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Optimization of polymerase chain reaction for direct detection of colibacillosis in infected chickens

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SUMMARY

A total of 33 local *E. coli* isolates were used in this study. These isolates were biochemically and serologically identified as O1, O2, O6, O78 and O126. Four sets of oligonucleotide primer sequences were designed specifically for 16S*rRNA*, *STX*, *Sth* and *eaeA* genes. DNA and Plasmids were extracted and polymerase chain reaction was optimized for each. 16S*rRNA* gene primer successfully amplified with all serotypes giving rise a product mass of 204 bp, while *STX* gene primer was amplified with O1, O2 and O78 serotypes in a specific band at 323 bp. At the same time the specific primers of *Sth* gene get a 171 bp molecular weight product only with O6 serotype meanwhile the *eaeA* gene primers successfully amplified only with O126 serotype giving rise a molecular weight band at 200 bp. In conclusion, PCR assay was able to differentiate between the different serotypes of *E. coli* in the suspected samples saving time, money and effort.

INTRODUCTION

E. coli infection in poultry is one of the principal causes of mortality and morbidity in chickens and turkeys resulting in significant economical losses in poultry industry due to retardation of growth, decreased feed conversion rate, decreased egg production, decreased fertility, reduced hatchability; which is one of the major problems in the hatchery industry (Wooley *et al.*, 2000), downgraded carcasses and condemnation of whole affected carcasses or organs after slaughter and finally the high cost of wide range of antibacterial agents used to control *E. coli* infection in many poultry farms. *E. coli* has unique complex antigenic structure; it has seven antigenic

structures, however only four are dominant and have important significance which are, somatic antigen (O), capsular antigen (K), flagellar antigen (H) and fimberial antigen (F). Accordingly, there are many serotypes based on this antigenic structure giving rise to many problems associated with the control and prevention of *E. coli* infection. These problems including; difficulty to prepare common antigen, difficulty to detect carriers, difficulty in vaccine production except in special cases like local vaccines prepared from local dominant serotypes and difficulty to treat cases due to great difference in their sensitivity to antibacterial agents. Epidemiological, pathological and microbiological characters of *E. coli* also affect the prevention and control of the infection as *E. coli* widely spread in nature (dust, feather, feces, and litter) and lives as a normal inhabitant in intestinal and respiratory tracts of poultry. Also *E. coli* bacteria differ in its pathogenicity from pathogenic, opportunistic to non pathogenic. *E. coli* isolates which are pathogenic for poultry, commonly belonging to certain serogroups, particularly serogroups O78, O1 and O2, and to some extent O15 (Gross, 1994 and Chart, *et al.*, 2000). Serological and bacteriological methods are not sensitive enough to differentiate all bacterial isolates. Molecular differentiation of different *E. coli* strains may aid in differentiating those that are specifically pathogenic for a certain host and give guidance for epidemiological studies of sources of infection and disease transmission. Those techniques of molecular and genetic differentiation involve polymerase chain reaction (PCR) (Whittam and Wilson 1988) and restriction fragment length polymorphism (RFLP) (Maurer, *et al.*, 1998 b).

Therefore, the present work was planned to differentiate between the isolated *E. coli* using genotypic identification by PCR.

MATERIALS AND METHODS

1. *E. coli* strains:

A total of 33 local isolates were used in this study. These isolates were identified biochemically using the API 20E® (Analytical Profile Index, BioMérieux, France) identification system and serologically using polyvalent and monovalent antisera (Hossain *et al.*, 2008).

2. Isolation of DNA from *E. coli* (Sambrook *et al.*, 1989):

A single bacterial colony was transferred into 2ml of nutrient broth and incubated over night at 37°C with vigorous shaking then poured into a microfuge tube and centrifuged at 10,000 xg for 5 min at 4°C. The supernatant was discarded and the pellet was dried as possible.

The bacterial pellet was resuspended in 400µl of TE buffer (pH 8.0) and 150µl of Lysozyme, mixed by inverting the tubes several times and incubated on ice for 15 min. 60µl of SDS 10% and 40µl of proteinase k were added, mixed and incubated in a shaking water bath at 37°C for 30 min then treated with phenol: chloroform: isoamyl (25: 24: 1). DNA precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and stored at -20°C for 2 h, then double volume of absolute ethanol (100%) was added and centrifuged at 12,000 xg for 30 min. DNA pellets were dried then washed by 1ml ethanol 70% and centrifuged at 12,000 xg for 30 min at 4°C. The supernatant was discarded and DNA pellets were dried as mentioned before. The pellets of DNA were dissolved in 50µl of TE buffer then 3µl of RNase were added.

