Benha Journal of Applied Sciences (BJAS) **print : ISSN 2356–9751**
Vol.(2) Issue(3) Dec.(2017), 81-88 **online : ISSN 2356–976x**
http:// bjas.bu.edu.eg

Studies on Different Recent Techniques for Diagnosis of Campylobacter in Rabbit

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Abstract

Rabbits can breed for the production of meat and fur. Their meat is considered as a source of human campylobacteriosis; caused by Campylobacter organism's has been recognized as the main etiological agent of human bacterial gastrointestinal disease.

Two hundred and thirty rabbit samples were collected including cloacal swabs from (130), liver (40), intestinal samples (40), water (10) and ration (10) from apparently healthy and diseased rabbits suffering from diarrhea in different farms. All rabbit samples were processed for isolation of Campylobacters. Each sample was homogenized in sterile Thioglycolate broth, incubated at 42 °C for 48 hrs under microaerophilic condition. All The isolates were subjected biochemical tests, such as catalase, oxidase, hippurate hydrolysis test, glycine, sodium chloride (NaCl) 3.5% tolerance test and susceptibility to cephalothin and nalidixic acid. Identified colonies were stored at -70 C in nutrient broths with 15% glycerol until subjected to molecular identification. The results of this study showed that overall Campylobacter isolates was 58 (25.22%) from the different sources sampled. The prevalence of C. jejuni was the most prevalent species 26 (11.30%) in samples taken from rabbits followed by C. coli was 15 (6.52%) then C. lari was 12 (5.22%) and C. hyointestinalis was 5(2.17%). The overall prevalence of C.jejuni and C.coli (74.3%) (25.70%); the difference was notably due to a positive hippurate test result for isolates identified as C.jejuni due to the absence of hippurate hydrolysis for C.coli. Multiplex PCR methods the genus specific (16S rRNA) revealed that 51 (22.17%) Campylobacter species isolates; 27 (52.94%) as C. jejuni specific at323 bp while, 17 (33.33%) produced the C. coli specific at 126 bp and 7 (13.73%) other Campylobacter species.

We concluded that C. jejuni and C. coli are highly prevalent in rabbit farms in Egypt. Control measures for contamination of the rabbit supply should be identified to protect human exposure to Campylobacter spp. Further analysis of rabbit samples by using PCR assay are needed to evaluate the applicability of the method for detection of Campylobacter organisms exposed to an environment.

1. Introduction

Campylobacter enteritis is a leading cause of acute bacterial gastrointestinal infection worldwide. The genus Campylobacter includes many species of which Campylobacter jejuni and C. coli are common pathogens and the majority of diagnosed human Campylobacter infections [15].

Campylobacteriosis is considered as the major important zoonotic gastrointestinal disease around the world and most of the cases are caused mainly by C. jejuni. Poultry play an important role in transmission of campylobacteriosis to human [10, 13].

Campylobacter species were isolated from the caecal contents of rabbits (Oryctolagus cuniculus). All strains were initially identified as belonging to the genus Campylobacter by means of genus-specific PCR, but were identified PCR for known thermophilic species [24,20] Cells were spiral shaped with bipolar unsheathed flagella, with no periplasmic fibres, and appeared coccoid after 10-12 days of incubation. Phylogenetic analyses based on 16S rRNA gene revealed that all strains recognized Campylobacter [17].

Routine detection of Campylobacter species in clinical laboratories is based on culture on selective media and subsequent phenotypic identification. Culture methods are based toward the detection of C. jejuni and C. coli. The antimicrobial agents incorporated into used selective media may inhibit growth of some Campylobacter species [7 , 25].

The true incidence of Campylobacter species may be under estimated because of the limitations of routine culture methods because conventional methods are relatively slow [9] .Presumptive results may be available after 2 days. However, definitive species-level identification based on phenotypic methods may require a further 3 to 4 [23].Phenotypic identification can be challenging because of the fastidious growth requirements and the distinguishing of biochemical characteristics by Campylobacter species [12].

Molecular methods based on PCR amplification may provide an alternative to culture methods for the detection of Campylobacter in different specimens. The application of PCR-based assays applied to the detection of Campylobacter species in rabbit specimens [19]. Amplification of DNA targets including the Campylobacter flagellin gene, 16S rRNA and the 16S/23S rRNA intergenic spacer region (Touzet, et al. 2009).

