Comparative Study Between Traditional Methods, Commercial Biochemical Test and PCR for Identification of Campylobacter Species Isolated from Poultry Farms


(1) Bacteriology, Immunology and Mycology Department, Faculty of Veterinary medicine, Suez Canal University
(2) Animal Health Research Institute, Mansoura Lab. Agriculture Research Center (ARC)
(3) Animal Reproduction Research institute.

Abstract:
Campylobacter spp. causes severe issues in chickens. In this investigation. Campylobacter species were isolated (182) in Dakahlia Governorate's from 440 broilers and 440 ballade breeds. Campylobacter coli 42 isolates (4%) and Campylobacter jejuni 140 isolates (16%). For C. jejuni, rates of recovery from various internal organs were 60%, 24%, 10%, and 5%, from the cloacal swab, heart, liver, and gizzard respectively. The morphological studies revealed that the majority of species from Campylobacter are motile, survive at 37-42°C, and are G-ve, slender spirally curled. They require 5%O2, 85%N2 and 10%CO2 to create a colony that looks like a dew drop on mCCDA media. By using API20, the suspected isolates were identical to typical Campylobacter

Keywords: Campylobacter jejuni, C. coli, Poultry, API 20E and PCR.

Introduction:
Campylobacter spp. were acknowledged as among the most frequent causative organisms of enteritis and, human gastroenteritis (Caprioli A et al., 1996). Campylobacteriosis cases now outnumber those brought on by traditional intestinal bacteria. The rate of detection of Campylobacter spp. from patients with infections of alimentary tract was 3-4 times higher than Escherichia coli and Salmonella (EFSA, 2017). Campylobacter infection rates have risen recently (Platts et al., 2014).
Family Campylobacteriaceae includes 22 species, where C. coli and C. jejuni are the primary cause of human gastroenteritis, despite other, "developing" species like C. concisus, C. hyointestinalis, C.
upsaliensis, C. sputorum and C. ureolyticus, were responsible for gastroenteritis and periodontitis (Fitzgerald and Nachamkin, 2011). Typically, diverse animal species, whether domestic or wild, become colonised by Campylobacter species, which are also present in food of animal origin (Man, 2011).
Campylobacter species could grow at temperatures ranged from 37° - 42° C with a pH ranged from 6.5 - 7.5. So they defined as "thermophilic". However, (Levin 2007) reported that these bacteria do not exhibit a true thermophilia and cannot thrive at temperatures equalled to or above 55° C, they are more appropriately referred to as "thermotolerant."

Aim of the work:
This work was done to identify and isolate Campylobacter spp. from poultry with traditional, commercial biochemical tests and using PCR to identify the most reliable Campylobacter Spp isolated from poultry.

Material and Methods: -
For isolation and identification of Campylobacter species, cloacal swabs and organ samples (heart, gizzard, and liver) from 110 apparently healthy Ballade breeds and 110 broilers chicken’s samples from poultry farms located at Dakahlia Governorate were collected. To avoid cross contamination, all samples were handled aseptically with sterile sampling equipment. and promptly carried in an ice box to the lab. for further bacteriological examinations. In sterile tubes, a loopful of each sample was cultivated for 24 to 72 hours onto Thioglycollate broth medium, Then, a loopful from each tube was streaked on a special antibiotic-infused Blood-free selective medium for Campylobacter. The inoculated plates were kept in anaerobic jars for, with kits that generate CO2, O2, and nitrogen at a temperature of 37 °C for 48 hours, and the motility of the bacteria was then observed under a phase contrast microscope.
The suspicious colonies were then purified on blood agar medium supplied defibrinated sheep blood for 24 hours. Oyarzabal and Battie (2012) suspected colonies were carefully examined for their morphological characteristics according to ISO (2006).