3. Isolation of plasmids:

The plasmids of different isolated *E. coli* serotypes were extracted using high pure plasmid extraction kits (SibEnzyme Ltd, Russia).

4. Polymerase Chain Reaction (PCR):

(a) Nucleotide primers: *E. coli* isolates were screened for the presence of 4 different virulence genes (*16SrRNA, STX, STh* and *eae*A) using specific primers and cyclic conditions for each gene primer. The primers used in the PCR reactions for *E. coli* serotypes were summarized as follow.

Gene	Primer		Sequence	Product	Reference
16SrRNA	*SRV3*	F	CGGCCAGACTCCTACGGG	204bp	Lee *et al.* (1996)
		R	TTACCGCGGCTGCTGGCAC		
STX	*Read*	F	GAGCGAAATAATTTATATGT	323bp	Read *et al.* (1992)
		R	CGAAATCCCCTCTGTATTTGCC		
STh	*STh*	F	ATTTCTGTATTGTCTTT	171bp	Ratchtrachenchar *et al.* (2004)
		R	ATTACAACACAGTTCACAG		
*eae*A	*eae*A	F	GTGGCGAATACTGGCGAGACT	200bp	Fagan *et al.* (1999)
		R	CCCCATTCTTTTTCACCGTCG		

(b) PCR amplification: The PCR mix was prepared as: 2µl DNA template, 1µl Taq DNA polymerase (IU/µl), 5µl 10X reaction buffer, 1µl DNTPs, 3µl 25mM $MgCl_2$, 1µl forward primer (25pmol), 1µl reverse primer (25pmol) and double distilled water up to 50 µl. The mixture was placed in the thermal cycler which was programmed according to Anand *et al.* (2006) for the 16SrRNA gene, *Read et al.* (1992) for *STX* gene, Chomvarin *et al.* (2005) for *STh* gene and Ji-Yeon *et al.* (2005) for *eae*A gene. PCR products were examined through agarose gel electrophoresis, visualized under

U.V transilluminator and photographed with a digital camera.

RESULTS

Results identification of local *E. coli* isolates:

According to the results of API 20E identification system and serodiagnosis, the isolates of *E. coli* were identified into 5 serogroups; O1 including 9, O2 including 3, O6 comprising 7, O78 comprising 9 while O126 comprising 5 out of 33 local isolates.

Results of DNA extraction from *E. coli* serotypes:

The estimated size of the genomic DNA of the different *E. coli* serotypes was more than 23 Kb as shown in photo (1).

Results of plasmids extraction:

The number of plasmids recovered from each isolate varied from three to eight. As shown in Photo (2), the molecular weight of the isolated plasmids varied from 2 to 12 Kb. From the plasmid profile of the isolated *E. coli*, it is clear that, there were large plasmids (> 17 Kb) were associated with all serogroups isolated from chickens.

