Evaluation of Different Methods in Detection of Campylobacter Infection in Poultry

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A total seventy six cloacal swabs were collected from different poultry species (spp.) (chickens, turkeys, ducks and pigeons) from different governorates in Egypt and examined to determine the presence of Campylobacter spp. Results revealed that 22 (27.6%) of cloacal swabs were positive for Campylobacter spp. after culture and biochemical tests. A higher rates of bacterial isolation were detected in chicken (40%) and pigeon (38.5%), the predominant isolated species were Campylobacter coli (C. coli) (54.6%) and Campylobacter jejuni(C. jejuni) (45.4%). Multiplex real time Polymerase Chain Reaction (mrtPCR) confirmed that 58(76.3%) of the 76 samples and also the predominant spp. was C. coli, and Ag capture ELISA showed 65(85.5%) out of 76 samples were positive for Campylobacter spp. The isolation using standard method is a gold tool for enumeration and obtaining viable cells but it is consuming longer time. mrt-PCR not only able to detect Campylobacter spp. but also was species specific and rapid test, while ELISA was rapid and more sensitivity than both methods.

Keywords: Campylobacter, spp., mrt-PCR, ELISA, Poultry spp, Isolation

INTRODUCTION

The genus Campylobacter has great importance in public health because it may cause diarrhea and human Campylobacteriosis. (Jamshidi et al. 2007),Campylobacteriosis is a self-limiting infection which characterized by a rapid onset of fever, severe abdominal cramps, and diarrhea that may include blood and leukocytes, with symptoms becoming apparent within 1- 7 days after consumption of the contaminated food or liquid(Robinson1981).C.jejuni is the most commonly found species which responsible for approximately 90% of Campylobacter infections in humans, C.coli was considered the second most predominant species and represented as 5–10% of the infections (Gillespie et al.2002). Also co-infections with multiple Campylobacter species in humans have been reported as well (Richardson et al.2001).The EU(European union) notify that the rate of infection was increased by 10% in 2014, compared with the previous year, and a statistically significant increasing trend was observed in the 7-year period from 2008 to 2014 (EFSA, 2015).The main reservoir of Campylobacter was poultry spp. which colonizes in small intestines of live poultry, including broiler chickens, turkeys, and ducks (Newell and Fearnley 2003). 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 (Logan et al. 2001).

Thus Phenotypic methods routinely used for Campylobacter detection often resulted in unreliable identification, requiring further testing, and need several days Therefore, interest has been growing in the development of molecular approaches to allow sensitive, unambiguous, and rapid detection and identification of
Molecular differentiation between Campylobacter spp

Campylobacter in retail poultry products and in samples from poultry processing (Melero et al. 2011). The molecular method especially quantitative (rt-PCR) is carried out to identify and discriminate between isolates of C. jejuni and C. coli (Toplak et al. 2012).

Also (Bailey et al. 2008) detected Campylobacter antigen using The TECRA Campylobacter Visual Immunoassay (CAMVIA) protocol by enriching the poultry meat sample in TECRA Campylobacter enrichment broth followed by an ELISA and compared the result with a conventional cultural method due to high importance of Campylobacter spp. in public health so our study aimed to compare Campylobacter spp. detection by molecular method mrt-PCR and Ag capture ELISA and the culture method. So we described a fast and reliable test for rapid and sensitive detection of Campylobacter spp. in poultry spp.

MATERIALS AND METHODS

Samples
A total of 76 cloacal swabs were collected from diarrheal poultry spp. (chicken, turkey, pigeon and ducks), the swabs were collected on transport media using Cary-Blair Medium; (Oxoid). All samples were kept refrigerated during transport to the laboratory and culture was performed immediately after reception.

Bacterial isolation and identification
The detection and identification of Campylobacter species in all samples were performed according to ISO 10272-1 2006 using Bolton broth; (Oxoid) as enriched and CCD agar and Karmali agar; (Oxoid). As a selective media.

Method for Ag capture ELISA (safe pathcataloge code; CYF-96)
Preparation of the samples: boiling Bolton broth containing samples for 10 minutes and allow cooling before testing. Add 100ul of negative control to well #1 and 100ul of positive control to well #2. Add 100ul of tested sample to the appropriate well Incubate at room temperature (15 to 25°C) for 30 minutes, then wash for 3 times. After last wash, slap the wells out on a clean absorbent towel, Add 2drops of enzyme conjugate (red solution) to each well. Incubate for 15 minutes then wash for 3 times. Add 2 drops of chromogen to each well. Incubate for 5 minute, Add 2 drops of stop solution, read the results visually or at 450/620-650nm using ELISA reader.

