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Differentiation between vaccinal and field strains of *E. coli* using phenotype and genotype characterization

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Abstract

Background: *E. coli* infection is considered as an important bacterial problem associated with significant economic losses and usually associated with a variety of disease conditions, including acute septicaemia, haemorrhagic enteritis, pericariditis, salpingitis and complicated air saculitis. These considerations suggest that control of *E. coli* by vaccination could be of great value especially live vaccine that based on defined mutations that impair and non-revering virulence.

Material and Methods: Wild type O78 strain, Live O78 *aroA* gene deleted vaccine and Live O78 *crp* gene deleted vaccine were used to accomplish this study. Phenotypic characterization was adopted by studying the cultural, biochemical and serological properties. Also genotypic characterization was studied for *16S rRNA*, *aroA* and *crp* genes.

Results: Growth pattern on different media differed among the used strains as the wild type and Δ aroA mutant were nearly similar while the Δ *crp* mutant strain was greatly differed. Biochemically the difference between the wild-type and Δ aroA mutant was inability of mutant strain to produce arginine dihydrase (ADH) and fermentation of saccharose. On the other hand the Δ *crp* mutant failed to produce ADH and to ferment any of the carbohydrates except glucose. A successful amplification of the *16S rRNA* gene at 585 bp was detected with the all tested strains while was 1206 bp with only wild type and Δ crp mutant strain when aroA gene primers were used. Regarding *crp* genes, the amplified products was at 1029 bp with the wild and Δ aroA mutant but not with Δ *crp* mutant strains.

Conclusion: Findings of this study prove the use of methods based on molecular techniques like PCR to differentiate between different types of E. coli either wild or vaccinal mutant type strains. Also it may help in exclusion or proving the return back to virulence of the mutant vaccinal strains.

Key words: *E. coli O78, chickens, phenotypic characterization, genotypic characterization, wild and vaccinal mutant strains.*

Introduction

Escherichia coli (*E. coli*) is a gram-negative bacteria that belong to the family Enterobacteriaceae and considered as a member of the commensal intestinal flora in animals. It may sometimes produce pathogenic strains (1). *E. coli* infection is one of the most important bacterial diseases affecting chicken, resulting in significant economic losses through mortality, morbidity, cost of treatment and condemnation at processing plant (2).

The infection has been associated with a variety of disease conditions, including acute septicaemia, haemorrhagic enteritis, pericariditis, salpingitis and complicated air sacculitis (3). More than one hundred *E. coli* serotypes have been reported but the infection in chickens was mostly belonged to serogroups O1, O2 and O78 (4). This infection is treated with expensive antibiotics, often resulting in the subsequent development of resistant strains that prevent continued use of effective treatment.

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These considerations suggest that control of such condition by vaccination could be of great value (5). Alive vaccine based on defined mutations that impair virulence and non-revering may meet the requirements for application in commercial operation. Deletion mutation (identified by the symbol Δ) in *aroA* gene (a gene that codes 5-enolpyruvylshikimate 3-phosphate synthase, one of enzymes that govern the shikimate pathway for synthesizing aromatic amino acids involved with the growth and metabolism of bacteria) and *crp* gene (gene for cAMP receptor protein) have been shown to reduce the virulence of *E. coli* strains (6, 7).

The present study is undertaken with the aim to differentiate between the field or wild *E. coli* strains and the recently widely used live vaccinal strain of *E. coli* among poultry clusters using either phenotypic specification or genotypic characterization.

Methods and materials

E. coli strains

A wild type strain and two mutant vaccinal strains used in this study. The wild type O78 strain was isolated and identified in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt. The mutant strains are: Live O78 *aroA* gene deleted vaccine (Δ *aroA E. coli* mutant vaccinal strain) and Live O78 *crp* gene deleted vaccine (Δ *crp E. coli* mutant vaccinal strain).

Phenotypic differentiation

Phenotypic differentiation was performed by colony morphology, microscopic examination, motility, biochemical identification and serological differentiation as follows:

Colonial morphology: The wild type O78 strain and their corresponding mutants (Δ *aroA* and Δ *crp* vaccinal strains) were cultivated onto nutrient agar, MacConkey agar, and Salmonella Shigella agar to differentiate their macroscopic colonial characters. Microscopic examination: Smears from freshly growing colonies were stained with Gram stain and examined microscopically. Motility: Motility was assured by growing and spreading of the wild type and vaccinal strains by stabbing in semisolid agar. Biochemical identification: Pure cultures of vaccinal and wild type strains were examined biochemically by using API 20E identification system according to (8) following the procedures of kit manual. Serological differentiation: Both vaccinal and wild type strains were subjected to serological differentiation according to procedures described by (9) using polyvalent diagnostic antisera (O6, O27, O78, O148, O159, O168) then each monospecific diagnostic antisera.