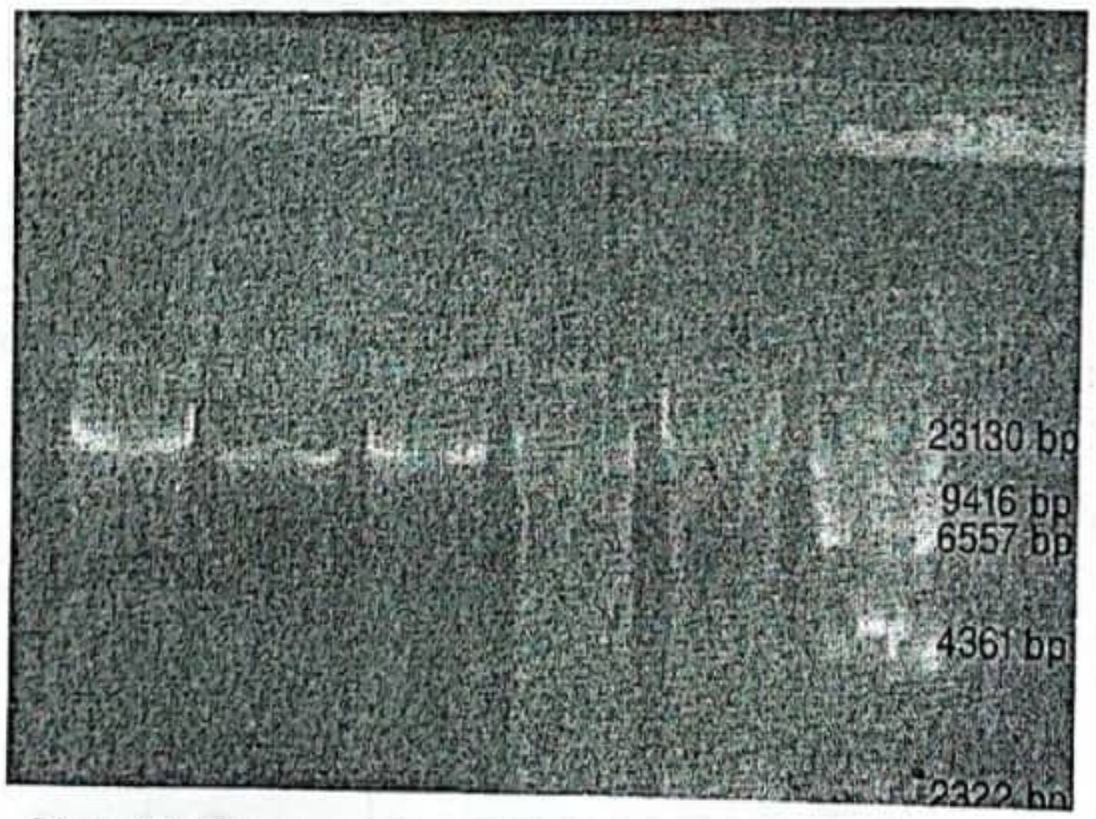

Photo (1): The extracted genomic DNA of *E. coli* serotypes.

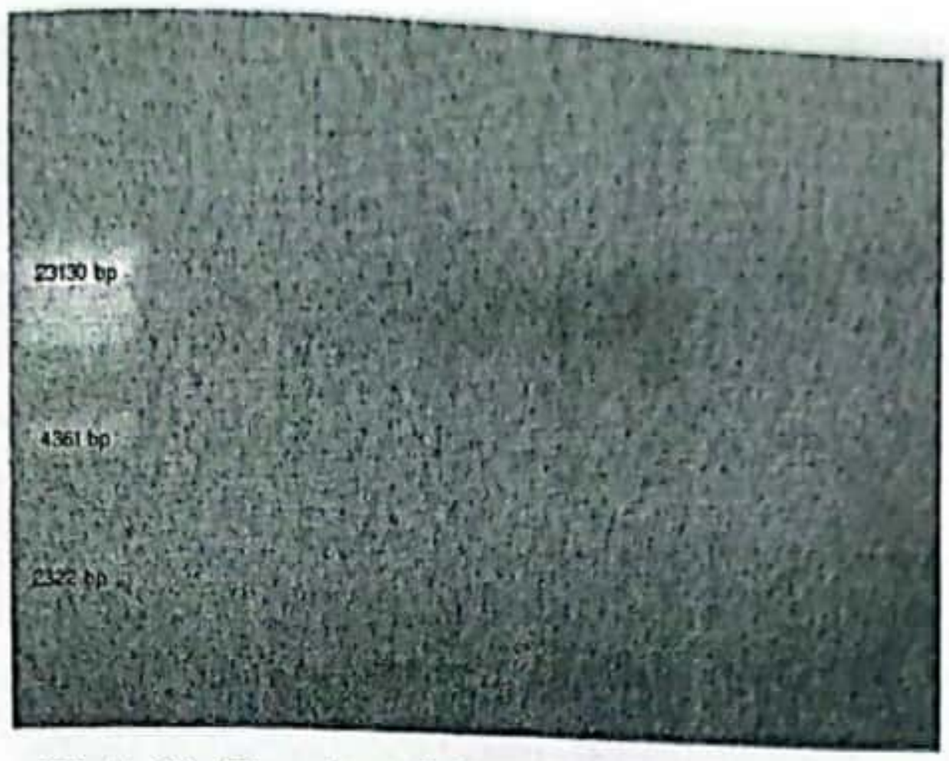

Photo (2): The extracted plasmids of *E. coli* serotypes.

Results of polymerase chain reaction:

(a) Amplification of *16SrRNA* gene of *E .coli* isolates:

The genomic DNA of *E. coli* serotypes were tested using specific primers for the *16SrRNA* gene. The *16SrRNA* gene was amplified in the five *E. coli* serotypes giving rise to a PCR product of 204bp as shown in photo (3).

Photo (3): PCR amplification of 16*SrRNA* gene of *E. coli* serotypes:

(b) Amplification of *STX* gene of *E. coli* serotypes:

- The genomic DNA of *E. coli* serotypes were tested using specific primers for *STX* gene. The *STX* gene was amplified at 323 bp in serotypes O1, O2 and O78 as shown in photo (4). On the other hand *E. coli* O6 and O126 were negative under the same condition.

