Alexandria Journal of Veterinary Sciences 2016, Apr. 49 (2): 141-148
ISSN 1110-2047, www.alexjvs.com
DOI: 10.5455/ajvs.219057

Comparison between Bacteriological Isolation and Molecular Detection of *E. coli* from Chickens Suffering from Colibacillosis and/or Diarrhea

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ABSTRACT

In the current study, we compared the efficiency of PCR detection to culture-dependent isolation of *E. coli*, as a model, from the liver and intestinal contents. Further, the incidence differences of *E. coli* isolation from the liver and intestine. Samples were collected from birds suffering from respiratory manifestation and/or diarrhea. Ninety *E. coli* isolates were recovered from 60 birds (52 intestinal and 38 liver samples) by bacteriological culture on selective broth and selective agar. PCR was performed using *pho-A* gene as a general marker for *E. coli* on DNA directly purified from the samples and assigned PCR (a). Negative PCR (a) samples were cultured on broth and another PCR was done, assigned PCR (b). Bacteriological isolation was more sensitive than PCR (a) indicating that inhibitors in the samples could have reduced or totally blocked the amplification capacity of PCR (a), which limited its diagnostic usefulness. PCR (b) was more sensitive than PCR (a) and more practical than bacteriological isolation. Detection of three virulence genes; *iut-A, iss* and *tsh*, showed genotypic variations of avian pathogenic *E. coli* (APEC) isolated from the liver and the intestine. In conclusion, an enrichment step results in more sensitive PCR than culture performed one. Further, general genes rather than virulence genes should be used as an indicator for *E. coli* detection in infected materials.

Key words: *E. coli*, PCR, *pho-A*, colibacillosis

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1. INTRODUCTION

Although microbial isolation progress results in advancement of the detection methods, sensitivity and specificity are still not sufficient and direct application to sample is still not practical *(Scholl et al., 1990)*. Low culture sensitivity is usually unable to estimate a low microbial load especially without a pre enrichment step *(Malorny & Hoofer, 2005)* and cannot distinguish bacteria on species level *(Kreader, 1995)*.

Culture identification of some bacteria may require several days and longer depending on the target microorganisms. Adding to that, the possible contamination that may interfere with microbial isolation, even with selective media. Further, rapid detection of microbes especially those of epidemiological significance is important to overcome time and costs of multiple cultures and confirmation. Detection of pathogenic *E. coli* by culture depending on its produced toxin makes their identification a laborious work that decreases the likelihood of finding the pathogenic among the majority of non-pathogenic isolates *(Wernars et al., 1991)*. Moreover, no biochemical markers exist to easily differentiate these isolates *(Sandrina et al., 1995)*. Serotyping remains the most frequently used diagnostic method in different laboratories, with only limited identification *(Catherine et al., 2012)*. Furthermore, antibiotics that are incorporated into selective media may inhibit the less commonly encountered serotypes. Cells may exist in a variety of metabolic states that may be unsuitable to culture *(Lawsoni et al., 1998)* and other bacteria can remain viable but in a non-cultivable form and storage conditions may induce revival of other non-target cells, which can hamper the isolation process *(Harkanwaldeep et al., 2011)*. In this study, to compare the efficiency of bacterial detection based on bacterial classical culture techniques to that of direct and enriched PCR, *E. coli*-biochemically positive samples were subjected to PCR directly (PCR (a)) on sample sediments to detect *E. coli* general gene; *pho-A*. Enrichment broth of negative PCR (a) samples were subjected to PCR (PCR (b)). Both positive direct PCR samples' sediments and positive enriched PCR incubated broth were subjected to detect three of the most prevalent

virulence genes of avian pathogenic *E. coli* (APEC); *iss, tsh* and *iut-A.*

2. MATERIALS AND METHODS:

2.1. Bird samples, bacteriological and biochemical Identification:

A total of 120 samples (60 liver and 60 intestine) were collected from 60 broiler chicken bird suffering from respiratory and digestive manifestations (diarrhea, respiratory rales, tracheitis, periorbital swelling and fibrinous air sacculits, pericarditis and perihepatitis). The whole liver and 5 cm of the large intestine of each bird were collected. Samples' surfaces were first sterilized (Saba et.al ;(2012) & Azza et al., (2012). From each sample, a G-ve selective broth culture; MacConkey broth, was performed, and then sub cultured on specific agar for E. coli, Levine eosin methylene blue agar (L-EMB), incubated aerobically at 37°C for 24 h and then examined for bacterial growth. Colonies from each sample were subjected to gram staining and biochemical testing as catalase test, urease test and IMViC (Cruickshank et al., 1975).

