.

The aim of this work was:

- To study the prevalence of *C. jejuni* and *C. coli* in the feaces of lambs with and without diarrhea.
- To characterize the whole cell and cytotoxin antigen of campylobacter isolated from clinical cases.
- Study the susceptibility of Campylobacter isolated from lambs to 9 antibiotics.

Material and Methods

Samples

A total of 75 faecal swab samples were collected from sheep. Out of these samples, 45 were obtained from lambs (1-3 months age) suffering from severe diarrhea characterized by dark brown faeces with mucous and blood while the remaining 30 samples were taken from apparently healthy lambs during the period from August 2011 up to January 2012. All samples were collected and transported to the laboratory in the transport broth with supplement to preserve the organism from drying during transportation (OIE 2008).

Isolation and identification of Campylobacter species

Samples in transport broth with supplement were cultured directly onto modified Campylobacter blood free selective medium with antibiotics supplement (cefoperazon charcoal deooxycholate agar) (C.C.D.A) .The inoculated plates were incubated in an atmosphere of 5% Oxygen, 10% CO_2 tension at 42°C for 48 hours using anaerobic gas generator kits (Gaspack kits). Suspected colony appeared flat, moist and translucent dew like when young.

Suspected colonies were picked up and purified onto thioglycolate media for further identification. Growing colonies were stained with Gram stain to demonstrate the characteristic morphology of the colonies (Cruckshank *et al*., 1975). Motility test was performed to demonstrate the cork screw like motion characteristic for campylobacter species. The isolates were identified biochemically and biotyped according to (Koneman *et al.,* 1995) and (Quinn *et al.*,2002).

Antibiotic sensitivity test

The technique was carried out using the disc diffusion method according to Bopp *et al*. (1985). Bacterial isolates were tested for resistance using gradient disk diffusion MIC to Neomycin (30µg), Gentamycin (10µg), Ampicillin (10µg), Streptomycin (10µg), Nalidixic acid (30µg), Erythromycin (15µg), Chloramphenicol (30µg), Sulfamethoxazole (25µg) and Tetracycline(30µg) (Oxoid). Three colonies of Campylobacter organisms were inoculated into tubes containing 5 ml Muller Hilton broth (Oxoid), then incubated for eight hours under reduced O_2 tension at 37°C. The turbidity was adjusted to match that of standard McFarland 0.5 barium sulphate tube (0.5 ml of 1.175% barium chloride hydrate at 99 ml of 1% sulfuric acid) by adding sterile saline solution. The suspension was then inoculated evenly on 150-mm Muller Hilton agar plates supplemented with 5% defibinated sheep blood. Different antibiotic discs were placed on the surface of agar plates. The plates were incubated for 72 h at 37°C under the microaerophilic conditions, and the inhibitory zone diameters were measured.

Antigen preparation

Whole cell antigen was prepared from fresh campylobacter strain grown on selective medium in a microaerophilic conditions at 42°C for 48h then the organisms were harvested in sterile saline containing 0.5% formalin, incubate at 37°C overnight, washed once with BPS (7.4 pH), suspended to concentration of 109 CFU in PBS then stored at 4°C in saline containing 0.5% formalin. For titration the turbidity of the antigen preparation was standardized to correspond to the McFarland tube No.1 (Kosunen *et al.,*1981).

Cytotoxin was prepared from Campylobacter strains which were grown at 42°C for 24h in selective medium supplemented with 1.0% Isovitalex under agitated condition in atmosphere of 10%CO_2 tension. For broth filtrates only treated with 2mg of polymyxinsulphate per ml at final 10 minutes. Cell free broth filtrates were obtained by centrifugation at 13000Xg for 10 minutes, and then the supernatants were sterilized by passages through 0.22 µm pore size membrane filter. The sterile filtrate was concentrated and precipitated with 70% zinc sulphate solution, then suspended in 10 mMTris hydrochloride buffer according to Jennifer *et al.* (1991).

Rabbit Immunization

Three groups each of 5 rabbits were used, Two groups were inoculated with two different prepared antigens (whole cell antigen and cytotoxin antigen)

cytotoxin was inoculated in the first group by injecting each animal with (1ml) via subcutaneous route (Derek *et al.*,1986) and injecting 109 CFU of whole cell antigen in the second group S/C while the third group were injected with physiological saline and kept as control serum (Yung *et al.,* 1987).

Neutralization assays

10µl sample of partially purified toxin (100µ) was mixed with 10µl of serially diluted antisera obtained from naturally infected sheep, after 1h incubation at 37°C (10^6) in 1ml of growth medium that was added, the cell showed morphological changes induced by cytotoxin (Yung *et al.,* 1987).

ELISA Assay

Campylobacter antigens (whole cell and cytotoxin antigens) were used as coating antigens in detecting antibody levels against these antigens in naturally infected sheep and experimentally infected rabbits. Horseradish peroxidase (HRP) conjugated rabbit antisheep IgG was diluted in PBS in case of ovine serum samples while Labeled sheep anti-rabbit IgG was diluted in PBS in case of rabbit serum samples according to Derek *et al.* (1989).