Biochemical identification:
A. Catalase production test
A positive results represented by bubbles formation due to release of O2 from the H2O2 in the presence of catalase. (C. jejuni, C. coli) (ISO, 2006).
B. Hydrogen sulphide production: (Bailey & Scott’s.2007)
Blackening of the medium mean H2S production.
C. Hippurate hydrolysis test. used to distinguish between C. jejuni and C. coli commercial kits (API20E):
API 20 E is a standard kit that is designed for the identification of bacteria that belong to the family *campylobacteriaceae* and it cannot be used to detect the absence or presence of any other microorganisms. Identification is obtained with the numerical profile.

**Determination of the numerical profile:**
The tests are divided into groups of three on the result sheet, and a value of 1, 2, or 4 is listed for each. By summing the values corresponding to favorable responses in each group, for each of the 20 tests of the API 20 E strip, a 7-digit profile number is obtained. The oxidase reaction makes up the twenty first test and, in the event that it is affirmative, has a value of 4. Finally, these seven-digit profile number for each strip were used for identification with identification software or the analytical profile index. In some cases, this seven-digit profile number was not enough and supplementary test need to be carried out by nitrate reduction and used to be formed eight-digit profile number.

**Molecular detection of campylobacter spp. common genes:**
Conventional PCR assay for *Campylobacter* isolate confirmation was performed. DNA was obtained through a QIA amp DNA mini kit (Germany). An Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit. was used to prepare the PCR Master Mix also the cycling conditions for primers during PCR. In PCR, oligonucleotide primers with specific sequences amplify a particular product. are shown in Table (1) and the cycling conditions of each primer are showed in table (2).

**Table (1). Sequences of oligonucleotide primers.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primersequence (5'-3')</th>
<th>Lengthof amplified Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rRNA</td>
<td>TATACCGGTAAAGGAGTGCTGGAG 650bp</td>
<td>Wang et al., 2002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCAATTAACCTTCGAGCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>GAA GAG GGT TTG GGT GGT G 735bp</td>
<td>Al Amri et al., 2007</td>
<td></td>
</tr>
<tr>
<td><em>hipO</em></td>
<td>AGC TAG CTT CGC ATA ATA ACT TG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>GGT ATG ATT TCT ACA AAG CGA G 500bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>asp</em></td>
<td>ATA AAA GAC TAT CGT CGC GTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table (2) Primers cycling conditions during cPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primary denaturation</th>
<th>Secondary Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>23SrRNA</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>55˚C 40 sec.</td>
<td>72˚C 45 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td>C. coli (asp)</td>
<td>94˚C 5 min</td>
<td>94˚C 3 sec.</td>
<td>49˚C 40 sec.</td>
<td>72˚C 45 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td>C. jejuni (hipO)</td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>55˚C 40 sec.</td>
<td>72˚C 50 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
</tbody>
</table>

**Results:**

**Prevalence of Campylobacter spp in poultry:**

A total of 182 Campylobacter spp. isolates were recovered out of 880 samples. The isolation rate of C. jejuni was 14% in broilers (65/440) and 17% in ballade breeds (75/440); this show that C. jejuni in ballade breeds was higher than in broilers breeds. The isolation rate of C. coli was 5% in broilers (22/440) and 4.5% in ballade breeds (20/440). According on the organ of sampling, there was a statistically significant variation in the presence of Campylobacter in chickens. The highest rate of C. jejuni and C. coli from different organs recovered from cloacal swaps followed by the heart, liver then gizzard in both broiler and Ballade breeds (Table 3 and 4).

