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Prevalence of *Campylobacter jejun/coli* in Poultry: Comparison between three Culture Media

الكشف عن انتشار بكتيريا الكامبيلوبكتر جيجوني *Campylobacter jejuni* والكامبيلوبكتر كولاي *Campylobacter coli* في لحوم الدواجن: مقارنة بين ثلاثة أنواع من الأوساط الزراعية

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Abstract: *Campylobacter jejun*i is a major etiological agent in human diarrheal disease. Differentiating between *C. jejuni* and *C. coli* represents a diagnostic challenge. Optimal culturing procedure for *Campylobacter* spp. from live broilers carcass, stool and liver tissue are needed for epidemiological studies. This study was conducted to assess the performance of different media for culturing and isolation of *Campylobacte*r spp. from different biological specimen obtained from commercial broilers. Three media selective for *Campylobacter* were assessed: campy-cefex (CD), modified charcoal cefoperazone deoxychocolate agar (mCCD) and Skirrow media. One hundred samples of skin rinse, liver tissue, and feces from broiler chicken were cultured into three selective media for *Campylobacter*. A semi-nested PCR assay was used for confirmation. Fifty five samples and two samples were positive for *C. jejuni* and *C. coli,* respectively. Selectivity of each medium after 48 hr incubation were 55% mCCDA, 45% Campy-cefex, and 24% Skirrow medium. The difference in performance of both mCCDA and Campy-cefex compared to Skirrow's medium proved to be statistically significant (P ≤ 0.01 and P ≤ 0.05, respectively). Performance of the different culture media was not influenced by the type of biological specimens tested. mCCDA was found in our hands to be more selective and specific than the other two media.

Keywords: *Campylobacter jejuni, poultry, Skirrow blood agar, Campy-Cefex agar, mCCDA.*

المستخلص: تعد بكتيريا الكامبيلوبكتر جيجوني من المسببات الرئيسية للإصابة بأمراض الإسهال عند الإنسان، ويعد التفريق بين نوعي بكتيريا الكامبيلوبكتر (جيجوني والكولاي) من التحديات في مجال الكشف عن مسببات الأمراض. أن التوصل إلى منهج مناسب لاستزراع فصائل الكامبيلوبكتر من لحوم الدواجن المغلفة وكبد الدجاج وعينات البراز لهذه الدواجن، يعد أمرا مطلوبا لدراسات انتشار الأمراض والأوبئة. لقد تم إجراء هذه الدراسة لاختبار أداء أنواع مختلفة من الأوساط الزراعية لاستزراع وعزل فصائل الكامبيلوبكتر من عينات حيوية مختلفة من مزارع تجارية. تم إستخدام ثلاث أنواع من الأوساط الزراعية الإنتقائية الخاصة

باستزراع الكامبيلوبكتر كالتالي: كامبي سيفيكس(CD) campy-cefex، الوسط الزراعي سيفوبيرازون دي أكيجوكوليت المطور باستخدام الفحم ((modified charcoal cefoperazone deoxy chocolate agar (mCCD) والوسط الزراعي سكايرو (Skirrow). تم الكشف عن 100 عينة لخلاصة غسيل جلد الدجاج وأنسجة كبد الدجاج وكذلك البراز من دجاج المزارع، والتي تم زرعها على الثلاثة أنواع من الأوساط الزراعية. أستخدمت تقنية تفاعل البلمرة المتعددة الشبه عشي لتأكيد النتائج. لوحظ أن 25 عينة أعطت نتيجة إيجابية لبكتيريا الكامبيلوبكتر جيجوني وعينتين فقط أعطيتا نتيجة إيجابية لبكتيريا الكامبيلوبكتر كولاي. وكانت النسب المئوية لدرجة العزل الانتقائي كما يلي: 55% لـ mCCD، 54% لـ campy–cefex ، و42% لـ Skirrow . كانت نسبة أداء الأوساط الزراعية الـ mCCD والـ campy-cefex بالمقارنة مع الوسط الزراعي الـ Skirrow ذات دالة إحصائية مرتفعة (P≤ 0.01 و P≤ 0.05، على التوالي)، ولم تثبت علاقة بين أداء الأوساط الزراعية ونوع العينة التي تم الكشف عنها. من النتائج السابقة تبين لنا أن الوسط الزراعي الـ mCCD والمتاح لنا استخدامه يعد أكثر انتقائية ودقة من الوسطين الآخرين.

