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CHARACTERIZATION OF CAMPYLOBACTER ISOLATES FROM SHEEP WITH PARTICULAR REFERENCE TO ENTEROTOXIN PRODUCTION

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SUMMARY

C. jejuni subsp. *jejuni, C. coli* and *C. laridis* were isolated from apparently normal and diarrhoeic sheep with an incidence of 10% and 20%, respectively. *C. jejuni* subsp. *jejuni* was the most predominant species with an overall incidence of 10.7%. All Campylobacter strains isolated from diarrhoeic animals were invasive to Hela cells and their cytotoxin produce degree ++ and +++ of changes in Vero cells and titer of toxins were high (64-128).

C. jejuni enterotoxin isolated from diarrhoeic sheep gave maximum secretion in rat ligated ileal loops. While enterotoxin of other strains evoked less fluid secretion in rat ligated ileal loops. SDS PAGE of heat labile enterotoxin extracted from isolated strains indicated protein profiles ranging from 49.3 to 59. 3 KD.

In immunized-challenged rats the fluid secretion was reduced by 70 to 80% less than the value for similar challenged unimmunized control rats. Antibody titers measured by ELISA showed four folds higher level than that in control animals.

INTRODUCTION

The importance of Campylobacter species as one of the major causes of diarrhoea in domestic animals and humans through the world is recognized (Butzler and Oosterom, 1991).

Moreover, epidemiological data have provided strong evidence that animals and food products of animal origin are the main sources for human infection. Hence, Campylobacter enteritis consti-

tutes a zoonosis of major concern in public health significant and indeed has been shown to be a greater problem (Nielsen et al., 1997).

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).

Invasiveness is compatible with the occurrence of bloody diarrhoea, often associated with endoscopic evidence of colitis or bacteraemia (Fernandez and Trabulsi, 1995).

The mechanism by which Campylobacter cause disease is not well known. The occurrence of infection appears to require adherence of *Campylobacters* to the gut mucosa, perhaps mediated by flageller adhesions or outer bacterial membrane components. The intestinal mucous gel seems to be a major site for colonization by Campylobacter species (Butzler and Oosterom 1991).

One of the mechanism by which *Campylobacter* species show its pathogenicity might be the production of toxins. Some of these toxins have a similarity to those of Vibrio cholera (Goossens et al., 1990).

Some *Campylobacter* strains produce cytotoxic response in tissue culture system as Vero cell lines and the pathogenic significance of this cytotoxins has not been well evaluated.

The purpose of this work was to investigate the pathogenic properties of Campylobacter species isolated from apparently normal and diarrhoeic sheep with particular reference to its enterotoxin production.

MATERIAL AND METHODS

Samples:-

A total of 75 faecal samples were collected from sheep from various private farms in Khafr EL-Sheik Governorate.

Out of these samples, 45 were obtained from animals suffering from severe diarrhoea characterized by dark brown faeces with mucous and blood. The remaining 30 samples were taken from apparently normal ones during the period from March 2001 up to January 2002.

All samples were collected and transported to the laboratory. In the transport broth with supplement (Firehammer and Myers, 1981).

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 (code: SR 155). The inoculated plates were incubated in 10% CO_2 tension at 37°C and 42°C for 3 days and were examined daily to demonstrate the characteristics colonies. Suspected

growing colonies were stained with Gramís stain to demonstrate the characteristic morphology of the isolates. Motility test was done by using the hanging drop technique to demonstrate the cork-screw like motion characteristic for Campylobacter species. The isolates were identified biochemically and biotyped according to (Koneman et al., 1995).

Hela cell invasion:-

Hela cell monolayer was prepared (Manninen et al., 1982). Monolayers were washed three times with warm PBS and then seeded with 1 ml of a viable bacterial suspension 6.8 X 10^8 cfu. After three hours incubation at 37°C in an atmosphere of 10% CO_2 tension in air, the infected monolayers were washed three times in PBS. Supplemented medium was added and incubated for further three hour. The monolayers were again washed three times in PBS. After being infected air dried monolayers were fixed with 95 % methanol and then stained for 10 min. in 1: 10 solution of Giemsa stain. The number and percent of infected Hela cell and dead Hela cell were counted and the number of bacteria associated with each cell was counted for 100 cells.