2.2. Detection of *E. coli phoA* gene and virulence genes by Polymerase Chain Reaction (PCR):

DNA was extracted by phenol-chloroform according to *Sambrook et al., (1989)*. PCR (Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit) was conducted by adding 12.5 µl of Emerald Amp GT PCR master mix (2x premix), 4.5 µl PCR grade water, 1 µl forward primer (20 pmol), 1 µl reverse primer (20 pmol) and 6 µl template DNA to a total volume of 25 µl. Primers used for the detection of the different genes are listed in Table (1). For PCR (a), DNA was extracted directly from 20 samples (10 fecal and 10 liver; one liver and one fecal sample from each bird) to detect *E.coli* general gene, *pho-A*, while for PCR (b), the negative PCR (a) tissue samples (2 intestinal and 3 liver) were bacteriologically broth cultured, and from this broth DNA was extracted and further subjected to *pho-A* gene detection. Both, *pho-A* positive PCR (a) samples and *pho-A* positive PCR (b) incubated cultured broth samples were subjected to the detection of the three selected genes; *iss, tsh* and *iut-A.*

Table (1). Oligonucleotide primers sequences used for PCR.

Gene	Primer Sequence 5'-3'	Amplified product	Reference
phoA	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720 bp	**Hu *et al.* 2011**
tsh	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	620 bp	**Delicato *et al*., 2003**
iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266 bp	**Yaguchi *et al*., 2007**
iutA	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300 bp	

3. RESULTS:

3.1. Incidence of *E. coli* isolation from liver and intestinal samples:

From a total of 120 samples collected from 60 broiler chicken, *E. coli* was confirmed to be positive biochemically in 75% (90/120) of the isolates; 86.6% (52/60) of them were from fecal samples and 63.3% (38/60) were from liver samples. *E. coli* negative samples were 13.4% (8/60) from fecal samples and 36.7% (22/60) from liver samples, with a total negative value of 25 % (30/120) of the isolates as shown in Table (2).

Table (2). Prevalence of *E. coli* isolation from liver and intestinal samples.

Samples	Biochemical tests	
	Positive	Negative
Intestinal samples	86.6% (52/60)	13.4% (8/60)
Liver samples	63.3% (38/60)	36.7% (22/60)
Total	75% (90/120)	25% (30/120)

3.2. Distribution of *E. coli* isolates among the examined birds:

It was found that 34 birds (56.5%) harbored *E. coli* in both organs, four birds (6.7%) were negative for both liver and intestinal *E. coli*, while 18 birds (30%) were positive for *E. coli* detection from the intestine only and 4 birds (6.7%) were positive for *E. coli* detection from the liver but their intestines were negative as shown in Table (3).

Table (3). Distribution of *E. coli* isolates among the examined birds.

Number of birds (%)	*E. coli* isolates	
	Intestinal	Liver
34/60 (56.6%)	+	+
18/60 (30%)	+	-
4/60 (6.7%)	-	+
4/60 (6.7%)	-	-

3.3. Prevalence of *E. coli* detection by PCR from liver and intestinal samples from chicken:

PCR was performed on DNA isolated from the samples' sediments directly to detect *E.coli* general gene; *pho-A*, and assigned PCR (a). As shown in Table (4), 8 out of 10 (80%) intestinal samples were positive for the *pho-A* gene, while, 7 out of 10 (70%) liver samples were positive for the *pho-A* gene. Negative PCR (a) samples were bacteriologically broth cultured, and from this broth, another PCR was done which was assigned PCR (b). The detection of the *pho-A* gene by PCR (b) showed 100% positive results from the negative samples that could not be detected by the first PCR; PCR (a), as shown in Table (4).

Table (4). Sensitivity of *E. coli* detection by PCR from liver and intestinal samples.

Samples	PCR (a)		PCR (b)	
	Positive	Negative	Positive	Negative
Intestinal samples	80% (8/10)	20% (2/10)	100% (2/2)	0% (0/2)
Liver samples	70% (7/10)	30% (3/10)	100% (3/3)	0% (0/3)
Total	75% (15/20)	25% (5/10)	100% (5/5)	0% (0/5)

3.4. Prevalence of individual *E. coli* virulence genes in liver and intestinal samples:

Both positive direct PCR (a) samples sediments and positive PCR (b) incubated cultured broth were subjected to the detection of the three selected genes; *iss*, *tsh* and *iut-A*. As shown in **Table (5)**, *iut-A* gene was found in 100% of both liver and intestinal samples, *tsh* gene was found in 50% of the isolates, and the incidence of *iss* gene was 90%, 100%, 80% in total samples, intestine and liver, respectively.