كلمات مدخلية : *ميكروب الكامبيلوباكتر جيجوناي، الدواجن، الوسط الزراعي سكايرو، الوسط الزراعي الخاص بميكروب الكامبيلوباكتر والمحتوي على المضاد الحيوي السيفيكسيتين، الوسط الزراعي المطور والمحتوي على المضاد الحيوي السيفوبرازون وفحم الديؤوكسيكوليت.*

INTRODUCTION

In recent years, *Campylobacter* is recognized globally as the major etiologic agent in human diarrheal disease (Friedman, 2000). *Campylobacter* genus is responsible for the highest number of cases of human enteritis. The natural habitat of most *Campylobacter* species are the intestines of warm-blooded animals, especially birds such as ducks, geese, and seagulls. The principle sites of colonization of the bacteria in chickens with highest incidence and numbers are the ceca of the large intestine and cloaca where *C. jejuni* cells pervade the lumina of crypts without attaching to the copt microvilli (Beery, *et al*. 1988). Several studies have shown that poultry, in particular chicken, is the major source of infection for human campylobacteriosis (Harris, *et al*. 1986; Humphrey, *et al*. 1993). The intestinal carriage of *Campylobacters* appears to be a major contamination factor for broiler carcasses (Oosterom, *et al*. 1983) affecting the microbial quality of the carcass after processing (Musgrove, *et al*. 1997) and it is a potential source of human campylobacteriosis (Grant, *et al*. 1980).

The occurrence of *Campylobacter* as causative agent of diarrhea illness in the Gulf Cooperation Council (GCC) countries has been shown to range from 1.6-28% of total causative agent of gastroenteritis (Sethi, *et al*. 1989; Al-Freihi, *et al*. 1993; Akhte, *et al*. 1994; Ismaeel, *et al*. 2002). In Bahrain, in a study made by Ismaeel, *et al*. (2005), *Campylobacter* rate was found to be 1.6% when compared to *Salmonella* (5.7%) and *Shigella* (3.2%).

Several culture media have been used for the isolation of *Campylobacter* from chicken using different samples (Jones, *et al*. 1991; Humphrey, *et al*. 1993; Pearson, *et al*. 1993; Shreeve, *et al*. 2000). Other studies used direct plating of fecal sample which proved to be the fastest method of isolation (Shanker, *et al*. 1990; Kazwala, *et al*. 1992; Kapperud, *et al*. 1993; Jacobs-Reitsma, *et al*. 1994). Modified charcoal cefoperazone deoxycholate agar (mCCDA) is more frequently used worldwide (Bolton, *et al*. 1984; Hutchinson and Bolton, 1984; Kazwala, *et al*. 1992; Jacobs-Reitsma, *et al*. 1994). However Preston agar (Bolton and Robertson, 1982; Kapperud, *et al*. 1993) modified camp-cefex agar (mCC) (Stern NJ, 1992) and a variation media containing different antimicrobials have also been used (Chattopadhyay, *et al*. 2001).

The aim of this study was to assess the performance of different common cultivation media for isolation of *Campylobacter* from fecal, carcass and liver tissues.