Results

Table 1 illustrated that isolates of *C. jejuni* recovered from 7 (15.6%) of the 45 diarrheic sheep which was higher than from 2 (6.66%) of the apparently healthy sheep. Total number of positive samples of *C. jejuni* was 9 (12%) of 75 rectal swab samples from sheep while *C.coli* was recovered from 2 (4.4%) of the 45 diarrheic sheep which was higher than the apparently healthy sheep (3.33%). Totally *C.coli* was isolated from 3 (4%) of examined sheep samples.

TABLE 1. Incidence of Campylobacter species isolated from apparently healthy and diseased sheep.

Isolated species and subspecies	Apparently healthy		Diseased		Total	
	Number of isolates/number of cases examined	%	Number of isolates/number of cases examined	%	Number of isolates/ number of cases examined	%
C. jejuni	2/30	6.66	7/45	15.6	9/75	12
C. coli	1/30	3.33	2/45	4.4	3/75	4
Total	3/30	10	9/45	20	12/75	16

In-vetro Antimicrobial sensitivity

As shown in Table 2, *C. jejuni* isolated from sheep were sensitive to chloramphenicol (90%) followed by gentamycin and neomycin. All *C. jejuni* isolated from sheep were resistant to penicillin, ampicillin, erythromycin and tetracycline.

TABLE 2. Antimicrobial sensitivity of *Campylobacter jejuni* isolated from sheep (n=10).

Antibiotic	*C. jejuni*					
	Sensitive		Moderate		Resistant	
	No	%	No	%	No	%
Neomycin	5	50	5	50	0	0
Gentamycin	7	70	3	30	0	0
Ampicillin	0	0	1	10	9	90
Erythromycin	0	0	2	20	8	80
Chloramphenicol	9	90	1	10	0	0
Nalidexic Acid	0	0	4	40	6	60
Sulphamethoxazole	0	0	2	20	8	80
Tetracycline	0	0	2	20	8	80
Pencillin	0	0	0	0	10	100

n=number of examined isolates.

As shown in Table 3 *C. coli* isolated were sensitive to chloramphenicol (70%) and gentamycin (60%) while resistant to penicillin, ampicillin, tetracycline, erythromycin and nalidexic acid. The percentages of resistant strains of *C. coli* were higher than *C. jejuni* strains.

TABLE 3. Antimicrobial sensitivity of Campylobacter coli isolated from sheep (n=10).

Antibiotic	*C. coli*					
	Senstive		Moderate		Resistant	
	No	%	No	%	No	%
Neomycin	5	50	3	30	2	20
Gentamycin	6	60	4	40	0	0
Ampicillin	0	0	1	10	9	90
Erythromycin	0	0	1	10	9	90
Chloramphenicol	7	70	3	30	0	0
Nalidexic Acid	0	0	1	10	9	90
Sulphamethoxazole	0	0	2	20	8	80
Tetracycline	0	0	1	10	9	90
Pencillin	0	0	0	0	10	100

n=number of examined isolates.

Table 4 illustrated that high anticytotoxic antibody levels in serum of rabbits were significantly greater than antibody titer to whole cell antigen from 1^{st} 2^{nd}, 3^{rd} and 4^{th} week post immunization.

TABLE 4. Antibody response of immunized rabbits to cytotoxin and whole cell antigen using ELISA.

Week post immunization/ Type of antigen	Cytotoxin	Whole cell
First week	0.514±0.0193	0.416±0.0166
Second week	0.663±0.0264	0.5532±0.0183
Third week	0.7514±0.0408	0.5814±0.0221
Fourth week	0.6928±0.0325	0.5492±0.0257

Table 5 demonstrated that neutralization antibodies against cytotoxin in naturally infected sheep were significantly higher than whole cell antibodies.

TABLE 5. Neutralization of cytotoxin and whole cell antigens of campylobacter from sheep (sera from naturally infected sheep).

Type of atigens/titer	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Cytotoxin	+	+	+	+	-	-	-
Whole cell antigen	+	+	-	-	-	-	-

Discussion

Campylobacter species have been recognized as a cause of diarrhea in cattle and sheep (Radoststits *et al.,* 2000). Campylobacter enteritis constitutes a zoonotic disease of major concern in public health and indeed has been shown to be a greater problem than Salmonellosis in several countries (Bacon *et al.,* 1999). In the present study, it was of interest to note that out of 75 sheep showing diarrhea and apparently healthy, the occurrence of campylobacter reach 12 cases (16%).These results agreed with findings of Yazicioghu (2000) who recorded 17.2% prevalence rate of campylobacter. While lower than 49% and 25% recorded by Garcia *et al.* (2010) and Rotariu *et al.* (2009) from sheep feces.