Genotypic characterization

DNA Extraction

DNA templates from the vaccinal and wild type *E. coli* strains were extracted using Isolate Genomic DNA Mini Kit. (Bioline) (6).

Primers pairs

Specific primers for *16s rRNA, aroA* and *crp* genes were used as listed in Table (1).

Table 1. Primers used for amplification of *16s RNA, aroA* and *crp* genes.

Genes	Primer Sequence	Product	Reference
16s rRNA	F5-GAC CTC GGT TTA GTT CAC AGA-3 R5-CAC ACG CTG ACG CTG ACCA-3	585 bp	10
aro A	F5-CAT GGT ACC TCG TGT CGA TGG CAC TAT TA-3 R5-GCC GAG CTC TCA AGA ATC GTC ACT GGT GT-3	1206 bp	6
crp	F5-CTG ACG ACC AGA GGC GGA TT-3 R5-CTA CCA GGT AAC GCG CCA CT-3	1029 bp	7

Polymerase chain reaction (PCR) assay

5 µl of genomic DNA, 12.5 µl of dream taq green master mix (Thermoscientific), 1 µl of each primer (10 pmole) and 5.5 µl of deionized water were added to 0.5 ml micro centrifuge tubes. The amplification reactions were performed under following conditions: 94°C for 4 min, then 29 cycles each at 94°C for 90 sec, 62°C for 90 sec and 72°C for 2 min.; lastly 72°C for 10 min for *16 SrRNA* (10), 94°C for 3 min, then 30 cycles each at 94°C for 45 sec, 60°C for 30 sec and 72°C for 45sec.; lastly 72°C for 7 min for *aroA* gene (6) and 94°C for 4 min, then 30 cycles each at 94°C for 1m, 60°C for 1 min and 72°C for 90 sec.; lastly 72°C for 6 min for *crp* gene (7). The PCR products were analyzed on 1% agarose gel.

Results

In the present study, the phenotypic and genotypic characterizations were used to differentiate between the wild-type O78 strain, Δ *aroA* and Δ *crp* mutant vaccinal strains.

Growth on different media: The pattern of growth on different media when incubated at 37C° for 24 hrs differed among the wild type and the two types of mutant colonies. As shown in Table 2, wild type and Δ *aroA* mutant demonstrated a large sized rounded, non-pigmented colonies on nutrient agar medium and large sized fermenting rounded, non-mucoid colonies on MacConkey and SS media. Concerning the Δ *crp* mutant strain, it

showed small sized rounded, non-pigmented colonies on Nutrient agar medium and small sized non-fermenting rounded, non-mucoid phenotype on MacConkey and SS median. Both wild and two mutant strains were Gram negative, motile, non-sporulated, medium sized bacilli.

Table 2. Colonial and microscopic characteristics of the *E. coli* strains.

***E. coli* strains**	**Media**			**Motility**	**Gram stain**
	Nutrient agar	MacConkey agar	SS agar		
O78 strain	+ve	+ve	+ve	+ve	Gram negative, non-sporulated, medium sized bacilli
***ΔaroA* mutant**	+ve	+ve	+ve	+ve	
***Δcrp* mutant**	+ve	-ve	-ve	+ve	

Biochemical Activity: Depending on the results of API 20E identification system as demonstrated in Table (3) and Figure (1), the wild type O78 strain showed positive results with ONPG,ADH, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative results with CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests meanwhile the Δ *aroA* mutant gave positive reaction with ONPG, LDC, ODC, IND,GLU, MAN,SOR, RHA, MEL and ARA tests and negative reaction with ADH, CIT, H2S, URE, TDA, VP, GEL, INO, SAC, AMY and OX tests. On the other hand, the Δ *crp* mutant give positive results with ONPG, LDC, ODC, IND, GLU and ARA tests and negative results with ADH, CIT, H2S, URE, TDA, VP, GEL, MAN, INO, SOR, RHA, SAC, MEL, AMY and OX tests.