MATERIALS AND METHODS

Poultry samples

A total of 100 chicken samples (including 35 whole chickens, 27 chicken livers, and 38 chicken

feces) were collected from poultry factories in the Kingdom of Bahrain and from the Kingdom of Saudi Arabia (KSA). Bahrain's specimens were gathered immediately after packaging at the factory site. Chicken and chicken livers from KSA were taken directly at the custom port at the King Fahad Causeway and transported to the laboratory by Public Health inspectors. Feces were freshly obtained by squeezing the chickens. Samples were kept under microaerophilic conditions generated with Camp Pack® (Oxoid, Cambridge, UK).

Processed broiler samples

The isolation procedure for *Campylobacter* from a whole chicken was based on the procedure described by Hunt and Abeyta (1998) with some modification. Chicken samples were prepared by adding 225 ml of 0.1 % (w/v) buffered peptone water in a sterile Stomacher bag (Nasco Whirl-Pak, Atkinson, WI, USA). Mixing was continued for 3 min to ensure that the entire external surface was well rinsed. A 45 ml rinse was poured into a sterile container, and centrifuged at 3500 rpm for 10 min. The supernatant was discarded, and the pellet was used for seeding the media. The feces were softened when necessary with sterile saline and vortexed for 15 seconds. After thoroughly mixing the pellets, each plate was inoculated with 15 μl of the pellet. All presumptive isolates from the plates were collected and stored at -80°C in tryptic soy broth (Difco) supplemented with 30% glycerol (vol/vol) and 5% blood.

Culture methods

All samples were plated on the selective media (Table 1); for each medium two plates were each spread with 0.1 ml of the samples. For initial isolation, plates were incubated for 48 hr at 42°C under microaerophilic conditions (5% O2, 10% CO2, 85% N2) generated by Campy Pack® Plus gas-generating envelopes (Oxoid, Cambridge, UK). Quality control strain *C. jejuni* ATCC 33291 was incubated with each lot of plates. Suspected colonies were sub-cultured on chocolate agar plates and incubated micro-aerobically at 37°C for 24 hr. Plates without *Campylobacter* growth after 48 hr, were incubated for additional 24 hr to facilitate the maximum recovery of *Campylobacte*r spp. from samples containing low numbers of cells.

Identification of *Campylobacter* on solid medium

Preliminary identification was based on colony morphology, Gram stain, and positive results from catalase and oxidase test (Ransom, 1998). Further identification to species level was made by standard biochemical tests comprising indoxyl acetate hydrolysis, susceptibilities to cephalothin, nalidixic acid and hippurate test. Additionally API Campy® identification Kit (Biomerieux, Marcy-1'Etoile, France) was performed to confirm the speciation. All *Campylobacter* isolates were stored at –80°C in 50% nutrient agar plus 50% glycerol for further analysis.

DNA extraction from whole chicken, liver tissue and stool

Bacterial DNA was extracted from 200 μl samples of a whole chicken and liver tissue by using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. After 5 min final incubation at room temperature, the resulting DNA extracts were divided in aliquots and stored at –20°C for the PCR test. For stool DNA an extraction QIAamp DNA stool Mini kit (Qiagen GmbH, Hilden, Germany) was used.

PCR amplification

The extracted DNA was subjected to three different sets of primers (Thermo Electron GmbH, Germany). The primers used are listed in Table (2). A semi-nested PCR was performed for the amplification of the 16S rRNA gene and hippuricase gene for *Campylobacter jejuni* and *Campylobacter coli* as described previously (Linton, *et al*. 1997). The amplification was made using a DNA thermal cycler (Gene Amp® PCR system 9700). The cycles were as follows: a hot start as initial denaturation at 94°C for 4 min, and 25 cycles of denaturation at 94°C for 1 min, annealing temperature suitable for each primer pair; for *C. jejuni*/*C. coli* specific gene amplification, the temperature used was 58°C, for hippuricase gene 66°C, and for *C. coli* was 60°C. Extension at 72°C for 1 min, followed by an additional extensional hold for 7 min.

Table 1. Composition of the media used in the study.