**Identification of Campylobacter isolates:**

On charcoal-based surfaces like mCCDA, the typical colonies have a tendency to spread and are greyish, flat, wet, and may have a metal sheen is characteristic for C. jejuni, while C. coli appear as colonies of a creamy-grey, wet, and more distinct form. The isolated colonies were catalase, H₂S positive, while Hippurate was positive with C.jejuni and negative in C.coli

**Result of commercial kits (API20E):**

As shown in figure (1) and (2)

**Molecular confirmation of campylobacter DNA**

Ten isolates were subjected to molecular examination for common gene (23srRNA). The results showed the presence of campylobacter DNA in the 9 isolates by using 23srRNA gene at 650 bp with percent 100% as detected in fig (3). C.jejuni and C.coli that previously identified by morphological methods were confirmed by specific primers for both species (hipO and asp respectively) as shown in figure (4,5).
Table (3) Recovery pattern of C. jejuni from positive samples

<table>
<thead>
<tr>
<th>Type of Birds</th>
<th>Number of +ve samples</th>
<th>Cloacal swab NO %</th>
<th>Heart NO %</th>
<th>Liver NO %</th>
<th>Gizzard NO %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broilers</td>
<td>65</td>
<td>40</td>
<td>61</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Ballade</td>
<td>75</td>
<td>44</td>
<td>58</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>84</td>
<td>60</td>
<td>34</td>
<td>24</td>
</tr>
</tbody>
</table>

Table (4) Recovery pattern of C. coli from positive samples

<table>
<thead>
<tr>
<th>Type of birds</th>
<th>No of +ve samples</th>
<th>Cloacal samples NO %</th>
<th>Heart NO %</th>
<th>Liver NO %</th>
<th>Gizzard NO %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broilers</td>
<td>22</td>
<td>9</td>
<td>40</td>
<td>8</td>
<td>36</td>
</tr>
<tr>
<td>Ballade</td>
<td>20</td>
<td>10</td>
<td>50</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>19</td>
<td>45</td>
<td>13</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure (1) Biochemical identification of C. jejuni by using API20E
Figure (2) Biochemical identification of C.Coli by using API20E

Figure (3) detection of 23srRNA gene (common gene) at 650 bp

Figure (3) 23Sr RNA primer-based amplification of a 650 bp fragment on an agarose gel. (common gene of campylobacter, L: 100-1000bp ladder, Lane (pos.): Positive control (Mycoplasma). Lane (neg.): Negative control (Saline), and Lane (1and 3-10): Positive samples (Campylobacter spp.)
Figure (4) detection of *asp* gene was presented in all campylobacter coli isolates with percentage (100%)

Figure (4) Agarose gel electrophoresis demonstrating *asp*-based 500 bp fragment amplification. (specific gene of *C. coli*) L: 100-1000bp ladder.
Lane (pos.): Positive control (*mycoplasma*). Lane (neg.): Negative control (Saline).
Lane (1,4,5,7 and 8): Positive samples (*Campylobacter coli*.)

![Agarose gel electrophoresis demonstrating *asp*-based 500 bp fragment amplification.](image)

Figure (5) detection of *hipO* gene was presented in all campylobacter Jejuni isolates with a percentage (100%)

Figure (5). Agarose gel electrophoresis showing amplification of 735bp fragment using *hip O* primer. (Specific gene of *C. jejuni*)
L: 100-1000bp ladder.
Lane (pos.): Positive control (*mycoplasma*). Lane (neg.): Negative control (Saline).
Lane (3,6,9 and 10): Positive samples (*Campylobacter jejuni*.)

![Agarose gel electrophoresis showing amplification of 735bp fragment using *hip O* primer.](image)

**Discussion:**
According to epidemiological research, handling and consuming raw or undercooked poultry items can cause up to 50–80% of all human *Campylobacter* infections. A 1 Log10 CFU/g decrease in the quantity of *Campylobacter* was found on carcasses could potentially cut the danger to the public health by 50 to 90%.

Similar to the findings of a surveillance research conducted in England and Wales, compared to *C. coli*, *C. jejuni* was discovered in more than 12 times the number of instances of human illness according to *Friedman et al. (2000)*. According to *Gillespie et al. (2002)*, who established comparable findings to those of Friedman et al. (2000), 93% of human sickness was caused by *C. jejuni*, and the majority of the
remaining cases were caused by *C. coli*. Because of this, the majority of research and studies have looked at the prevalence and physiological traits of *C. jejuni* and *C. coli*, two representative *Campylobacteraceae* organisms.