Table (5). Prevalence of individual virulence genes in liver and intestinal samples.

Samples	Genotype groups		
	A *+iut-A, + iss, +tsh*	B *+iut-A, +iss, -tsh*	C *+iut-A, -iss , -tsh*
Intestinal samples	50%	50%	0%
Liver samples	50%	30%	20%
Total	50%	40%	10%

3.5. Genotype differences of *E. coli* isolates from liver and intestinal samples:

As shown in Table (6), 50% of the samples (50% of both intestinal and liver samples) showed the *+iut-A, + iss, +tsh* genotype; genotype A, of the selected virulence genes. While, 40% of the samples (50% of intestinal samples and 30% of liver samples) showed the *+iut-A, +iss, -tsh* genotype; genotype B. Further, 10% of the samples (20 % of liver samples) showed the *+iut-A, -iss, -tsh*; genotype C. All samples expressed at least one of the examined virulence genes. Genotype similarity of *E. coli* from liver and intestinal samples from the same bird (Table 7) showed that 6 out of 10 birds (60%) harbored similar *E. coli* genotypes in both liver and intestinal samples, while 4/10 (40%) showed different genotypes of *E. coli* isolated from both samples from the same bird.

Table (6). Genotype difference of *E. coli* isolates between liver and intestinal samples:

Genotype	Number (%)
Similar	**6 (60%)**
Different	**4 (40%)**

Table (7): Similarity of *E. coli* isolates from liver and intestinal samples from the same bird.

Samples	Individual Genes		
	iut-A	***iss***	***tsh***
Intestinal samples	100%	100%	50%
Liver samples	100%	80%	50%
Total	100%	90%	50%

4. DISSCUSION:

In our study, bacteriological examination of two samples (liver and intestine) from 60 birds was performed in order to investigate the possible differences in the rate of isolation of E.coli between the liver and the intestine. The biochemically confirmed isolates of E.coli were higher in the intestinal than the liver samples. This may be because feces is a source of both pathogenic and nonpathogenic E.coli, where bacterial isolation alone cannot differentiate between both organisms (Wernars et al., 1991). It is known that E.coli isolated from liver are pathogenic as liver does not contain normal E.coli microflora (Davidoliver, 2003).

The presence of E.coli in the liver without isolation from the intestine in our samples may indicate that both air sacs and lungs can be the portal of entry for E.coli into the systemic circulation, probably via damaged epithelium. And that the digestive system is not only the source of colibacillosis, which agrees with Pourbakhsh et al., (1997) who recovered E.coli from blood and all tested extra-respiratory organs of experimentally infected birds with gradual increase in bacterial counts in trachea, lungs, air sacs, and liver. Dho-Moulin & Fairbrother, (1999) also concluded that experimental infection studies have shown that air-exchange regions of the lung and air sacs are important sites of entry of E.coli into the bloodstream of birds during the initial stages of infection. Birds negative for E.coli isolation from the intestine and positive from the liver as well as birds negative for both liver and intestinal E.coli may have administrated antibiotics on the same day of sampling before laboratory examination that could have affected E.coli survival in both liver and intestine, where growth of microorganisms may be impaired as a result of sublethal cell injuries due to antibiotic therapy (Sethabutr et al., 1994).

PCR eliminates the need for isolation and further biochemical identification, especially with fastidious microorganisms that need unique medium requirement (Bayatzadeh et al., 2011). We found that five out of ten samples failed to show up E.coli content in PCR (a), which indicates that bacterial culture was more sensitive than the PCR (a). Sandrina et al., (1995); Holland et al., (2000) and Dhanashree & Shrikar, (2008) preferred bacterial culture over the direct PCR, while Lawsoni et al., (1998); Gong et al., (2002); Estibaliz et al., (2004) and Catherine et al., (2012) preferred the opposite.

Also, Louie et al., (1994) and Louie et al., (1998) preferred the PCR applied directly on the samples as the results are obtained within 24 hour, while the culture requires at least 4 days, which make PCR assay more practical for routine laboratory use especially with complex samples as fecal material.

