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# Serological and Molecular Typing of Avian Pathogenic *E. Coli* Originating from Outbreaks of Colibacillosis in Chicken Flocks

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**Abstract:** ***Avian pathogenic Escherichia coli (E. coli) is the etiological agents of colibacillosis, which is one of the principal causes of morbidity, mortality in poultry worldwide and responsible for significant economic losses to the poultry industry. Some outbreaks consistently associated with specific serotype, O111 causing mortality, septicemia and polyserositis with the chickens. Detection of Shiga toxin1, Shiga toxin 2 and Intimin genes that encode virulence factors, by multiplex PCR is an efficient method for identifying virulent strains of isolated E. coli. The objective of this study was serotyping of isolated E. coli, measurement of cytotoxicity of E.coli on Vero cell culture, detection of Shiga toxin1, Shiga toxin 2 and Intimin genes of isolated E. coli by using of Polymerase Chain Reaction. E.coli isolates was recovered from 510 samples with overall prevalence 75.2%. The most prevalent E. coli serovar isolates recovered from different sources of poultry broiler farms were Untypable E.coli serovare; followed by O26; then O2, O124, O125, and O115. Isolated E .coli caused third degree of the cytopathic effect of Vero cell sheet. Out of 20 E. coli isolates recovered from various broiler samples, 5 isolates were positive for Shiga toxin 1 (Stx1) gene, 3 for Intimin gene, and 3 were positive for the Shiga toxin 2 (Stx2) gene.***

**Keywords:** *Escherichia coli*, Shiga toxin 1, Shiga toxin 2, Intimin gene, broiler, Colibacillosis, Multiplex PCR.

## 1. Introduction

Colibacillosis is one of the most important diseases threatening the poultry industry**.** Pathogenic serogroups of *E.coli* are ubiquitous in environments in which poultry is raised. *E. coli* is a common inhabitant in the intestinal tract of poultry at higher concentrations in younger birds, birds without established normal flora and in the lower intestinal tract [1]. Coliform bacteria can be found in the litter and fecal matter.

However, *E. coli* accounts for only a small number of total bacteria in litter [2]. Feed ingredients and water are often contaminated with pathogenic Coliform and a common source of introducing new serovares into a flock [3]. Among healthy, normal chicken 10 to 15% of intestinal Coliform may be, belong to potentially pathogenic serotypes.

In Egypt, Ismailia proince, air sacculitis caused by *E .coli* is common among broiler chickens and layers and its serotypes O78, O26, O1, O2, O157 and O111 were the most commonly encountered serotypes [4]. Although, molecular methods for identifying specific virulence genes are available, serotyping remains a useful tool for epidemiologic studies.

Pathogenic *E. coli* strains have been broadly classified into two major categories: the Enteric pathogens and the Extra-intestinal pathogens. Enteric pathogenic *E. coli* have been broadly divided into Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Shiga toxin producing *E.coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E.coli* (EAEC) And diffusely adherent *E. coli* (DAEC) [5].

The virulence mechanisms that characterize *Escherichia coli* were genetically coded by chromosomal, plasmid, and bacteriophage DNAs and include heat-labile (LTI, LTIIa, and LTIIb) and heat-stable (STI and STII) toxins, Verotoxin types 1, 2 and 2e (VT1, VT2 and VT2e, respectively), cytotoxic necrotizing factors (CNF1 and CNF2), attaching and effacing mechanisms (eaeA), Enteroggregative mechanisms (Eagg), and Enteroinvasive mechanisms (Eniv) [6].

From a zoonotic point of view, STEC is the only *E. coli* pathogenicity group of major interest, as the shiga toxin-producing strains are able to cause severe disease in humans when being transmitted through the food chain from their animal reservoirs. [7] Avian *E. coli* strains from broiler flocks in Egypt, show similar sequence types to *E. coli* associated with human infection [8].

Escherichia coli colonies isolated from fresh dead hens of laying flocks with the E. coli peritonitis syndrome (EPS) were genotyped using pulsed-field gel electrophoresis (PFGE). Typing is important from an epidemiological point of view and also if the use of autogenous (auto)vaccines is considered [9].

DNA probes and polymerase chain reaction have been developed to detect specific genes important in virulence [10]. Multiplex PCR assays were developed for the detection

of the genetic characterization of (Shiga Toxin Escherichia coli) STEC in cultures of feces or foodstuffs.

Assay utilized four PCR primer pairs and detected the presence of $stx_1$, $stx_2$, (including variants of $stx_2$) and Intimin (eaeA). Multiplex PCR allowed the simultaneous amplification of more than one target sequence in a single PCR reaction, saving considerable time and effort and decreasing the number of reactions to be performed in order to assess the possible presence of microorganisms in sample [11]. Multiplex PCR–DNA probing assay provides rapid and specific detection of four major virotypes of *E. coli* [12].