Photo (4): PCR amplification of *STX* gene of *E. coli* serotypes.

M, marker; lane 1, *E. coli* serotype O1; lane2, serotype O2; lane 3, serotype O6; lane4, serotype O78 and lane 5, serotypes O126.

(c) Amplification of *STh* gene of *E. coli* strains:

The genomic DNA of *E. coli* serotypes were tested using specific primers for the *STh* gene. The *STh* gene was amplified only in serotype O6 giving a PCR product of 171bp as shown in photo (5). Meanwhile other serotypes were negative on using *STh* gene specific primers.

(d) Amplification of *eaeA* gene of *E. coli* strains:

The genomic DNA of *E. coli* serotypes were tested using specific primers for the *eaeA* gene. The *eaeA* gene was amplified only in serotype O126 giving a PCR product of 200bp as shown in photo (6). On the other hand, the remaining serotypes gave a negative reaction against *eaeA* gene specific primers.

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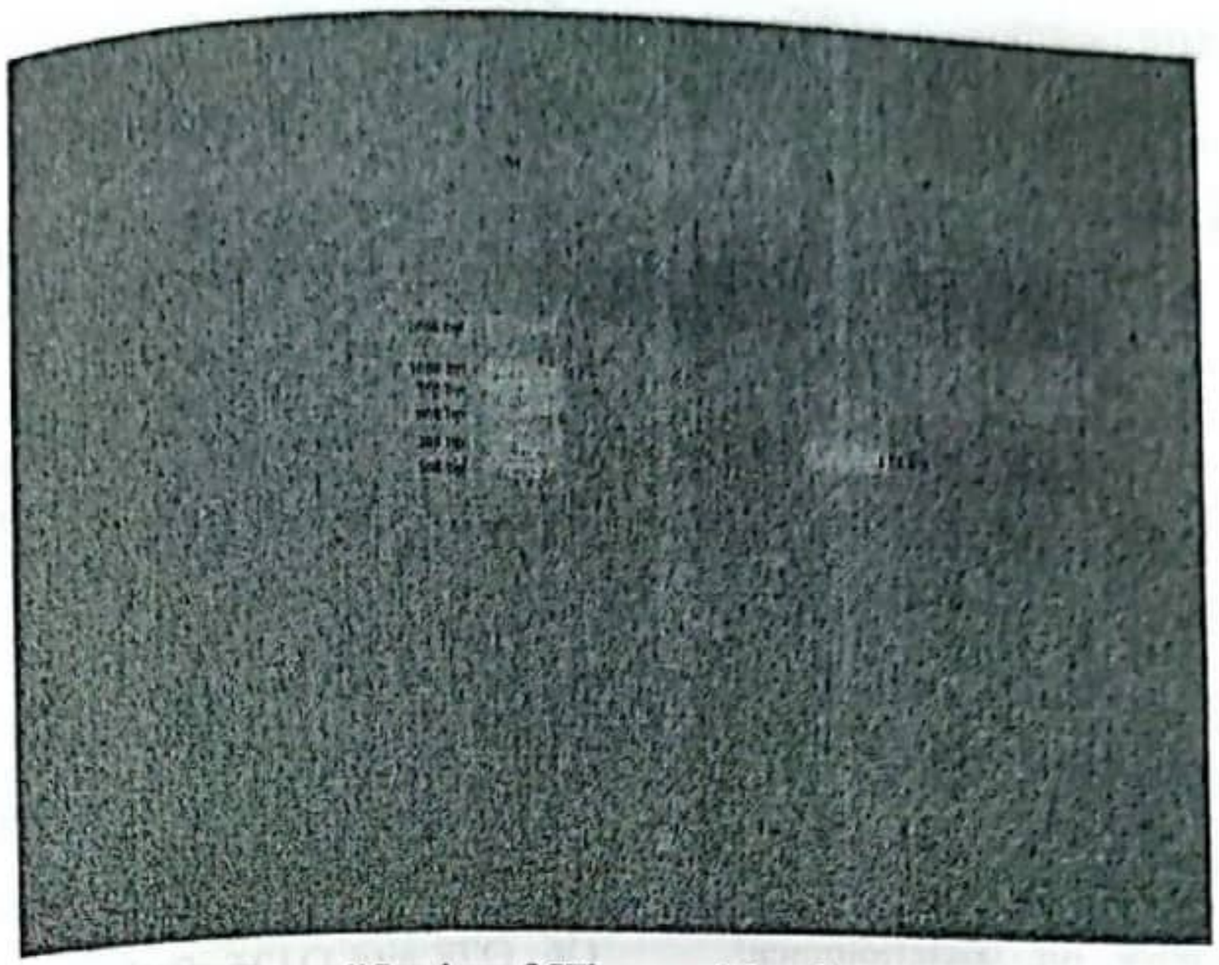