The aim of this study was to identification of Campylobacter species isolated from rabbit specimens by conventional methods. Molecular characterization for Campylobacter isolates by using PCR and amplification of DNA including the Campylobacter flagellin gene, 16S rRNA, virulence gene and cytolethal genes in rabbit specimens.

2.Materials and methods

2.1Sampling

Two hundred and thirty rabbit samples were collected including cloacal swabs from (130), liver (40), intestinal samples (40), water (10) and ration (10) from apparently healthy and diseased rabbits suffering from diarrhea in different farms. All rabbit samples were collected in sterile sample collection vials, transferred to the lab. As quick as possible, all samples kept at 4 °C and processed for isolation of campylobacters Table (1).

2.2 Isolation and identification of campylobacter species

About 10 g of each sample were homogenized in sterile Thioglycolate broth, incubated at 42 °C for 48 hrs under microaerophilic condition (5% O2, 10% CO2 and 85% N2). Microscopic examination for the incubated samples for detection of Campylobacter microorganisms identified under phase contrast microscope using (4 00 x) magnification for detection of characteristic motility [31]. All The isolates were subjected to Gram's staining and biochemical tests, such as catalase, oxidase, urease, nitrate reduction and indole acetate hydrolysis, hippurate hydrolysis test, glycine tolerance test, sodium chloride (NaCl) 3.5% tolerance test and susceptibility to cephalothin and nalidixic acid by the disc diffusion method according to [15] Identified colonies

were stored at -70 °C in nutrient broths with 15%glycerol until subjected to molecular identification

3.Molecular characterization of Campylobacter species.

3.1 Isolation of DNA

DNA extracts were prepared for each isolate by 8 minutes boiling of colonies in 10% Chelex 100 (Bio-Rad) in 10 mM Tris/HCl, 1 mM EDTA, pH 8. The crude DNA preparation was stored at 4°C until used [12].

3.2 Oligonucleotide primers used in cPCR.

Six pairs of primers were supplied from (Metabion). They have specific sequence and amplify specific products as shown in Table (2).

3.3 Extraction of DNA: according to QIAamp DNA mini kit

20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml micro centrifuge tube. 200 µl of the sample was added. 200 µl buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56˚C for 10 min. The 1.5 ml micro centrifuge tube was centrifuged. Ethanol (96%) 200 µl were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml micro centrifuge tube was briefly centrifuged. The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube. The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 was added without wetting the rim. The cap was closed, and centrifuged at full speed for 3 min. Centrifugation at full speed for 1 min was done. The QIAamp mini spin column was placed in a clean 1.5 ml micro centrifuge tube. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was Incubated at room temperature (15-25˚C) for 1 min, and then centrifuged at 8000 rpm for 1 min.

3.4 Preparation of duplex PCR Master

Mix for each of (C. coli glyA and C. jejuni hipO), (cdtB and cdtC) and (virB11 and flaA) genes according to GoTaq® Hot Start Green Master Mix.

3.5 Agarose gel electrophoreses [16]

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, 0.5µg/ml Ethidium bromide was added and mixed thoroughly. Twenty µl of each PCR product samples, negative control and positive control were loaded to the gel. The gel was photographed by a gel documentation system and the data was analyzed.

4. Results

Out of 230 rabbit samples screened for the presence of Campylobacters, 58 samples yielded characteristic Campylobacter colonies on CCDA plates after 48 hours of incubation Table (4). They had characteristic corkscrew motility observed by the phase contrast microscope. The biochemical tests for isolates were found positive for catalase, oxidase, and nitrate. None of the isolates revealed positive reaction for urease activity Table (5).

Out of the 51 (22.17%) Campylobacter isolates, 27 (52.94%) were identified as C. jejuni (323bp) , 17 (33.33%) isolates as C.coli (126 bp), and 7 (13.73%) isolates as other Campylobacter species based on the hippurate hydrolysis test and all were found to be sensitive to nalidixic acid and resistant to cephalothin Tables (6) & Fig (1,2 and 3).

Discussion

Rabbits can breed for the production of meat and fur. Their meat is considered as a source of human campylobacteriosis; caused by Campylobacter organism's has been recognized as the main etiological agent of human bacterial gastrointestinal disease [1,17, 16]. Campylobacter will have a positive impact on consumers' perceptions related to food safety, the food industry and public health agencies [20].