Medium	Base	Supplement
Skirrow[a]	Proteose peptone (15.0g), Liver digest (2.5g), Yeast extract (5.0g), Sodium chloride (5.0g), Agar (15.0g), Deionized water (1 litre)	Lysed horse blood (50.0 ml), Vancomycin (0.01g), Polymyxin B (2500.0 i.u.), Trimethoprim (0.005g)
Campy-Cefex	*Brucella* agar[b] (43 g/liter), ferrous sulfate[c] (0.5 g/liter), sodium bisulfite[c] (0.2 g/liter), sodium pyruvate[c] (0.5 g/liter), deionized water (1 liter)	Laked horse blood[b] (50 ml), cefoperazone[c] (33 mg), cycloheximide[c](0.2 g)
mCCDA[d]	Nutrient broth no. 2 (25 g/liter), bacteriological charcoal (4 g/liter), casein hydrolysate (3 g/liter), sodium desoxycholate (1 g/liter), ferrous sulfate (0.25 g/liter), sodium pyruvate (0.25 g/liter), agar (12 g/liter), deionized water (1 liter)	Cefoperazone (32 mg), amphotericin B (10 mg)

[a] Columbia Blood Agar Base (CM0331) and Skirrow supplement (SR0069)

[b] Oxoid, Inc., New York, NY.

[c] Sigma-Aldrich, St. Louis, MO.

[d] *Campylobacter* selective blood-free agar (CM0739) and CCDA selective supplement (SR0155, Oxoid).

. Extension at 72°C for 1 min, followed by an additional extensional hold for 7 min.

Table 2. Primer sets for the detection of *Campylobacter* species.

Primer designation	Nucleotide sequence	Amplicon size (bp)	spp detected
CCCJ609-F	5'- AATCAATGGCTTAACCATTA-3'	854 bp	*C. jejuni & C. coli*
CCCJ1442-R	5'-GTAACTAGTTTAGTATTCCGG-3'		
HIP400-F	5'GAAGAGGGTTTGGGTGGTG-3'	735 bp	*C. jejuni*
HIP1134-R	5'AGCTAGCTTCGCATAATAACTTG-3'		
CC18 F	5'-GGTATGATTTCTACAAAGCGAG-3'	500 bp	*C. coli*
CC519	5'-ATAAAAGACTATCGTCGCGTG-3'		

Detection of PCR products

The amplified DNA (16 μl) was run on 1.5% Agarose (Sigma type I-A, low EEO) gels, stained with ethidium bromide, and photographed under UV light. A positive control, 16 μl of DNA from *C. jejuni* ATCC 33291, *C. coli* CCUG 11283, and a negative reagent control (sterile UV- irradiated distilled water) were included in all runs. Samples were considered positive for the 16S rDNA-based PCR assay specific for *Campylobacter jejuni* and *Campylobacter coli* when one of the three bands were detected: band of 854 bp and 735 bp band for the *Campylobacter jejuni* PCR assay based on the hippuricase gene and 500 bp band for the *Campylobacter coli* PCR assay based on the aspartokinase gene and no similar bands were seen on the negative control.

Statistical Analysis

Data were analyzed using SPSS Statistical Package Version 12. Chi square (χ^2) test was used to analyze data for statistical significance and a p value of <0.05 was considered significant.

RESULTS

During the study, a total of 100 samples - including 38 feces, 27 livers, and 35-skin rinse from broiler chickens, were collected from Bahrain and Saudi slaughterhouse (Table 3). Fifty five samples were *C. jejuni* as determined by positive reaction to the selected biochemical tests and two were *C. coli*. All were confirmed by both API and PCR (Figure 1). Selectivity of each medium after 48 hr incubation were 55% mCCDA, 45%

Campy-cefex, and 24% Skirrow's medium (Table 4). No further colonies were detected beyond 48 hr incubation. The differences in performance of both mCCDA and Campy-cefex compared to Skirrow's proved to be statistically significant ($P \leq 0.01$ and $P \leq 0.05$, respectively). The performance of the different culture media was not influenced by the type of biological specimens tested. Table (4) also shows the semi-qualitative growth of *Campylobacter* from specimens on each of the selective media.