Photo (5): PCR amplification of *STh* gene of *E. coli* serotypes

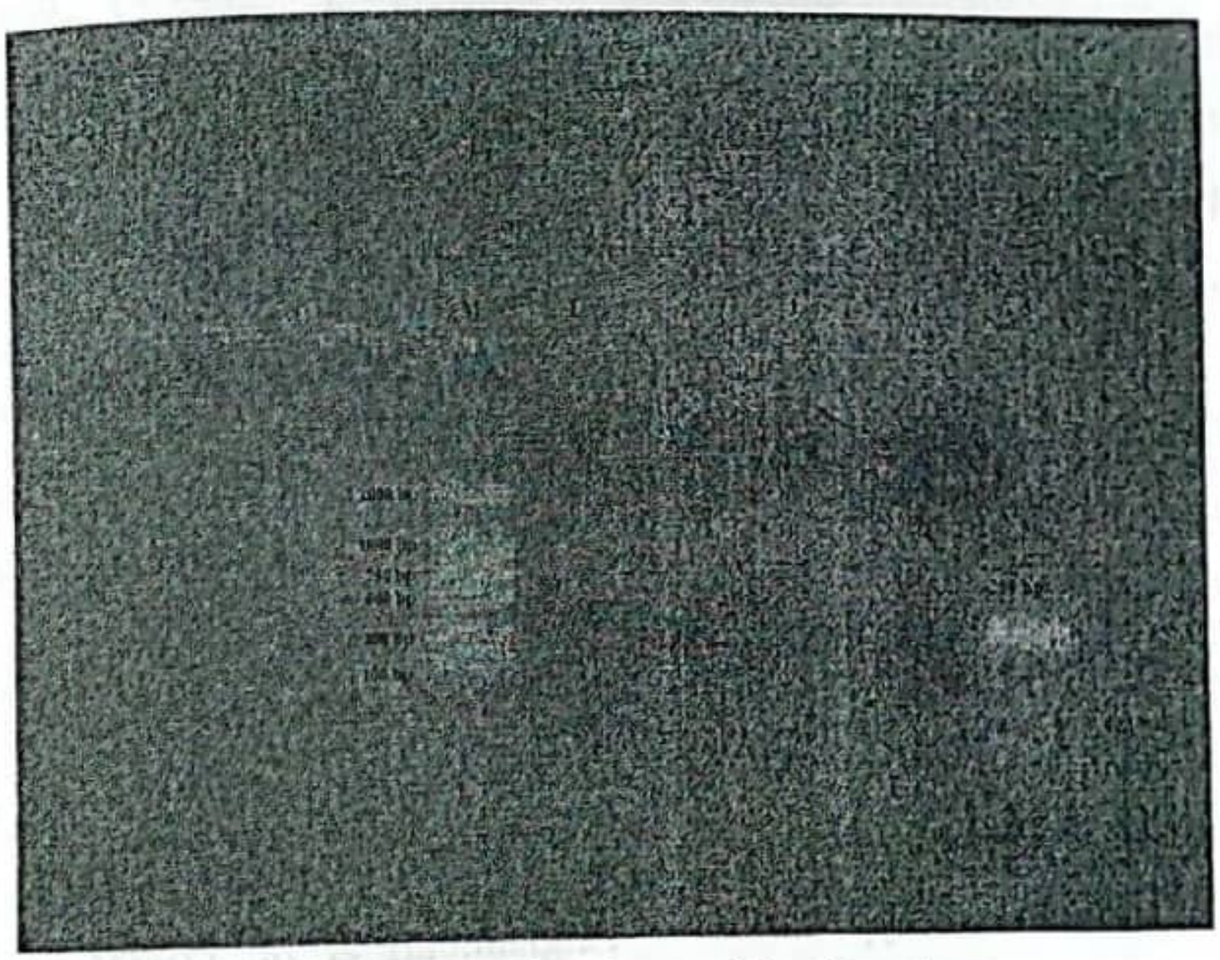

Photo (6): PCR amplification of *eaeA* gene of *E. coli* serotypes

M, marker; lane 1, *E. coli* serotype O1; lane2, serotype O2; lane 3, serotype O6; lane4, serotype O78 and lane 5, serotypes O126.