The results of this study showed that overall Campylobacter isolates was 58 (25.22%) from the different sources sampled. The prevalence of C. jejuni was the most prevalent species 26 (11.30%) in samples taken from rabbits followed by C. coli was 15 (6.52%) then C. lari was 12 (5.22%) and C. hyointestinalis was 5(2.17%) (Table, 4 & fig. 1). The overall prevalence of C.jejuni and C.coli (74.3%) (25.70%); the difference was notably due to a positive hippurate test result for isolates identified as C.jejuni due to the absence of hippurate hydrolysis for C.coli [22, 25].

Data recorded in table (4) revealed the high incidence of Campylobacter in intestinal content (40%) followed by water (30%) then in cloacal swabs was (26.92%) and in liver (10%). The high incidence of Campylobacter in intestinal content and cloacal swabs may be due to the normal inhabitant of Campylobacter organisms in intestine of rabbits without any diseased signs [4,35].The high incidence of Campylobacter in diseased rabbit samples was (32.04%) followed by apparently health rabbit samples (19.69%). The high incidence of Campylobacter isolates in apparently health rabbit samples was in the intestinal content (46.67%) may be due to the normal inhabitant of Campylobacter organisms in intestine without any diseased signs. Followed by cloacal swabs was (21.33%) then in drinking water samples (14.29%) and liver (7.69%). Also, Campylobacter contamination increases during untreated drinking water [27, 5].

In this present study, according to the Multiplex PCR methods and amplification parameters, 58 Campylobacter isolates yielded the genus specific (16S rRNA) revealed that 51 (22.17%) Campylobacter species isolates; 27 (52.94%) as C. jejuni specific at323 bp while, 17 (33.33%) produced the C. coli specific at 126 bp and 7 (13.73%) other Campylobacter species (Table, 5 and Fig. 1). C. jejuni isolates from liver and water (100%), followed by cloacal swabs (50%) and intestinal samples (42.86%) were confirmed by mPCR, while C. coli isolates were confirmed by the amplification of glyA gene as cloacal swabs (37.5%) and intestinal samples (35.71%). The hippurate hydrolysis assay is dependent upon the inoculums size of the bacterium, which means that the assay is unable to detect low level of hippuricase product [18] .Therefore, the detection of the gene by PCR instead of the phenotypic detection of the hippuricase product is considered a reliable alternative method for the discrimination of C. jejuni isolates [21, 3].

Vir B11 is a pathogenic gene responsible for the expression of invasion. In the present study, the confirmed C. jejuni isolates Vir B11 gene was high incidence in liver and intestinal samples 50% while in cloacal swabs was 35.71%. A putative virulence gene associated with adhesion of the pathogen to intestinal epithelial cells [26] .This gene is 100% conserved among C. jejuni and C. coli isolates of diverse sources; therefore, it was used to detect virulent isolates of both species [8]. Cytolethal distending toxin (CDT) is widely distributed among Gram-negative bacteria [11] and is the best characterized of the toxins produced by Campylobacter spp. It has been described as an important virulence factor of this pathogen [2].CDT holotoxin, composed of three subunits encoded by the cdtA, cdtB and cdtC genes, causes eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and consequently leading to cell death [35] .C.jejuni cytolethal distending toxins showed at 555 bp for CdtC and at 495 bp for CdtB of the genus Campylobacter 555 bp and 495 bp amplicons were confirmed.

C. lari strains were about the same size as the C. jejuni and C. coli products during the PCR step did not amplify this product. Thus, the assay could be used to discriminate between C. lari and the C. jejuni-C. coli isolates. The remaining seven Campylobacter strains were belonging to other Campylobacter species in PCR assays. However, reducing the primer concentration from 0.25 to 0.2 µm during the first PCR step was sometimes necessary to reduce the number of nonspecific amplicons [33].

We concluded that C. jejuni and C. coli are highly prevalent in rabbit farms in Egypt. Control measures for exposure and contamination of the rabbit supply should be identified so that methods can be developed to protect human exposure to Campylobacter spp. Further analysis of rabbit samples by using PCR assay are needed to evaluate the applicability of the method for detection of Campylobacter organisms exposed to an environment.