Enterotoxin production:- Klipstein and Engert, 1985

Strains were grown at 42°C for 24 h in Gc medium (Difco laboratories, Detroit, Mich) supplemented with 1.0%. Isovitalex under agitated condition in the presence of 8% CO_2, for broth filtrates only, treated for final 10 min. with 2 mg of polymyxin sulphate per ml. Cell free broth filtrates were obtained by centrifugation at 13.000 X g for 10 min. at 4°C, and then the supernatants were sterilized by passages through 0.22 µm pore size membrane filter. The sterile broth filtrate was concentrated and precipitated with 70% zinc sulphate solution as described by (Klipstein and Engert, 1985). The amount of toxin used were based on their protein concentration determined by the method of Lowry et al. (1951).

SDS- PAGE

Analytical discontinuous was performed by the technique of Samboork et al. (1989) with 3% acrylamide stacking gels and 10% of acrylamide running gel.

Assays for cytotoxin:-

The Vero cell assay was performed by a modification of the method of Klipstein et al. (1985) freshly trypsinized cells were diluted 1:5 in Eagle minimal essential medium plus 2% foetal calf serum; 100 µl samples were placed in 96 well microtiter plates to which 100 µl of bacterial free supernatant were added to each well. The plates were incubated for 18 h at 36°C in 5% CO_2 and then fixed with methanol and stained with Giemsa stain. The results are reported as the last dilution that showed > 50% rounded cells.

Rat ligated ileal loop:-

Toxins preparation in 250µl of buffer were placed

for 16 h into single 10 cm ligated ileal loops of fasting Sprague Dawely rats weighing about 75 to 200 grams. The results reported for each data point are the mean T ± the standard error of the mean for the volume / length ratio V/L in four rats (Klipstein et al., 1983).

Immunization and challenge procedure:- (Klipstein and Engert, 1984)

Sprague Dawely rats about (150-175 grams) were given primary immunization of enterotoxin preparation 100 µg I/P with Freund complete adjuvant per kilogram of body weight followed by two per-os (P.O.) booster 500 µg immunization at 4 days intervals (500 µg for each peroral booster). Immunization P.O. was given via intragasteric tube 2 h after the P.O. administration of cimetidine at the dosage of 50 mg/kg of body weight to ablate gastric acid secretion.

Immunized and control animals were challenged 4 to 7 days after the final booster immunization 6 mg instillation of 0.1 ml of a broth culture counting 109 viable organism. The significance of the difference in secretion (as volume/ length ratio) between immunized and control group was determined.

Antitoxin response:-

At the time of challenge, serum was obtained from immunized animals and antitoxin titers were determined by ELISA as previously reported techniques Klipstein et al. (1983).

RESULTS

Data presented in table (1) shows that out of 30 apparently normal sheep, *Campylobacter jejuni* subsp. *jejuni*, *C. coli* and *C. laridis* were recovered in an incidence of 3.33% each. Therefore the total incidence among apparently normal sheep was 10%. Out of 45 diarrhoeic sheep *Cambylobacter jejuni* subsp. *Jejuni* and *C. coli* were isolated in an incidence of 15.6% and 4.4%, respectively. The total incidence among diarrhoeic sheep was 20%. On identification, *C. jejuni* subsp. jejuni was the most predominant species with an overall incidence of 10.7%.

Results in table (2) revealed that all *Campylobacter* species isolated from diarrhoeic sheep were invasive to Hela cells and cause deaths to these cells with different degree (Photo No. 1&2). Whereas the strains isolated from apparently normal animals were found to be non-invasive.

Determination of Verotoxin activity among Campylobacter isolates, the obtained results in table (2) revealed that all Campylobacter isolates were produce cytotoxin. It is clear that 9 isolates from diarrhoeic sheep produced degree ++ and +++ of changes which included elongation of Vero cells and vacuolation between cells (degree ++) in addition to stillate shape cells with detachment (degree +++) and titers of their toxins were high (64, 128). While 3 isolates recovered from apparently

normal sheep produced degree + of changes which include slight rounding of Vero cells and titre of their toxins were low (32 and 16).

Campylobacter jejuni enterotoxin isolated from diarrhoeic sheep gave maximum secretion in rat ligated ileal loops. While enterotoxin of other strains evoked less fluid secretion in rat ligated ileal loops.