Table (1) Results of RCR amplifications of different genes of *E. coli* serotypes:

Serotypes	PCR results								Pathotype
	16SrRNA	Product	*STX*	Product	*STh*	Product	*eaeA*	Product	
O1	+	204bp	+	323bp	–	171bp	–	200bp	EHEC
O2	+		+		–		–		EHEC
O6	+		–		+		–		ETEC
O78	+		+		–		–		EHEC
O126	+		–		–		+		EPEC
Recovery rate	33/33 (100%)		21/33 (64%)		7/33 (21%)		5/33 (15%)		

EHEC: Enterohaemorrhagic *E. coli.*, ETEC: Enterotoxigenic *E. coli.* & EPEC: Enteropathogenic *E. coli.*

DISCUSSION

This work was planned for bacteriological characterization of chicken *E. coli* isolates using PCR. In birds *E. coli* infections cause many clinical manifestations, the most common is being airsaculitis, pericarditis, septicemia and death (Sojka, 1965 and Vidotto, *et al.*, 1990). Colibacillosis due to virulent *E .coli* in chickens is characterized by a respiratory disease which is frequently followed by a generalized infection (Gross, 1984).

In the present study, only a restricted number of serotypes O1, O2, O6, O78, and O126 have been biochemically and serologically identified that represent the most common serotypes in the Egyptian poultry farms. The most predominant serotypes were O1 and O78 where each represent 9 isolates out of 33 followed by O6 (7 isolates), O126 (5 isolates) and finally O2 (3 isolates). These results were confirmed by Salama, *et al.* (2007) who recovered 5 different *E. coli* serotypes identified as O1, O2, O6, O78 and O126. Pathogenic *E. coli* isolates for poultry commonly belong to certain serogroups, particularly O78, O1 and O2, and sometimes O15 (Gross, 1994 and Chart, *et al.*, 2000). The relation between biochemical and serological identification of *E. coli* confirmed that the variation of reactions within the same source of samples was related to the differences in serotypes and also revealed the similarity between serotypes O1 and O2 in their biochemical reactions. Similar serotypes (O1, O2 and O78) were obtained by Ibrahim (1997) and McPeake *et al.* (2005). In addition Peighambari *et al.* (1995) recorded that the most common serogroupes of *E. coli* encountered in avian disease condition were O78, O2 and O1 which were associated with septicemic *E. coli* infection in poultry.

The bacteriological method for detecting pathogens, typically involves culturing the

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Table (1) Results of RCR amplifications of different genes of *E. coli* serotypes:

Serotypes	PCR results								Pathotype
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O2	+		+		–		–		EHEC
O6	+		–		+		–		ETEC
O78	+		+		–		–		EHEC
O126	+		–		–		+		EPEC
Recovery rate	33/33 (100%)		21/33 (64%)		7/33 (21%)		5/33 (15%)		

EHEC: Enterohaemorrhagic *E. coli*., ETEC: Enterotoxigenic *E. coli*. & EPEC: Enteropathogenic *E. coli*.

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The bacteriological method for detecting pathogens, typically involves culturing the

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amplification of heat stable enterotoxin (*sth*) encoding gene using specific primers. The gene was detected only in serotype O6 which was enterotoxigenic *E. coli* with a product of 171bp as shown in photo (5). This result isn't in conformity with that of Chomvarin *et al.* (2005) who examined 11 *E. coli* isolates for the presence of enterotoxin encoding gene and non of them were positive for this gene.

As regards to the *eaeA* amplification using its specific primers, a PCR product of 200bp was obtained only in serotype O126 which was enteropathogenic *E. coli* as shown in photo (6). Ji-Yeon *et al.* (2005) obtained a PCR product of 890bp on studying the same gene using PCR while Kilic *et al.* (2009) stated that *eaeA* gene was correctly amplified in 48% of the samples as *E. coli* with a product of 384bp, and reported that these results were confirmed by the results of biochemical tests.

From Table (1) it was clear that *STX* gene was present in 21 out of 33 isolates (64%) followed by *STh* gene (21%) which was present in 7 isolates then *eaeA* gene (15%) present in 5 isolates and all 33 isolates carried *16SrRNA* gene. Similar results were obtained by Debroy and Maddox (2001) who claimed that the most commonly observed virulence factor in bovine isolates was the presence of *eaeA* genes, which occurred in about 30% of the isolates. The same researchers reported a 13.2% isolation rate for *eaeA* from chickens. The isolation rates of *eaeA* from different *E. coli* strains have been reported in epidemiological studies from various locations worldwide. Meanwhile Bi, *et al.* (1999) detected the the *eaeA* gene in 60% of the strains. Also Kariuki, *et al.* (2002) and Kilic, *et al.* (2009) detected the *eaeA* gene in 60.9% of *E. coli* strains in chickens in Kenya and in 48% of *E. coli* strains in chickens in Elazig, Turkey, respectively. According to PCR results, by using *STX*, *STh*, *eaeA* and *16SrRNA* genes, one can differentiate between the different serotypes of *E. coli* isolated from chicken or other animal species.