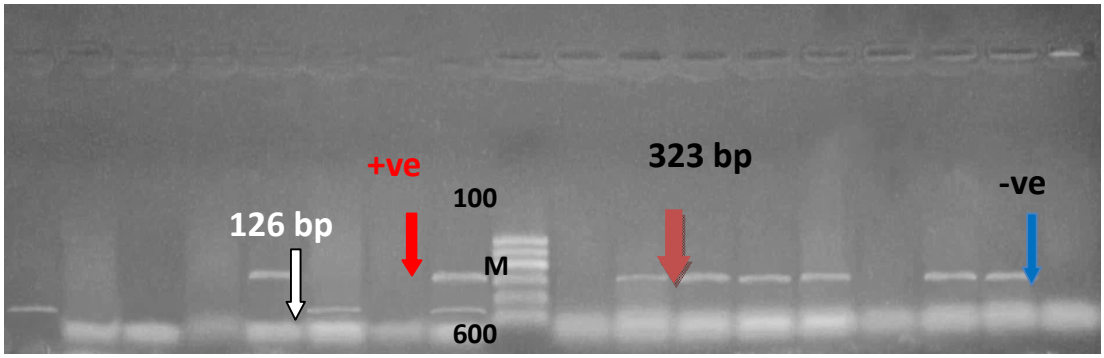

Fig (1) Multiplex PCR on 1.5% agarose gels. +ve= positive -ve= negative
All the 58 Campylobacter isolates yielded the genus specific (16S rRNA)
C. jejuni 323 bp, *C. coli* 126 bp. LaneM: 100-600 bp ladders; Lane: 10, 15 *C. coli* at 126 bp; Lane: 1, 2, 4, 5, 6,7,11 *C. jejuni* at 323 bp.

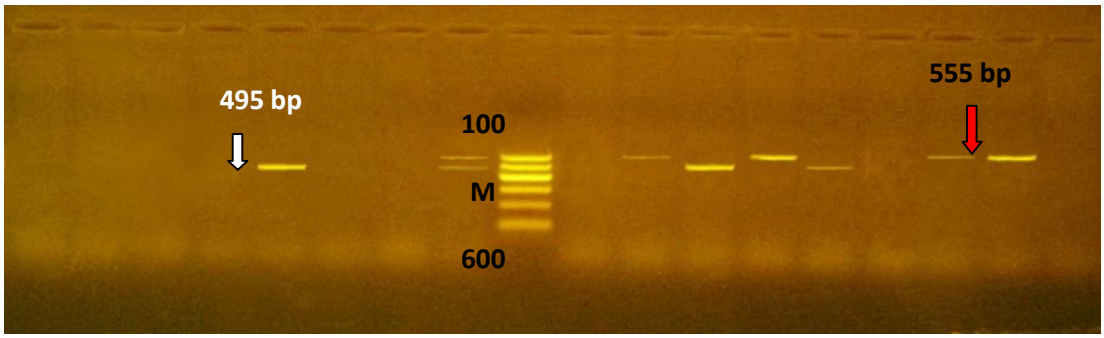

Fig (2) C.jejuni toxins showed at 555 bp for CdtC and at 495 bp for CdtB. LaneM: 100 bp – 600 bp ladder;
Lane: 2, 4, 7 *C. jejuni* CdtC at 555bp Lane: 1, 5, 6, and 11
C.jejuni CdtB at 495b.

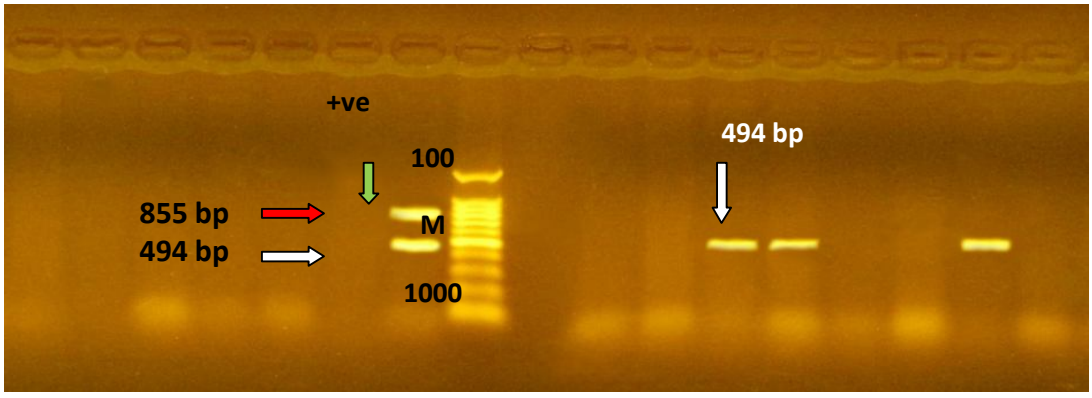

Fig (3) The virulence strains of *C. jejuni* virB11 showed at 494 bp and FlaA at 855 bp
LaneM: 100 bp –1000 bp ladder; Lane: 1, 4, 5 C. jejuni virB11 at 494bp.