Extraction of DNA
DNA from 76 cloacalswabs were extracted using a QIAamp DNA mini kit (Qiagen) according to the instructions. The DNA obtained was stored at -20 °C.

mrt-PCR protocol.
Component of Master Mix 1-2x QuantiTect Probe RT-PCR Master Mix (12.5 μl), forward primer (50 pmol) (0.5 μl), reverse primer (50 pmol)( 0.5 μl), probe (30 pmol)( 0.125 μl), RNase Free Water(4.125 μl) and template DNA(5 μl).

Three genes were targeted in the multiplex PCR protocol, namely 16S rRNA (Campylobacter spp. gene), hipO (hippuricase gene for C. jejuni) and orfA (C. coli). The primer sets used, as described in table [1].

Table No (1) oligonucleotide primers and probes used in real time PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>CTGCTTAACACAAAGTGGTAGGG</td>
<td>Lübeck et al, 2003</td>
</tr>
<tr>
<td></td>
<td>TTCCTTAGGTACCGTCAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-FAM-TGTCATCCTCCACCACGGCGT \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \</td>
<td></td>
</tr>
<tr>
<td>C. jejunihipO</td>
<td>5′- TGG TGC TAA GGC AAT GAT AGA AGA -3′</td>
<td>Benson et al, 2002</td>
</tr>
<tr>
<td></td>
<td>5′-TGA CCA CCT CTT CCA ATA ACT TCA -3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′ HEX – AAC TAT CCG AAG AAG CCA TCA TCG CAC CTT – BHQ-1-3′</td>
<td></td>
</tr>
<tr>
<td>C. coli orfA</td>
<td>5′- GCA CTC ATC CAA TAC TTA CAA GA-3′</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS
Table (2) illustrated comparison of Campylobacter spp. detection by molecular method (mrt-PCR) and by Ag capture ELISA to the culture method.

The results showed that 22 out of 76 samples were identified as Campylobacter spp. by standard methods of isolation, the percentage of isolation was 40%, 38.5% and 6% respectively in chicken, pigeon and duck while turkey showed 0% for isolation of Campylobacter.

Out of 76, 65 samples were identified as positive Campylobacter spp. by ELISA test. The percentage of Campylobacter Ag detection in chicken was 100% in pigeon was 73.1% , in duck was 82.3% and in turkey was 87.5%. While by mrt-PCR, 58 out of 76 were identified as Campylobacter spp. The percentage of detection in chicken, pigeon, duck and turkey were 88%, 73.1%, 58.8% and 87.5 respectively.

Ag capture ELISA test was more sensitive than mrt-PCR test and both were more sensitive than culture method with percentage (85.5%, 76.3% and 27.6%) respectively. While mrt-PCR more specific than culture method. The Campylobacter spp. identified by culture were 10 (45.4 %%), 12 (54.4 %) C. coli, while mrt-PCR revealed 17 (29.3%) C. jejuni, 33 (56.9%) C. coli, (6.1%) specimens had mixed infection (C. jejuniand C. coli) and also 4/58(6.1%) untying. The Campylobacter spp. was identified by culture and multiplex PCR while The Ag capture-ELISA test did not allow differentiation between C. jejuniand C. coli

Table (2): Comparison between different methods for diagnosis of campylobacter spp using (standard method, Ag capture ELISA and mrtPCR)

<table>
<thead>
<tr>
<th>Type of bird</th>
<th>Standard method</th>
<th>ELISA</th>
<th>mrtPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of positive samples</td>
<td>%</td>
<td>No of positive samples</td>
</tr>
<tr>
<td>Chicken</td>
<td>10/25</td>
<td>40%</td>
<td>25</td>
</tr>
<tr>
<td>Pigeon</td>
<td>10/26</td>
<td>38.5%</td>
<td>19</td>
</tr>
<tr>
<td>Duck</td>
<td>1/17</td>
<td>6%</td>
<td>14</td>
</tr>
<tr>
<td>Turkey</td>
<td>0/8</td>
<td>0%</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>22/76</td>
<td>27.6%</td>
<td>65/76</td>
</tr>
</tbody>
</table>

The best methods for identification of Campylobacter spp was mrt-PCR.

DISCUSSION
Campylobacter species, especially C. jejuniand C. coli, are a major cause of human bacterial enteritis. In our mrt-PCR protocol, three genes, namely 16S rRNA (specific for Campylobacter spp), hipOand orfA (species specific for C. jejuniand C. coli, respectively), (Ivanova et al. 2014) indicated that the molecular detection of Campylobacter is equally reliable in chicken, pigeon, duck and turkey were 88%, 73.1%, 58.8% and 87.5 respectively.