The present study was comparable to the outcome reported by *(Nahed et al., 2021)* 20% and 17.75%, respectively, *C. jejuni* were found in of the intestinal contents of laying and broiler chickens, this predominance may be related to the fact that several *Campylobacter* species are thought to be more prone to the digestive system of hens, particularly the caecum and colon *(Jokinen et al., 2011)*.

**Conclusion:**
Poultry meat is the leading source of animal protein for human consumption in many countries. Biochemically identified *Campylobacter* were molecularly confirmed by the amplification of 23S rRNA(common gene of campylobacter, *hipO* gene (specific gene of *C. jejuni*), *aspgene* for *C. Coli*

**References:**

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**European Food Safety Authority (EFSA), (2011):** EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. EFSA Journal 9(4):2105.


الملخص العربي

الكامبيلوباكتر هي مشكلة صحية هامة وتحديا كبيرا في جميع انحاء العالم. يتم التعرف على أنواع الكامبيلوباكتر كسبب للاسهال حيث أن ميكروب الكامبيلوباكتر موجود في الجهاز الهضمي في الدواجن ويتنقل إلى الإنسان بسبب الأسهال وتتعرج الدم والتهاب المفاصل ومتلازمة غيلان باريه والاضطرابات العصبية. تعتبر الدواجن من المصادر الهامة للعدوى البشرية الدواجن الكامبيلوباكتر هي سلالة الجرام حزازونية لها سياق أحادي أو ثنائي وتضم أكثر من 10 أنواع أكثرها شيوعا كامبيلوباكتر كولي وكامبيلوباكتر جيجوني. يمكن أن تقلل من انتشار استعمار الكامبيلوباكتر بالقرب من عمر السوق من 40 إلى 80٪ من خلال الحواجز الفيزيائية والكيميائية. يمكن أن يساهم تنظيف وتطهير بيوت الدواجن بين القطعان بشكل فعال في الحد من استعمار الكامبيلوباكتر. علاوة على ذلك، التدابير الصحية الصارمة والقيد العامة على الأمن البيولوجي.

وذلك تهدف هذه الدراسة إلى تحديد الإصابة بهذا المرض الضار وكيفية عزل الميكروب المسبب له ولتحقيق هذا الهدف قد تم القيام بهذه الخطوات:

1- تم عزل 182 عطرة من الكامبيلوباكتر من 440 دجاجة من بداري التسمين و440 دجاجة بلدي من محافظة الدقهلية بنسبة 16% (140) كامبيلوباكتر كولي.
2- أظهرت معدل عزل الكامبيلوباكتر من الأعضاء الداخلية المختلفة بنسبة عالية من محتويات الأمعاء الداخلية والقلب والكبد والقونصة بنسبة 60% و24% و10% ثم 5% للكامبيلوباكتر جيجوني وابضا بنسبة 45% و30% و14% ثم 4% لكامبيلوباكتر كولي.
3- أظهرت نتائج التصنيف أن أنواع الكامبيلوباكتر المعزولة من الدواجن تاجد كامبيلوباكتر كولي بنسبة 16% ممثلة في 140 عينة وكامبيلوباكتر جيجوني بنسبة 4% ممثلة في 42 عينة.
4- تم إجراء الاختبارات المورفولوجية التي اثبنت أن ميكروب الكامبيلوباكتر سلالة الجرام أسطوانية وغالبيتها متحركة تنمو عند درجة حرارة 37-42 درجة مئوية وتحتاج 15% أكسجين و10% ثاني أكسيد الكربون و85% نيتروجين و10% ثاني الأكسجين.
5- تم إجراء تفاعل البلمرة المتسلسل باستخدام البادئ المستقبلي المستخدم للجينات المختصر لكل جين عن جينات الضراوة (hipO&asp) وتم في البداية أجراء اختبار تفاعل البلمرة المتسلسل باستخدام البادئ العام تكشف عن جين 23SRN.