Table 3. API 20E characteristics of different types of *E. coli* strains.

	API 20E RESULTS																				
Type of samples	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**	**TDA**	**IND**	**VP**	**GEL**	**GLU**	**MAN**	**INO**	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**
O78 wild type strain	+	+	+	+	-	-	-	-	+	-	-	+	+	-	+	+	+	+	-	+	-
Δ *aroA* mutant	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-
Δ *crp* mutant	+	-	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-

ONPG: OrthoNitroPhenyl-βD-Galactopyranosidase, ADH: Arginine Dihydrolase, LDC: Lysine Decarboxylase, ODC: Ornithine Decarboxylase, CIT: Citrate utilization, H2S: Hydrogen sulfide, URE: Urease, TDA: Tryptophane Deaminase, IND: Indole, VP: Vages Proskauer, GEL: Gelatin, GLU: Glucose (fermentation/oxidation), MAN: Mannitol (fermentation /oxidation), INO: Inositol (fermentation/oxidation), SOR: Sorbitol (fermentation/oxidation), RHA: Rhamnose (fermentation/oxidation), SAC: Saccharose (fermentation/oxidation), MEL: Melibiose (fermentation/oxidation), AMY: Amygdalin (fermentation/oxidation), ARA: Arabinose (fermentation/oxidation), OX: Oxidase.

Figure 1. API20E identification system reactions. (A) The wild O78 give positive reaction with ONPG, ADH, LDC, ODC, IND, GLU, MAN, SOR, RHA, SAC, MEL and ARA tests and negative reaction with CIT, H2S, URE, TDA, VP, GEL, INO, AMY and OX tests. (B) aroA deleted vaccinal strain give positive reaction with ONPG, LDC, ODC, IND,GLU, MAN,SOR, RHA, MEL and ARA tests and negative reaction with ADH, CIT, H2S, URE, TDA, VP, GEL, INO, SAC and AMY tests. (C) crp deleted vaccinal strain give positive results with ONPG, LDC, ODC, IND, GLU and ARA tests and negative results with ADH, CIT, H2S, URE, TDA, VP, GEL, MAN, INO, SOR, RHA, SAC, MEL and AMY tests.

Serotyping: Positive results were obtained when *E. coli* polyvalent antisera was used with both wild and mutant *E. coli* strains. In the same concern a positive reaction was obtained when monovalent O78 antisera was used confirming the results of biochemical identification.

Genotypic characterization: The genomic DNAs of the wild type and vaccinal *E.coli* strains were extracted, electrophoresed on 0.7% agarose gel and the estimated size of the genomic DNA which was more than 23 kbp.

A successful amplification of the *16S rRNA* gene were occurred giving rise to a PCR product of 585 bp with the all tested strains as shown in Figure (2) and Table (4) and this confirm that all of the used strains as *E.coli*.

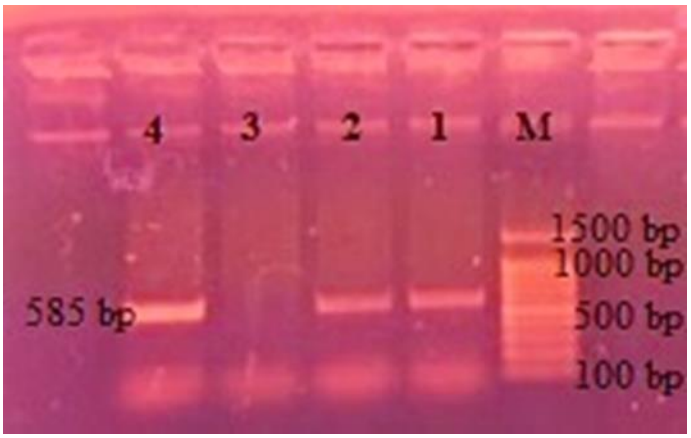

Figure 2. Result of PCR using specific primers of 16s rRNA gene of *E. coli*. M: marker, Lane 1: Δ *aroA* mutant, Lane 2: Δ crp mutant, Lane 3: Negative control and Lane 4: Wild O78 strain.

As regards to the specific *aroA* gene primers, it is successfully amplified giving rise aspecific PCR product of 1206 bp with the DNA of wild type and Δ *crp* mutant vaccinal strain but not amplified with the DNA of Δ *aroA* mutant as shown in Figure (3) and Table (4).