Culture provided the possibility of C. jejuni and C. coli discrimination. Also (Sternet et al. 1992) confirmed that the hipOgene is highly conserved in C. jejuni strains and represents the most widely validated gene for the identification of C. jejuni.

Our results showed that out of the 76 cloacal swabs collected from different poultry spp, 22

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(27.6%) were positive by culture and 58 (76.3%) were positive by a molecular method (mrt-PCR) and 65 (85.5%) were positive in Ag capture ELISA test. The *Campylobacter* spp. identified by culture were 10 (45.4%) C. jejuni, 12 (54.4%) C. coli, while mrt-PCR revealed 17 (29.3%) C. jejuni, 33 (56.9%) C. coli, 4 (6.1%) samples had mixed infection (C. jejuni and C. coli) and 4 (6.1%) untyping isolates. Our result nearly closer to (Schnider et al., 2010) who recorded that (71.5%) of the samples were PCR positive for *Campylobacter*.

The same result recorded by (Poelzler et al., 2011) who recorded that the *Campylobacter* spp. prevalence from 61% (quantitative culture) to 71.7% (mrt-PCR) in Austria. (Mayr et al., 2010) established that the real-time PCR assay, 55.4% of the samples were recognized as positive for thermophilic *Campylobacter* spp. where with the conventional method only 40.3% of the samples were positive. Although (Boer et al., 2015) confirmed that the molecular *Campylobacter* spp. detection was the highest incidence of *Campylobacter* spp. in poultry samples (~90%) and the species specific PCR showed the simultaneous presence of C. jejuni and C. coli in ~24% of the samples. (Randall et al., 2010) compared culture to PCR assay detected 45 to 47 positives, who confirmed that the PCR has sensitivity 93.6% and specificity 80.1%; however, it had the advantage over culture in that it could detect mixed infections of C. coli and C. jejuni from the infected cecal samples which by culture would have been more laborious and required multiple colonies also was capable of giving a result for a sample within a day. In spite of (Lund et al., 2004) showed no statistically significant difference in performance between real-time PCR and culture by selective enrichment.

(He et al., 2010) and (Mayr et al., 2010) the multiplex qPCR assay combined with an enrichment step is a sensitive, species-specific, and non-labor-intensive method suitable for rapid detection of C. jejuni, C. coli, and C. lari in chicken samples. (Arnold et al., 2015) reported that the sensitivity of the culture method was higher than that of PCR in detecting both species when the samples were derived from populations infected with at most one species of Campylobacter. However, from a mixed population, the sensitivity of culture for detecting both C. jejuni or C. coli is reduced while PCR is potentially able to detect both species, although the total probability of correctly identifying at least one species by PCR is similar to that of the culture method.

Polymerase chain reaction inhibitors as heme degradation products, such as bilirubin, as well as bile salts, polysaccharides, and large amounts of irrelevant DNA (Lund et al., 2003) are substances that inhibit enzyme activity. They are present in many biological samples and can limit the advantages of PCR detection. Various strategies can be applied to circumvent the problem of inhibitors; for example, optimization of the DNA isolation procedure to exclude inhibitors prior to the amplification and use of Taq DNA polymerase with a high capacity for amplification in the presence of the actual inhibitors (Lund et al., 2003). We found that the inhibitory removal solution present in the commercial DNA isolation kit performed very well with fecal samples. Combined with amplification using a high-yield Taq DNA polymerase containing glycerol that neutralizes the effect of inhibitors (Nagai et al., 1998), the problem of inhibitors in fecal material was greatly reduced in PCR.

Our results nearly closer to. (Bailey et al., 2008) who used Ag capture ELISA for detection of *Campylobacter* from poultry carcasses. A total of 350 carcasses (88%) were found to be positive for *Campylobacter*. Also (Zaghoul et al., 2012) reported that the sensitivity of ELISA was 95.6% and the specificity was 98.1. So ELISA was rapid and had acceptable performance sensitivity.

Also (Bessède et al., 2011) had reported that the specificities and negative predictive values (NPV) of the different methods were all in the range of 95 to 100% while the sensitivity of ELISA was higher than ImmunoCard Stat Campy and multiplex PCR it was (96%, 92% and 88.7% respectively).

**CONCLUSION**

Ag capture ELISA was rapid and more sensitivity than mrt-PCR and isolation, but the multiplex RT-PCR is more specific than both culture and Ag capture ELISA test.

**CONFLICT OF INTEREST**

The authors declared that present study was performed in absence of any conflict of interest.

**ACKNOWLEDGEMENT**

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AUTHOR CONTRIBUTIONS
MM: collected the samples, Ag capture ELISA and wrote the paper. EA: bacterial isolation and identification. OM: mrt-PCR. MI and SN: group supervisors and reviewed the results. All others read and approved the final version.

REFERENCES