Overall, this study demonstrated the importance and advantages of PCR in comparison with the traditional isolation and identification procedures in the rapid identification and differentiation of *E. coli* serotypes as it is more clearly that the use of PCR assay is able to differentiate serotypes of *E. coli* in the suspected samples saving time, money and effort. Also multiplex PCR can be optimized using different primers used in this work to discriminate the serotypes of *E. coli* in samples in one step.

REFERENCES

Aabo, S.; Rasmussen, O. F.; Rossen, L.; Sorensen, P. D. and Olsen, J. E. (1993). *Salmonella* identification by PCR. Mol. Cell. Probes, 7: 171.

Anand, S. K.; Fatima, S.; Joshi, C. G. and Purohit, J. H. (2006). Molecular charachterization of *E. coli* using PCR single strand conformation polymorphism analysis of *16S rRNA* gene. Indian J. Poult. Sci. 41(1): 1-5 research article.

Bi, Z.; Nagayama, K.; Akeda, Y.; Cantareli, V.; Kodama, T.; Takarada,Y.; Shibata, S. and Honda, T. (1999). Development of an enzyme-labeled oligonucleotide probe for detecting the *E. coli* attaching and effacing A gene. Microbiol. Immunol. 43: 663-667.

Chart, H.; Smith H. R.; La ragione, R. M. and Woodward, M. J. (2000): An investigation into the pathogenic properties of *Escherichia coli* strains BLR, BL21, DH5α, and EQ1. J. Appl. Microbiol. 89: 1048-1058.

Chomvarin, C.; Ratchtrachenchai, O. A.; Chantarasuk, Y.; Srigulbutr, S.; Chaicumpar, K.; Namwat, W. and Kotimanusvanij, D. (2005). Characterization of diarrheagenic *E. coli* isolated from food in Khon Kaen, Thailand. Southeast Asian J. Trop Med Public Health. 36(4): 931-939.

Debroy, C. and Maddox, C. W. (2001). Identification of virulence attributes of gastrointestinal *E. coli* isolates of veterinary significance. Anim. Health Res. Rev., 2: 129-140.

Fagan, P. K.; Hornitzky, M.A.; Bettelheim, K.A. and Djordjvic, S.P. (1999). Detection of shiga-like toxin (*stx1* and *stx2*), intimin (*eaeA*) and Enterohaemorrhagic *E. coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol., 65: 868- 872.

Ghanbarpour, R.; Salehi, M. and Oswald, E. (2010). Virulence genotyping of *E. coli* isolates from avian cellulites in relation to phylogeny. Comp Clin. Pathol. 19:147-153.

Gross, R. J. (1984). Acute enteritis P.458-476. In Topley and Wilson's principles of Bacteriology, Virology and Immunity. 7th Ed. Vol.3. Bacterial diseases Butley, Tanner Ltd. Forme.

Gross, W. B. (1994): Diseases due to *Escherichia coli* in poultry. In: Gyles, C. L., (Ed.), *Escherichia coli* in Domestic Animals and Humans. CAB International Library Wallingford UnitedKingdom. 237- 260.

Hossain, M. T.; Siddique, M. P.; Hossain, F. M. A.; Zinnah, M. A.; Hossain, M. M; Alam, M. K.; Rahman, M. T. and Choudhury, K. A. (2008). Isolation, identification, toxin profile and antibiogram of *E. coli* isolated from broilers and layers in Mymensingh District of Bangladesh. Bangl. J. Vet. Med. 6(1): 1- 5.

Ibrahim, I. S. (1997). Prevalence of *E. coli* in slaughtered broilers and their products. Ph.D. thesis (meat hygiene), Fac. Vet. Med. Cairo Univ.

Ji-Yeon, K. ; So-Hyun, K.; Nam-Hoon, K.; Won-Ki, B.; Ji Younlim; Hye-Cheong, K.; Jun-Man, K.; Kyoung- Min, N.; Woo-Kyung, J.; Kun-Taek, P. and Yong-Ho, P.(2005). Isolation and identification of *E.. coli* O157:H7 using different detection methods and molecular determination by multiplex PCR and RABD. J.Vet. Sci, 6(1): 7-19.