Data presented in Table 1 revealed that *C. jejuni* was isolated from examined sheep samples at a percentage of (12%) while *C.coli* was isolated at a percentage of (4%), so the prevalence of *C. jejuni* was higher than *C.coli*, these results support the observation of Busato *et al.* (1999) and Governor and Governor (2002) who reported that *C. jejuni* was most predominant isolates from diarrhoeic sheep followed by *C. coli*. As shown in Table 1 the prevalence of *C. jejuni* and *C.coli* was significantly higher in sheep suffering from diarrhea, this provided evidence of significant association between diarrhea and infection with *C. jejuni* and *C. coli*, this supported by the observation of Dodson and Lejeune (2005) who isolated *C. jejuni* from enteric diseased calves.

Furthermore in experimental study, Kerr (2004) found that oral inoculation of campylobacter was able to produce mild to moderate enteritis. In vitro antibiotic sensitivity test was done against strains of campylobacter using 9 antibiotics (Tables 2,3) illustrated that *C. jejuni* and *C. coli* were sensitive to

chloramphenicol, this support the data reported by (Oporto *et al.*, 2009) who treated cases of campylobacter infection successfully with chloramphenicol. In addition, high percentage of isolates of *C. jejuni* and *C. coli* were resistant to Ampicillin, tetracyclin, Erythromycin, Nalidexic acid and sulfamethoxazole but drug resistance was more frequent in *C. coli* than *C. jejuni*. These findings are agree with that recorded by Chuma *et al.* (2001) and Bae *et al.* (2005), they demonstrated that there was a very low prevalence of resistance among *C. coli* isolates, while (Anthony and Ellen, 2000) and (Shakespeare, 2002) explained that using of antibiotics in animal treatment may promote the emergence of multi-antibiotic resistant mutant of campylobacter species.

The virulence of Campylobacter species based mainly on the adherent, invasiveness and cytotoxin production which has been detected in several different strains of *C. jejuni* and has been confirmed to be toxic. These toxins play an important role in the pathogenesis of *Campylobacter jejuni* infection (Klipstin *et al.*, 1985). Cytotoxin produced by campylobacter was considered to be the primary virulence factor in the pathogenesis (Wassenaar, 1997) and (Schulze *et al.,* 1998). Cytotoxin facilitates invasion and spread of the organism following entry via the alimentary tract (Wong *et al.,* 1983) and (Pickett, 2000) Serum antibody titer have been associated with increase resistance to campylobacter in sheep while whole cell antigen stimulate somatic cell immune response which doesn't consistently provide high protective antibody titer. (Gurturk *et al.,* 2007).

To evaluate the humoral immune response of rabbits immunized with cytotoxin and whole cell antigen using ELISA, data presented in table (4) illustrated the significant increase in antibody titre during different intervals post immunization in rabbits immunized by cytotoxin than rabbits immunized by whole cell antigen according to (Bacon *et al.*,1999) and (Blaser and Duncan, 1984). These results indicated that the protective immunity could be manifested by cytotoxin antigen than the whole cell antigen, this could be attributed to higher immunogenic feature of cytotoxin as concluded by (Coote *et al.,* 2007).

The results of this study suggest that exposure to cytotoxin antigen may be necessary to produce an anticytotoxin immune response and that response is better predictor of resistance to campylobacter than the immune response to whole cell antigen. ELISA cytotoxin antibody response was significantly higher than those for the whole cell antigen recorded by (Blaser and Perez, 1993) and (leunk *et al*., 1988) found that cytotoxin activation of peripheral blood monocytes, macrophages and when incubated with neutrophil resulted in neutrophil activation.

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(Received 24/12/2013;
accepted 14 / 9/2015)

الاسهال المعوى فى الأغنام خاصةً العدوى ببكتريا الكامبيلوباكتر

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قسم البكتريولوجى - معمل الجيزة - معهد بحوث صحة الحيوان – القاهرة – مصر.

تم تجميع ٧٥ عينة من براز الأغنام منهم ٣٠حيوان سليم ظاهرياً و ٤٥ حيوان مصاب بالاسهال و بعد اجراء الاختبارات البكتريولوجية تم عزل ٩ عترات من ميكروب الكامبيلوباكتر جوجنى (١٢٪) وعدد ٣ عترات من ميكروب الكامبيلوباكتر القولونى (٤٪) و قد بلغت نسبة الاصابة بالكامبيلوباكتر جوجنى (١٥,٦٪) والكامبيلوباكتر القولونى (٤,٤٪) فى الأغنام المصابة بالاسهال و هى أعلى من النسبة فى الأغنام السليمة ظاهرياً و التى كانت (٦,٦٦٪) بالنسبة لميكروب الكامبيلوباكتر جوجنى و(٣,٣٣٪) بميكروب الكامبيلوباكتر القولونى.
وقد تم تحديد العترات باجراء الاختبارات البيوكيمائية و السيرولوجية للميكروب بعد عزله كما تم القيام باختبار الامراض باستخدام اثنين من أنتيجينات الكامبيلوباكتر (سيتوتوكسين و أنتيجين الخلية الكلى) و قياس الاستجابة المناعية لهذه الأنتيجينات باستخدام اختبار المعادلة واختبار الاليزا. كما تم اجراء اختبار الحساسية للعترات المعزولة باستخدام ٩ من المضادات الحيوية.