Table (1) Samples from diseased and apparently healthy rabbits

Rabbit cases	No. of rabbit samples	Type of samples				
		Cloacal swabs	Liver	Intestinal samples	Water	Ration
Apparently health samples	127	75	13	15	7	7
Diseased Samples	103	55	27	25	3	3
Total	230	130	40	40	10	10

Table (2) Oligonucleotide primers sequences

Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
C. jejuni hipO	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	323 bp	Wang *et al*., 2002
C. coli glyA	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	126 bp	
FlaA	AATAAAAATGCTGATAAAACA GGTG TACCGAACCAATGTCTGCTCT GATT	855 bp	Datta *et al*., 2003
virB11	TCTTGTGAGTTGCCTTACCCCT TTT CCTGCGTGTCCTGTGTTATTTA CCC	494 bp	
CdtB	GTTAAAATCCCCTGCTATCAA CCA GTTGGCACTTGGAATTTGCAA GGC	495 bp	Bang *et al*., 2003
cdtC	TGGATGATAGCAGGGGATTTT AAC TTGCACATAACCAAAAGGAAG	555 bp	

Table (3) Cycling conditions of the different primers during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	Reference
HipO and *glyA*	94˚C 6 min.	95˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.	Wang *et al*., 2002
FlaA and virB11	94˚C 5 min.	94˚C 30 sec.	53˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.	Datta *et al*., 2003
cdtB and *cdtC*	94˚C 5 min.	94˚C 30 sec.	42˚C 45 sec.	72˚C 30 sec.	35	72˚C 10 min.	Bang *et al*., 2003

Table (4) Detection of *Campylobacter* species in the examined rabbits by using conventional methods.

Type of Samples	No. of samples	Apparent health samples	Diseased Samples	Positive Campylobacter spp.	Campylobacter isolates			
					C. jejuni	*C. coli*	*C. Lari*	*C. hyointestinalis*
Cloacal swabs	**130**	**75**	**55**	**35 26.92%**	**14 10.77%**	**10 7.69%**	**8 6.15%**	**3 2.31%**
Liver samples	**40**	**13**	**27**	**4 10%**	**4 10%**	**-**	**-**	**-**
Intestinal samples	**40**	**15**	**25**	**16 40%**	**6 15%**	**4 10%**	**4 10%**	**2 5%**
Water	**10**	**7**	**3**	**3 30%**	**2 20%**	**1 10%**	**-**	**-**
Rations	**10**	**7**	**3**	**-**	**-**	**-**	**-**	**-**
Total	**230**	**127**	**103**	**58 25.22%**	**26 11.30%**	**15 6.52%**	**12 5.22%**	**5 2.17%**

Table (5) Biochemical tests to differentiate between Campylobacter species isolated from rabbit samples

Characteristics	*C. jejuni*	*C. coli*	*C. Lari*	*C. hyointestinalis*
Oxidase	+	+	+	+
Catalase	+	+	+	+
Nitrate reduction	+	+	+	+
Urease	--	--	--	--
Hippurate hydrolysis	+	--	--	--
Growth at:				
25◦C	--	--	--	**D**
37◦C	+	+	+	+
43◦C	+	+	+	+
Growth at 1% glycine	+	+	+	+
%3.5 NaCl	--	--	--	--
H_2S, lead acetate strip	+	+	+	+
H_2S, TSI	--	**D**	--	+
Susceptibility to:				
Nalidixic acid	**S**	**S**	**R**	**R**
Cephalothin	**R**	**R**	**R**	**S**

Table (6) Detection of *Campylobacter* by multiplex PCR in rabbit samples

Type of Samples	No. of examined samples	Positive Campylobacter spp. by PCR	*C. jejuni*	*C. coli*	*Other Campylobacter species.*
Cloacal swabs	130	32 24.62%	16 50%	12 37.5%	4 12.5%
Liver samples	40	4 10%	4 100%	--	--
Intestinal samples	40	14 35%	6 42.86%	5 35.71%	3 21.43%
Water	10	1 10%	1 100%	--	--
ration	10	--	--	--	--
Total	230	51 (22.17%)	27 52.94%	17 33.33%	7 13.73%

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