Johnson, R. P.; Clarke, R. C.; Wilson, J. B; Read, S. C.; Rahn, K.; Ren-Wick, S. A.; Sandhu, K. A.; Alves, D.; Karmali, M. A.; Lior, H.; McEwen, S. A.; Spika, J. S. and Gyles, C. L. (1996). Growing concerns and recent outbreaks involving non-O157:H7 serotyoes of verotoxigenic *E. coli*. J. Food Prot. 59: 1112-1122.

Kariuki, S.; Gilks, C.; Kimari, J.; Muyodi, J.; Getty, B.; Hart, C. A. (2002). Carriage of potentially pathogenic *E. coli* in chickens. Avian Dis. 46: 721-724.

Kilic, A.; Muz, A.; Ertaşh, B. and Özbey, G. (2009). Random Amplified Polymorphic DNA (RAPD) Analysis of *Escherichia coli* Isolated From Chickens. F.Ü. Sağ. Bil.Vet. Derg. 23 (1): 1- 4.

Lee, D. H.; Zo, Y. G. and Kim, S. J. (1996). Appl. Environ. Microbiol. 62: 3112-3120.

Maurer, J. J; Lee, M. D. ; Lobsinger, C.; Brown, T.; Maier, M. and Thayer, S. G. (1998): Molecular typing of avian *E. coli* isolates by random Amplification of Polymorphic DNA.. Avian DIS., 42: 431-451.

McPeake, S. J. W.; Smyth, J. A. and Ball, H. J. (2005). Characterization of avian pathogenic *E. coli* (APEC) associated with colicepticemia compared to fecal isolates from healthy birds. Vete. Microbiol. 110: 245-253.

Newcomb, J.; Dyer, S.; Blackwell, L.; Cartwright, K.; Palmer, W. H. and McFadden, J. (2000). J. Clinic. Microbiol., 35: 1809-1812.

Parreira, V. R. and Gyles, C. L. (2002). Shiga toxin genes in avian *E. coli*. Vet. Microbiol. 87: 341-352.

Peighambari, S. M.; Vaillancourt, J. P.; Wilson, R. A and Gyles, C. L. (1995). Characteristics of *E. coli* isolates from avian cellulites. Avian Diseases, 39: 116-124.

Ratchtrachenchar, O. A.; Subpasu, S.; Hayashi, H. and Bathein, W. (2004). Prevalence of childhood diarrhea as sociated *E. coli* in Thailand. J. Med. Microbiol. 53: 237-243.

Read, S. C.; Clarke, R. C.; Martin, A.; Degrandis, S. A.; Hii, J. and Gyles, C. L. (1992). PCR for detection of verocytotoxigenic *E. coli* isolated from animal and food sources. Mol. Cell. Probes., 6: 153-161.

Riyaz- UI- Hassan, S.; Verma, V. and Qazi, G. N. (2004). Rapid detection of *Salmonella* by PCR. Mol. Cell. Probes., 18,333.

Salama, S. S; Afaf A. K.; Elham A. E. and Taha, M. M. (2007). Molecular Strategies for the differentiation and identification of local *E. coli* isolated from chicken: I. characterization of protein profile. B.S. Vet. Med. J. January, 17 (1): 25-28.

Sambrook, J.; Fritsch, E. F. and Maniatis (1989). Molecular cloning a laboratory manual. 2nd Ed. Cold spring Harbor laboratory press.

Sojka, W. J. (1965). *E. coli* in domestic animals and poultry.1st Edition Commonwealth Agriculture. Bureau, Farnham, Royal Buck, England.

Tutenel, A. V.; Pierard, D.; Hoof, J. V.; Comelis, M. and De Zutter, L. (2003). Isolation and molecular characterization of *E. coli* O157 isolated from cattle, pigs and chickens at slaughter. International J. Food Microbiol., 84: 63-69.

Vidotto, M. C.; Muller, E. E.; De Freitas, J. C.; Alfieri, A. A.; Guimaraes, I. G. and Santos, D. S. (1990). Virulence factors of avian *E. coli*. Avian Diseases. 34:531-538.

Whittam, T. S. and Wilson, R. A. (1988): Genetic relationships among pathogenic strains of avian *E. coli*. Infect. Immun., 56(9): 2458-2466.

Wooley, R. E.; Gibbs, P. S.; Brown, T. P. and Maurer, J. J. (2000): Chicken embryo lethality assay for determining the virulence of avian *Escherichia coli* isolates. Avian Dis. 44: 318-324.

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