SDS PAGE of heat labile enterotoxin extracted from the isolated strains indicated 2 protein profile in C. jejuni (54.9- 49.3), in C. coli (55.7 ñ 51.9) and 3 protein profiles in C. laridis (59.3 ñ 55.7- 49.5) as shown in table (3) and photo (5).

Table (4) shows that fluid secretion in immunized- challenged rats was reduced by 70% to 80% less than the value for similarly challenged unimmunized control rats. On the other hand, the absorbance value in sera of immunized animals

Table (1): Incidence of Campylobacter species and subspecies isolated from apparently healthy and diseased sheep.

Isolated species and subspecies	Apparently healthy		Diseased		Total	
	Number of isolates/ number of cases examined	Percentage	Number of isolates/ number of cases examined	Percentage	Number of isolates/ number of cases examined	Percentage
C.jejuni subsp. *jejuni*	1/30	3.33%	7/45	15.6%	8/75	10.7%
C.coli	1/30	3.33%	2/45	4.4%	3/75	4%
C.laridis	1/30	3.33%	0/45	0%	1/75	1.3%
Total	3/30	10%	9/45	20%	12/75	16%

Table (2): Pathogenic properties of Campylobacter strains isolated from diarrhoeic and apparently normal sheep

Isolated species and subspecies	General healthy condition	Cytotoxicity		Hela cell invasion		Secretion in ileal loop V/L
		Degree of cytotoxicity	Verotox in titer	% of dead cells	% of infected cells	
C.jejuni subsp. *jejuni*	diarrhoeic	+++	128	70%	30%	321±1
C.jejuni subsp. *jejuni*	diarrhoeic	+++	128	85%	15%	289±11
C.jejuni subsp. *jejuni*	diarrhoeic	+++	128	85%	15%	210±7
C.jejuni subsp. *jejuni*	diarrhoeic	+++	128	80%	20%	295±14
C.jejuni subsp. *jejuni*	diarrhoeic	+++	128	75%	25%	190±
C.jejuni subsp. *jejuni*	diarrhoeic	++	64	80%	20%	265±15
C.jejuni subsp. *jejuni*	diarrhoeic	++	64	70%	30%	230±9
C.jejuni subsp. *jejuni*	Apparently normal	+	32	-	-	95±9
C.coli	Diarrhoeic	++	64	40%	60%	132±2
C.coli	Diarrhoeic	++	64	30%	70%	164±9
C.coli	Apparently normal	+	16	-	-	84±10
C.laridis	Apparently normal	+	32	-	-	70±3

V/ L volume length ratio of challenged rats ligated ileal loop by enterotoxin
+ slight rounding of Vero cells.
++ elongatin and vacuolation.
+++ rounding, elongation, vacuolation and satillate shape.

Table (3): Analysis of heat labile enterotoxin of ***Campylobacter*** isolates by SDS PAGE.

Marker		*C.jejuni*		*C.coli*		*C.laridis*	
M.W*	Amount	M.W*	Amount	M.W*	Amount	M.W*	Amount
97.4	15.4	59.32	31.7	55.72	61.3	54.90	48.1
66	18.8	55.72	34.1	51.95	38.7	49.34	51.9
45	17.6	49.52	34.2				
36	17.2						
29	13.4						
14	17.3						

* M.W. = Molecular weight

Table (4): Antitoxin response and protection against challenge with viable bacteria in immunized animals

Strains	ELISA*		Secretion in ileal loop V/L	
	Immunized	Control	Immunized	Control
C.jejuni subsp. *jejuni*	0.868	0.230	37 ± 2	37 ± 2
C.coli	0.593	0.195	26 ± 5	26 ± 5
C.laridis	0.561	0.181	21 ± 2	21 ± 2

* Absorbance value in sera of immunized and control animal.