This study was aimed to the isolation and identification of *E.coli* from different organs of broiler chicken as well as from feed, water and litter. Serotyping of isolated *E. coli* strains. Detection of Verotoxin of *E. coli* by using Vero cell culture. Detection of virulence genes of isolated *E. coli* by using of Polymerase Chain Reaction.

## 2. Material and Methods

**Samples:** A total of 918 samples (feed, water, litter and organs of recently dead chicken) was collected from four commercial broiler farms at Mashtol El-Souk and Abou-Hamad districts Sharkia Governorate, Egypt, as shown in Table (1).

**Table 1:** Distribution of samples of recently dead chicken (23-43 days old) as well as feed, water and litter samples from investigated farms.

| *Farm Names* | *Location* | *Feed* | *Water* | *litter* | *Number of recently dead chickens* | *Number of samples from freshly dead chickens* | | | | | | | | | *Total number of different samples* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *Liver* | *Lung* | *Kidney* | *Brain* | *Small intestine* | *Air sac* | *Bone marrow* | *Fresh heart blood* | *Total* | |
| Al-Afifi | Mashtol El-Souk district | 20 | 20 | 20 | 22 | 22 | 22 | 22 | 22 | 22 | 3 | 22 | 22 | 157 | 195 |
| Al-Tuhami | Abou-hamad district | 20 | 20 | 20 | 32 | 32 | 32 | 32 | 32 | 32 | 7 | 32 | 32 | 231 | 259 |
| Al-Khalawi | Mashtol El-Souk district | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 2 | 20 | 20 | 142 | 182 |
| Al-Habaiba | Mashtol El-Souk district | 20 | 20 | 20 | 21 | 21 | 21 | 21 | 21 | 21 | 1 | 21 | 21 | 148 | 187 |
| **Total** | | 80 | 80 | 80 | 95 | 95 | 95 | 95 | 95 | 95 | 13 | 95 | 95 | 678 | 918 |

**Isolation and identification of isolated *E. coli* [13]:**
Biochemical characterization of isolated *E. coli* by using conventional methods [14] and API20 E strips for confirmation of the isolated *E. coli* [15].

**In vitro virulence assay of *E. coli*:**
i. Haemolysis assay [16].
*E. coli* isolates were propagated on blood agar base supplemented with 5% washed sheep erythrocytes. Blood agar plates were incubated at 37°C for 24 hours, then examined for the presence of haemolysis or not.

ii. Congo red binding assay [17].
Each isolate was cultured on a separate plate of Congo red media and incubated at 37°C for 24 hours. After incubation, the cultures were left at room temperature for 48 hours to facilitate reading and separation of results. Invasive *E. coli* was identified by their ability to take up the Congo red dye. The positive isolates produced red colonies. The negative isolates appeared colorless.

**Serological identification of the isolates *E. coli*:**
Twenty isolates from different sources identified biochemically as *E. Cole* was subjected to serological identification according to [18], by using of polyvalent somatic antisera, for application of the slide agglutination test.

**Detection of cytotoxicity of *E. coli* on Vero cell culture.**
Thirty six isolates of *E. coli* recovered from examining samples randomly selected from organs of diseased freshly dead chicken, water, feed and water ,were subjected to testing their cytotoxic effect on Vero cell culture**.** The procedure of the experiments was carried out according to [19].

**Detection of $Stx_1$, $Stx_2$ and Intimin genes of isolated *E. coli* by using Multiplex PCR [20]:**
2.5.1. Oligonucleotide primers used for amplification of $Stx_1$, $Stx_2$ and Intimin of isolated *E. coli*: were summarized in Table (2).

**Table 2:** Oligonucleotide primers of $Stx_1$, $Stx_2$ and Intimin genes of *E.coli* [18].

| Virulence Factors | Accession number | Primer sequence 5'-3' | Position in open reading frame | Size of product (bp) |
|---|---|---|---|---|
| *Stx1* | Z36899 | TTCGCTCTGCAATAGGTA<br>TTCCCCAGTTCAATGTAAGAT | 125-142 of A subunit<br>659-679 of A subunit | 555 |
| Intimin | Z11541<br>S90827 | ATATCCGTTTTAATGGCTATCT<br>AATCTTCTGCGTACTGTGTTCA | 992-1013 of *eaeA*<br>1395-1416 of *aeaA* | 425 |
| *Stx2* | L11078 | GTGCCTGTTACTGGGTTTTTCTTC<br>AGGGGTCGATATCTCTGTCC | 30-53 of A subunit<br>128-147 of A subunit | 118 |

Extraction of DNA from *E. coli* isolates [21].