Fecal matter contains many PCR inhibitors such as; bilirubin, bile salts, and complex carbohydrates (Monteiro et al., 1997). Therefore, sample treatment prior to amplification would clear or lower the PCR-inhibitory components allowing sufficient concentration of target DNA thus enhancing the performance of the PCR assays (Iang, 1997; Eckhart et al., 2000; Scipioni et al., 2008) and thus avoiding the requirement for over dilution of the original DNA template in direct PCR detection (Hornes et al., 1991; Brian et al., 1992; Stacy-Phipps et al., 1995). Further, pre-PCR enrichment increases PCR sensitivity even for samples with low E.coli level through increasing live bacterial cells number and eliminating the false-positive results (Wernars et al., 1991) and this agrees with our positive PCR (b) results. Thus, it is recommended to use culture broth as a PCR template and this agrees with Bolton, (1984); Aspinal, (1993); Sethabutr et al., (1994); Skirrow, (1994); Linton, (1997); Holland et al., (2000); Shantta et al., (2001) and Dhanashree & Shrikar, (2008). Negative PCR (a) results may be due to the presence of inhibitors, which reduced or blocked the amplification capacity of PCR in comparison to pure solutions of nucleic acids thus limiting the usefulness of diagnostic PCR (Lantz et al., 2000), and purification steps may be insufficient to get rid of such inhibitors (Wernars et al., 1991). Better DNA extraction methods are necessary to avoid false negative result as reported by Sandrina et al., (1995). Presence of PCR inhibitors in fecal extracts complicated the interpretation of PCR as reported by Flekna et al., (2007); St-Pierre et al., (1994); De Lomas et al., (1992) and Dhanashree & Shrikar, (2008). Another explanation for the negative PCR (a) results is that bacterial cells were few or the DNA concentration could have been less than the detection threshold of the PCR assay (Lawsoni et al., 1998; Da Silva et al., 1999).

PCR efficiency depends on the type of used samples where our results in Table (4) indicated that PCR was more inhibited with the liver, a blood condensed organ, more than the intestinal samples. Blood is rich with PCR inhibitors, which inhibited the Taq DNA Polymerase as reported by Panaccio et al., (1991) and Abu Al-Soud & Radstrom (1998) in contrast to Schrader et al., (2012), while Waleed & Peter, (2000) showed that feces are less inhibitory to PCR than blood.

Considering the extreme diversity encountered in E. coli species, a single set of virulence factors (VFs) (Elaine et al., 2003; Catherine et al., 2012) or even a combination of any pair of them is insufficient to diagnose the majority of APEC strains. Further, Elaine et al., (2003) reported that 27.5% of the APEC isolates were negative for all seven virulence genes examined. So, in our study, a general E.coli pho-A gene was selected to confirm the presence of E. coli species (Hu et al., 2011), then the samples were subjected to detect three of the most prevalent virulence genes in APEC; iut-A, iss and tsh genes. No sample was reported negative for all of the examined genes, so the detected E. coli within the examined liver and fecal samples is most likely to be pathogenic. Holland et al., (2000) concluded that the detection of only virulence genes without species-specific gene might indicate the presence of microorganisms that harbor the same virulence trait rather than the target microorganism itself. All of our examined samples were positive for the pho-A gene and at least one virulence gene.

E.coli isolates from the liver were different from those from the intestine, which is evidenced by the differences in the detected virulence genes. Further, this difference where iss gene was detected in 100% of the intestinal samples but in 80 % of the liver samples, supports our assumption that E.coli could have reached the liver from the respiratory system instead of the intestine in the birds that were negative for E.coli isolation from the intestine. On the contrary, 60% of the birds had similar E.coli genotypes from both liver and intestine regarding the types of examined virulence genes, while 40% showed difference in E.coli isolated from both organs.

PCR rely on genetic determinants rather than the phenotypic traits as the culture does, allowing the identification of a variety of species instead if gone not detected by using only the classical phenotypic-dependent culture assay (Linda et al., 2010). Furthermore, it is simple, highly specific, can provide results on the same day of sample submission for evaluation, which is a potential advantage in outbreaks and epidemiological studies (Shanta et al., 2001). Further, PCR can detect target genetic sequences regardless the growth stage of the target cells (Gong et al., 2002) in contrast to the bacterial culture where cells in samples may exist in a variety of different growth stages or in small number, some of them may not be fit to grow in culture (Lawsoni et al., 1998). However, culture is still always recommended to determine the

antimicrobial susceptibility of isolated strains (Shanta et al., 2001).

Our results indicate that even though PCR (b) is more sensitive than PCR (a) in the detection of E.coli, bacteriological culture was more sensitive for the initial detection and the confirmation of the bacterial presence in the samples. Further, the pre-enrichment step before PCR (b) could have increased the PCR sensitivity over the bacterial culture through reducing the endogenous bacterial background that could have affected the PCR (a) step as reported before by Greenfield & White, (1993) and Gumerlock et al., (1993). Thus, it is recommended to apply the PCR detection of APEC after a specific enrichment step and to perform general gene detection parallel to or in combination with the virulence genes in order to confirm the E. coli infection as well as the pathogenic genotype.

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