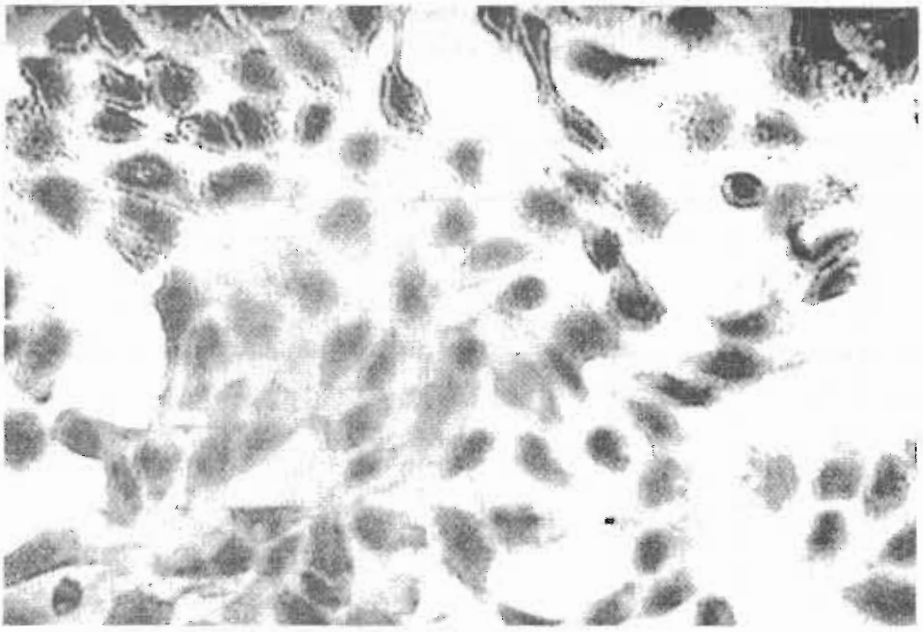

Photo (1): Hela cells showing no invasion (control -ve).

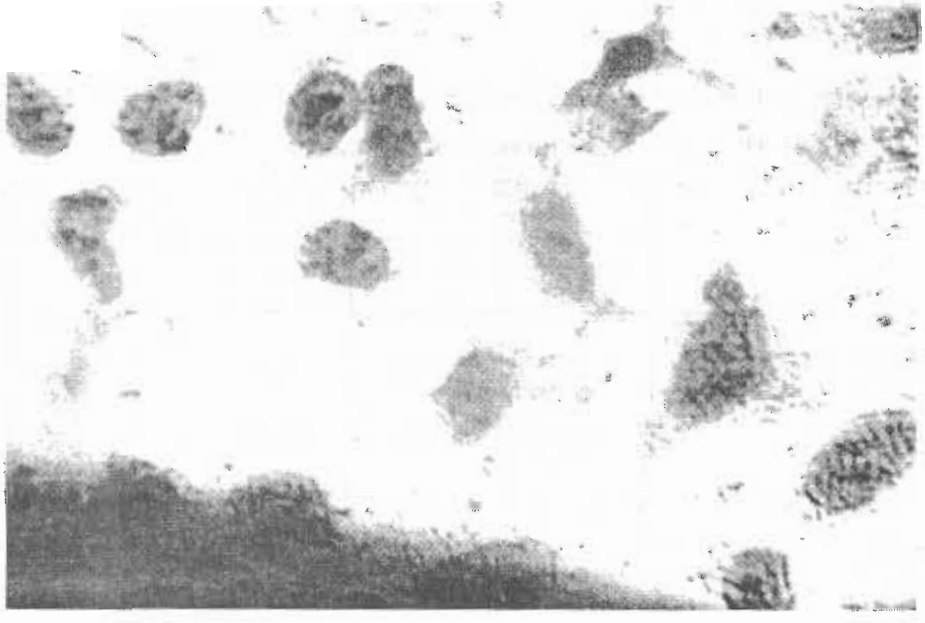

Photo (2): Hela cells showing invasion by bacteria, death and detachment from sheet.