Estimation of purity and concentration of extracting DNA from isolated *E. coli* [19]: The concentration and purity of DNA were determined by measuring the optical density (OD) at a wavelength of 260 and 280 nm by using a spectrophotometer.

Amplification and cycling protocol of multiplex PCR [19]: The PCR cycling protocol was applied in a thermal cycler as follows, Initial denaturation at 94ºC/5min, followed by 30 cycles of denaturation at 95ºC/1 min, annealing at 55ºC/1min, extension at 72ºC/ 1 min and Final extension at 72ºC/ 5 min.

Screening PCR products by agarose gel electrophoresis [19]. Two grams of agarose were added to TAE buffer, heat in heating microwave, allowed to cool to 70ºC then 0.5 µg/ml ethidium bromides were added. The warm agarose was poured into the gel tray with the comb and allowed for polymerization at room temperature, then the comb was removed and electrophoresis track filled with TAE buffer. Ten µl of each PCR product samples were loaded to the gel after mixing with 1 µl loading buffer. A 1kd molecular size ladder was run with each gel. The power supply was adjusted to 10 Volt/cm of the tank length. The run stopped and the gel was transferred to UV transilluminator. The gel was photographed by using a digital camera.

**Statistical analysis** Data were statistically analyzed using Statistical Analysis Systems (SAS) program.

## 3. Results

**Prevalence of *E. coli* isolated from investigating broiler poultry farms.**

The prevalence rate of recovered *E. coli* isolates from all investigated poultry farm samples, including freshly dead broilers, feed, water and litters were presented in Table (3).

**Table 3:** Prevalence of *E. coli* isolates from all examined samples (feed, water, litter and organs of recently dead birds).

| *Type of samples* | *Incidence* | |
|---|---|---|
| | *No. of positive samples* | *%* |
| Feed (n=80) | 72 | 90 |
| Water (n=80) | 22 | 27.5 |
| Litter (n=80) | 64 | 80 |
| Fresh heart blood (n=95) | 72 | 75.79 |
| Liver (n=95) | 88 | 92.63 |
| Lung (n=95) | 47 | 49.47 |
| Kidney (n=95) | 65 | 68.42 |
| Brain (n=95) | 68 | 71.58 |
| Small intestine (n=95) | 77 | 81.05 |
| Air sac (n=13) | 11 | 84.61 |
| Bone marrow (n=95) | 82 | 86.32 |
| Total (n=918) | 668 | 72.77 |

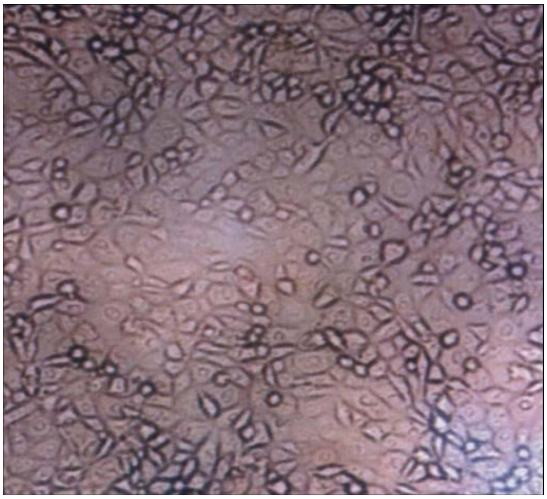
**Biochemical characterization of isolated *E. coli***
Conventional techniques are not sufficiently sensitive to distinguish between different strains and they are affected by physiological factors, while API20E system was identified about 98% of the isolated *E.coli.*

**In vitro virulence assay of *E.coli*:**
*E.coli* colonies produce clear zones of haemolysis as haemolysin positive pathogenic strains, while giving red colonies on Congo red medium (CR+ve).

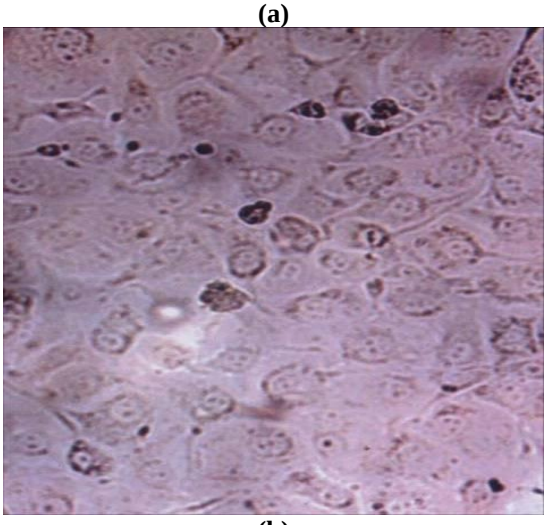
**Detection of verotoxin of *E. coli* by using Vero cell culture:**
Cytopathic effect on Vero cells was recorded as shown in Table (4) and Figure (1).