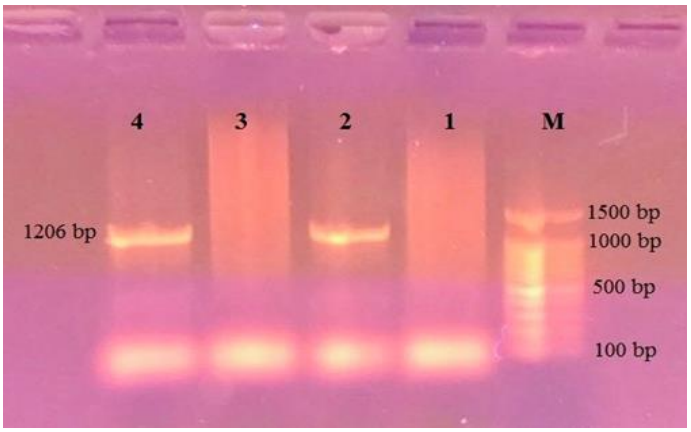

Figure 3. The result of PCR product of *aroA* gene from whole genomic DNA of *E. coli* strains. M, marker; Lane 1, Δ *aroA* mutant; Lane 2, Δ *crp* mutant; Lane 3, -ve control; Lane 4, wild O78 strain.

Regarding *crp* gene of the examined strains, the specific primer pair was successfully amplified and the PCR product of the amplified products were appeared under the UV transluminator at 1029 bps with only DNA of the wild and Δ *aroA* mutant but not with Δ *crp* mutant strains as shown in Figure (4) and Table (4).

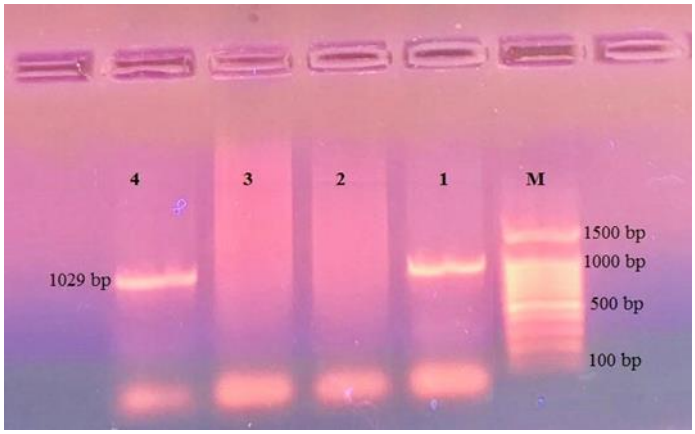

Figure 4. The result of PCR product of crp gene from whole genomic DNA of E. coli strains. M, marker; Lane 1, Δ aroA mutant; Lane 2, Δ crp mutant; Lane 3, -ve control; Lane 4, wild O78 strain.

Table 4. PCR amplification of wild and vaccinal *E. coli* strains.

Samples	No. of sample	Result of PCR		
		16S rRNA	aroA	crp
O78	1	+	+	+
Δ *aroA*	2	+	-	+
Δ *crp*	2	+	+	-
Total	5	3	2	2

Discussion

In birds *E. coli* infections cause many clinical manifestations with the most important are being airsaculitis, pericarditis, septicemia and may deaths (11). Colibacillosis due to virulent *E. coli* in chickens is characterized by a respiratory manifestation which is frequently followed by a generalized infection (12) resulting in significant economic losses through mortality, morbidity, cost of treatment and carcasses condemnation at processing plant. Live vaccine based on defined mutations that impair virulence is considered one of the most important methods to control the disease. Therefore, proper hygienic measures, accurate vaccination, good surveillance programs and most importantly, reliable detection method to differentiate the wild-type and vaccinal strains of *E. coli* are needed to control this disease (13). In the present study, the phenotypic and genotypic characterizations were used to differentiate between the

wild-type O78 strain, Δ aroA and Δ crp mutant vaccinal strains.