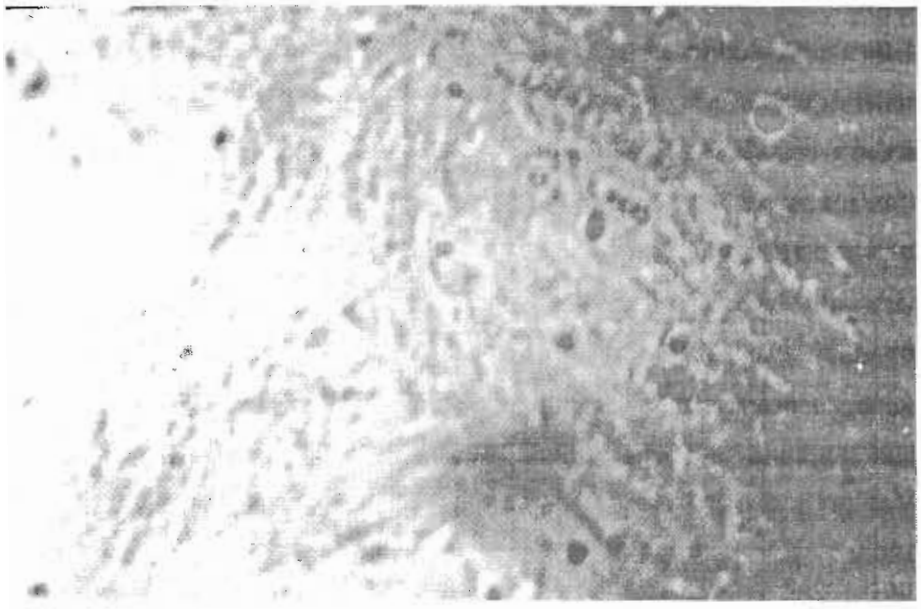

Photo (3): Vero cell showing no alteration control -ve.

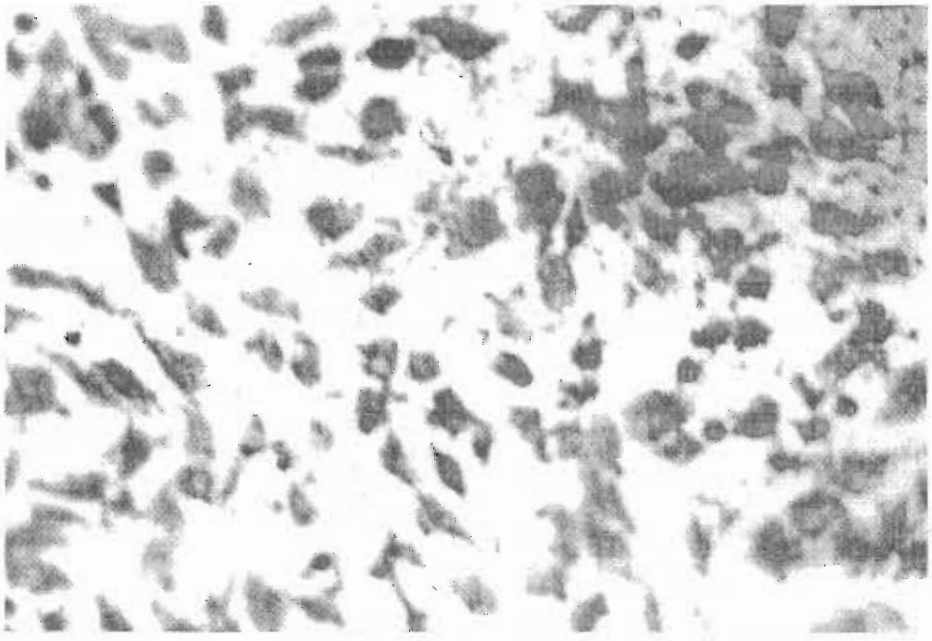

Photo (4): Vero cell cytotoxicity showing all degree of changes.

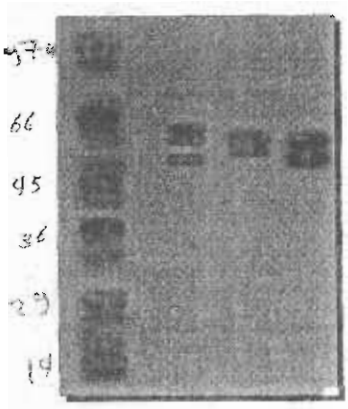

Photo (5): analysis of heat labile enterotoxin of different Campylobacter isolates.

showed four fold higher than in control animals.

DISCUSSION

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 (Stanley et al., 1998).

In the present study, it was of interest to note that out of 75 sheep showing diarrhoea or apparently normal, the occurrence of *Campylobacters* reached 12 cases with the recovery rate reached up to 16%.

These results agreed to large extent with finding of Yazicioglu (2000) who found that the prevalence rate of *Campylobacter* species in sheep was 17.2%.

In the present work such incidence was considerably significant lower than that recorded by Schiavo et al. (1987) who reported that the incidence of Campylobacter in faecal samples obtained from sheep were 33.1%.

On identification, *C. jejuni* was most predominant isolates from diarrhoeic sheep in an incidence of 10.7% followed by *C. coli* (4%).