The mCCDA yielded the greatest number of *Campylobacter jejuni* isolates, and was proven to be more selective in inhibiting overgrowth of contaminants (Table 5). On this medium, most of the plates were contaminated. Campy-cefex also yielded good isolation rates; however, it was less efficient in suppressing the growth of contaminants. On the other hand, Skirrow's medium allowed abundant growth of contaminants making examination of screening plates very cumbersome. An index of the performance (PI) for each of the selective media has been calculated as the ratio of the total number of plates yielding *Campylobacter* to the total number of plates showing contamination. The calculated PI index for each medium were: Skirrow's 0.25, Campy-cefex 0.47, and mCCDA 0.59.

Table 3. Isolation of *C. jejuni* from various chicken specimens.

Source	No. specimen tested	No. Positive samples	No. Positive on the tested media		
			Campy-cefex	mCCDA	Skirrow's
Skin rinse	35	22*	18	21	12
Liver	27	10*	7	10	2
Feces	38	25*	20	24	10
Total	100	57*	45	55	24

* = Some specimens were positive in one media only.

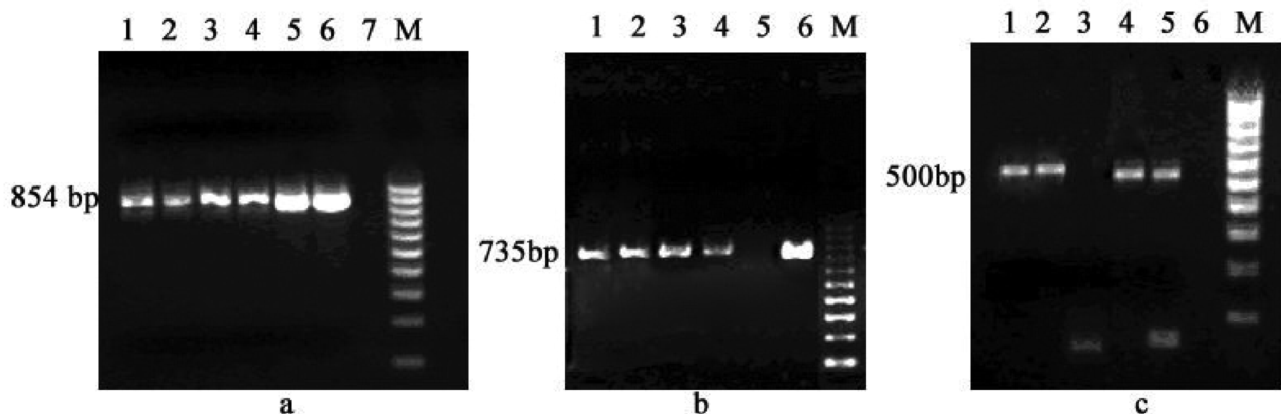

Fig. 1. Selected results from PCR tests.

Fig. 1a and Fig. 1b test for *C. jejuni* products size 854 and 735bp. Lane 1, 2 faecal sample; 3, liver sample; 4 -5 skin rinse; lane 6 positive control (ATCC 33291); 7 negative control.
Fig. 1c test for *C. coli* product size 500 bp. Lane 1 - 4 faecal samples (lane 3 negative sample); 5 positive control (*C. coli* CCUG 11283); 6 negative control.

Table 4. Semi-qualitative growth of *Campylobacter* isolated after 48 hr incubation.

Growth	Numbers of specimens showing growth of *Campylobacter*		
	Skirrow's	Campy-cefex	mCCDA
+++	5	17	30
++	3	10	15
+	6	14	8
+/-	10	4	2
Total	24	45	55

+++ = Growth over all the inoculated area.
++ = Growth over two thirds of the inoculated area.
+ = >10 colonies on the primary inoculated area.
+/- = < 10 colonies on the primary inoculated area.