The pattern of growth on different media incubated at 37C° revealed that, the wild type and Δ *aroA* mutant demonstrated a large sized rounded, non-pigmented colonies on nutrient agar medium and large sized fermenting rounded, non-mucoid colonies on MacConkey and S.S media as shown in Table (2). These results were confirmed by (14) who stated that *aroA* deleted bacterial strains such as *E. coli* and Salmonella are unable to self-produce aromatic amino acids and p-aminobenzoate that are necessary for their growth and they cannot grow unless such nutrients are supplied from the outside so the *aroA* gene deleted E. coli strain formulates colonies after 24 hrs of culturing at 37C° on MacConkey and trypticase soya agar media but not on the minimum agar media. On the other hand, the Δ *crp* mutant strain showed small sized rounded, non-pigmented colonies on Nutrient agar medium and small sized non-fermenting rounded, non-mucoid phenotype on MacConkey and S.S media. Also (7) constructed and characterized the avian *E. coli* Δ *cya crp* mutants and confirmed the same difference of colonial size and fermentation characters on MacConkey agar between the wild and mutant strains. Both wild and two mutant strains were Gram negative, motile, non-sporulated, medium sized bacilli as illustrated in Table (2).

As regards to the biochemical activity of the used strains, the API 20E system was used because of the availability of large number of tests which was beneficial when comparing between the wild-type and the two type's mutant strains, in addition to its proven accuracy (15). Depending on the results illustrated in Table (3) and Figure (1), it was clear that the difference between the wild-type and Δ *aroA* mutant was inability of the mutant strain to produce ADH (argenine dihydrase) and fermentation of saccharose. On the other hand the Δ *crp* mutant failed to produce ADH and to ferment any of the carbohydrates (MAN, INO, SOR, RHA, SAC and MEL) except glucose (GLU). The same finding obtained by (7).

Positive results were obtained when *E. coli* polyvalent antisera was used with both wild and mutant E. coli strains. In the same concern a positive reaction was obtained when monovalent O78 antisera was used confirming the results of biochemical identification.

The wild *E. coli* strains are usually identified by detection of a specific virulence factor (16) such as *aroA* that codes 5-enolpyruvylshikimate 3-phosphate synthase, one of enzymes that govern the shikimate pathway for synthesizing aromatic amino acids involved with the growth and metabolism of bacteria and *crp* gene (gene encode for cAMP receptor protein) which is a regulatory molecule that are required for satisfactory operation of many genes involved in transport and breakdown of nutrients in bacteria. Deletion of such genes impairs the growth and metabolism of bacteria so the live vaccines based on defined mutations of these genes have been shown to reduce the virulence of *E. coli* strains. In the present study these genes were amplified to differentiate between the wild and vaccinal strains.

Firstly the genomic DNAs of the wild type and vaccinal *E. coli* strains were extracted, and its estimated size was more than 23 kbp. The used strains were confirmed as *E. coli* by successful amplification of the 16S rRNA gene giving rise to a PCR product of 585 bp with the all tested strains as shown in Figure (2) and Table (4). The same product obtain by (17) who identified *E. coli* isolated from naturally healthy broiler chickens in Bangladesh by PCR using the same primer and (10) who used PCR to detect the pathogenic *E. coli* through pathological study of the colibacillosis affected birds.

Regarding the amplification using specific *aroA* gene primers, it is successfully amplified giving rise aspecific PCR product of 1206 bp with the wild type and Δ crp mutant vaccinal strain DNAs but not amplified with Δ *aroA* mutant DNA as shown in Figure (3) and Table (4). These results has been found by (6) who constructed a genetically defined *aroA* mutant of a native *E. coli* O78:K80 isolated from avian colibacillosis in Iran. On the same concern, *crp* genes of the examined strains were tested and successfully amplified using its specific primers and the PCR amplicon was 1029 bps with the DNAs of wild and Δ *aroA* mutant strains but not with Δ *crp* mutant strain as shown in Figure (3) and Table (4). (7) obtain the same product when constructed and characterized avian E. coli *cya crp* mutants.

Multiplex PCR using the different three primers sets can be optimized and this may support results obtained in this work as the analyst can achieve his request in only one step and this will save either more time or effort to detect the presence or absence of the *aroA*, *crp* or *16S rRNA* genes.

Conclusion

From the obtained findings in this study, the use of methods based on molecular biological techniques such as PCR provides accurate, specific and sensitive discrimination of wild type from vaccinal mutant strains (13). Also, this study can help to differentiate between different types of *E. coli* strains either wild or vaccinal mutant type strains. In addition to, this study may help in exclusion or proving the return back to virulence of the mutant vaccinal strain of *E. coli* through following epidemiologically these scheme or plan of work.

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Informed Consent: N.A.
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