C. laridis isolated from apparently normal sheep only with this respect, the present results are in accordance with those reported by Yazicioglu (2000) who showed that *C. jejuni* (12.2%) was the most predominant isolates from diarrhoeic sheep

Furthermore, Kim et al. (1987) isolated *C. laridis* from healthy animals. These finding might supported the assumption that different species of Campylobacter were the part of the normal flora of the intestinal tract of ruminant.

Using the clinical status of animals from which the *Campylobacter* strains were isolated, it was found that a correlation between pathogenic properties of specific isolates and the clinical status of animals. All of the isolates from diarrhoeic sheep were invasive and produced cytotoxins and enterotoxins. The pathophysiology of *Campylobacter* strains induced diarrhoea has not yet established, but it is tempting to speculate that two pathogenic mechanisms now recognized, invasion and enterotoxin production, may account for the variable clinical pattern of diarrhoeal disease caused by this organism.

Results of invasion of Hela cells by different strains revealed that strains isolated from apparently normal animals were non-invasive. Other strains isolated from diarrhoeic sheep were varied in the invasive capacity to Hela cells. This result nearly similar to that recorded by Fernandez and Trabulsi (1995) who found that all Campylobacter strains isolated from diseased sheep were invasive and the number of invaded Hela cells reached 46%.

correlated with the invasion of the gastrointestinal mucosa by the organism which is an essential and early step in the pathogenesis caused by the organism (Portillo et al., 1997). Previous works suggested that certain types of fimbriae could facilitate adhesion to enterocytes. Adherence is presumed to be a requisite for invasion (Ernst et al., 1990).

SDS PAGE of extracted heat labile enterotoxins were detected after staining with silver nitrate indicated 2 protein bands in *C. jejuni* and *C. coli* and 3 bands in *C. laridis*. The most common bands were 54.9 KD and 55.7 KD.

These results are nearly similar to that recorded by Klipstein et al., (1985) who discovered the presence of several molecular bands predominant component had a molecular weight of 57.4 KD.

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 to Chinese hamster ovary, Vero, Hela cells and intestinal tissue cultures. These toxins play an important role in the pathogenesis of Campylobacter jejuni infection (Klipstein et al., 1985).

The obtained results in table (2) and figure (2) showed that the toxins of isolates of Campylo-

bacter species from diarrhoeic sheep produced distinct cytopathic effect on Vero cells (++ 2nd and +++ 3rd degrees) with high titre (64-128) while toxins of isolates obtained from apparently healthy sheep produced slight cytopathic effect 1st degree + with lower titre 16-32. These findings are supported by results of Lee et al. (2000) who provided evidence of significant association between diarrhoea and infection with *Campylobacter* and the presence of Verotoxin. The same results recorded by Schulze et al. (1998) who discussed the role of produced toxins with its degrees and development of diarrhoea in diseased animals.

The obtained results in table (2) showed that *C. jejuni* enterotoxins of strains isolated from diarrhoeic sheep gave maximum secretion in rat ligated ileal loop while fluid secretion obtained by heat labile enterotoxins of other stains. This result nearly similar to that recorded by Chattopadhyay et al. (1991) who found that all *C. jejuni* from diseased animal and human proved to be toxin positive and cause fluid accumulation in ligated rat ileal loop.

Moser et al. (1992) indicated that once colonization has been established *Campylobacter* multiply and may produce toxins especially enterotoxins. Bacterial colonization of mucosal surfaces depends on bacterial being able to maintain close proximity to associate with the mucosa and adhere to mucosa membranes.

The results of the present study indicated that heat labile enterotoxins of *Campylobacter* achieved a strong (greater than four folds) serum IgG antitoxin response and a significant degree of protection (reduced fluid secretion) in immunized rats. This result agrees with Klipstein et al. (1983) who concluded that parentral primary immunization with labile toxin must be followed by p. o. boosters because parentral priming is a prerequisite for strongly effective p. o.booster immunization and that p.o. immunization also raises the mucosal IgG antitoxin titers that provide extended protection. Acute diarrhoeal disease continues to be a major cause for morbidity and mortality among animals (Atabay and Corry, 1998). Toxigenic strains of *C. jejuni* eventually prove to be a significant cause of diarrhoea hence vaccines containing heat labile enterotoxin might also provide some degree of protection against diarrhoea caused by toxigenic strains of *C. jejuni* (Klipstein and Engert, 1984)

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