Table 5. Semi-qualitative analysis of growth of contaminants from all the collected specimens.

Growth	Media (No. of specimens showing the observed growth)		
	Skirrow>s	Campy-cefex	mCCDA
+++	17	0	0
++	36	13	0
+	19	29	23
+/-	25	53	71
Total	97	95	94

+++ = Growth over all the inoculated area.
++ = Growth over two thirds of the inoculated area.
+ = >10 colonies on the primary inoculated area.
+/- = < 10 colonies on the primary inoculated area.

DISCUSSION

As food safety has become an increasing concern for consumers, there is a growing need for fast and sensitive methods for specific detection and identification of zoonotic microorganisms. Infections caused by *Campylobacter* is considered a main Public health problem in many developed countries (Heuer, *et al*. 2001). Since isolation of *C. jejuni* is essential for food monitoring and clinical diagnosis, it is important to study the effectiveness of different culture media for isolation of the organisms. Several agar plates have been modified for the recovery of *Campylobacter* species from poultry samples (Oyarzabal, *et al*. 2005). Though, few have been used for direct enumeration of *Campylobacter* species from poultry carcass rinse. In this study three selective media were compared, charcoal-based selective media, modified charcoal cefoperazone deoxycholate agar (mCCDA), and two blood-based media (Skirrow's [SKM] and Campy cefex media).

The purpose of this evaluation was to determine, which single medium performed most satisfactorily, when tested under the same conditions. Culture plates were incubated under the same conditions of time, temperature and atmosphere. Therefore, the differences in selectivity between the three media can be attributed to the influence of their major components: basal medium, growth promoting additives and inhibitory supplements. Skirrow's and Campy-cefex's media have the same basal base but different antibiotics. On the other hand mCCDA media has different growth promoting

additives and inhibitory supplements. Skirrow's medium lacked selectivity, heavy growths of *Proteus* species interfered with the isolation of *Campylobacter.* Our finding shows that Campy-cefex medium was more selective than Skirrow's in isolating *Campylobacter*, but it exhibited more contaminants that interfered with the appreciation of *Campylobacter* colonies. Although, Campy-cefex, and Skirrow's media contain multiple antibacterial compounds (polymyxin and trimethoprim, selective against Gram negatives; and vancomycin, selective against Gram positives), they did not perform as effectively as the mCCDA medium.

The mCCDA, which contains cefoperazone, yielded better isolation rates, with more selectivity than the other two media which was consistent with other previous studies (Oyarzabal, *et al*. 2005; Merino, *et al*. 1986). On the other hand, the presence of blood in Skirrow's and Campy-cefex, favored the growth of other confounding organisms. Therefore, the antimicrobial substances contained in the media are not the only factor in the successful isolation of *C. jejuni* and *C. coli*. Our findings are consistent with those of Oyarzabal, *et al* (2005), that charcoal-based medium (mCCDA) is the best medium for enumeration of *Campylobacter* species from poultry carcass rinse. Our findings were also consistent with the results of previous study made by Engberg, *et al*. (2000), in which mCCDA was found to be as effective as Skirrow and Campy-cefex media in recovering thermophilic *Campylobacter* species, but was much more selective than the other media. In addition, mCCDA produced significant suppression of other organisms, and has the highest performance index, as reported by Karmali, *et al*. (1986) and Bolton, *et al*. (1986). However, we suggest that by adding another media such as Campy-cefex to the culture protocol would increase chances of isolations. Thus, in our setting and in similar low resources communities we recommend the use of two culture media for processing food specimens of poultry origin. The contamination rate of Campylobacter from broiler chicken in our setting is considered significant. Education and increasing awareness about it is a major public health challenge.

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