**Table 4:** Detection of verotoxin of *E. coli* by using Vero cell culture.

| *Type of Samples* | *No. of randomly E. coli isolates* | *No. of isolates positive for verotoxin* | *Degree of Positive Sign* |
|---|---|---|---|
| I-Feed | 4 | 2 | 25% |
| II-Water | 5 | 3 | 50 – 75 % |
| III-Litter | 3 | 2 | 25 – 50 % |
| IV-Heart Blood | 3 | 1 | 25% |
| V-Liver | 3 | 1 | 25% |
| VI-Kidney | 3 | 1 | 25% |
| VII-Brain | 3 | 2 | 25% |
| VIII-Small intestine | 4 | 3 | 50 – 90% |
| IX-Lung | 3 | 2 | 25% |
| X-Air sac | 3 | 2 | 50% |
| XI-Bone marrow | 2 | 1 | 50% |
| Total | 36 | 20 | - |



**(a)**



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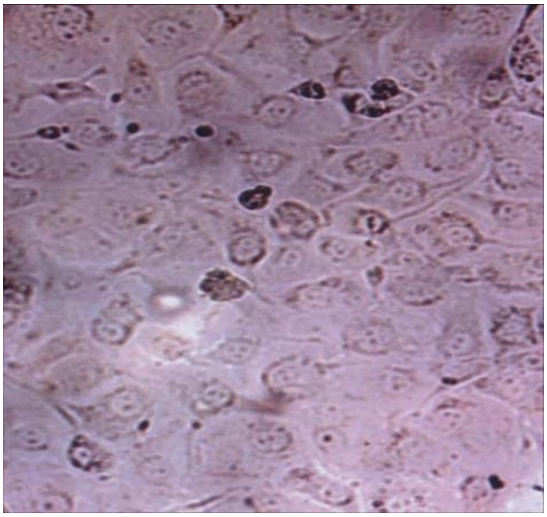

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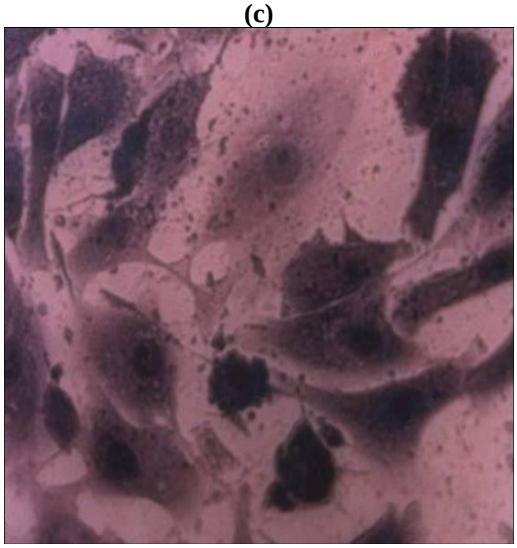


**(d)**

**Figure 1** (a) Normal Vero cells before inoculation, using inverted microscope. (b) Rounding of Vero cells after inoculation. (Cytopathic effect, first degree 25-50%). (c) Elongation and vacuolation of Vero cells (Cytopathic effect, second degree 50-75%). (d) Elongation and vacuolation with detachment of the part of cell sheet, lysis. (Cytopathic effect, third degree 75-90%).

**Serological identification of randomly selected *E. coli* isolates:**

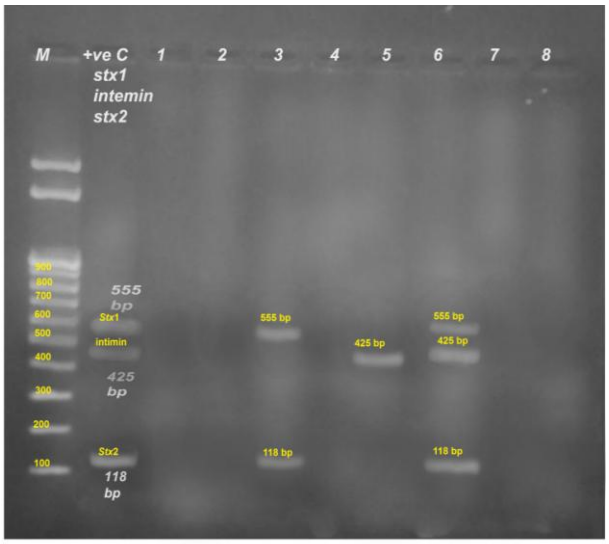
Twenty *E. coli* strains were examined serologically as shown in Table (5).

**Table 5:** Serotyping of *E. coli* isolates recovered from examined samples.

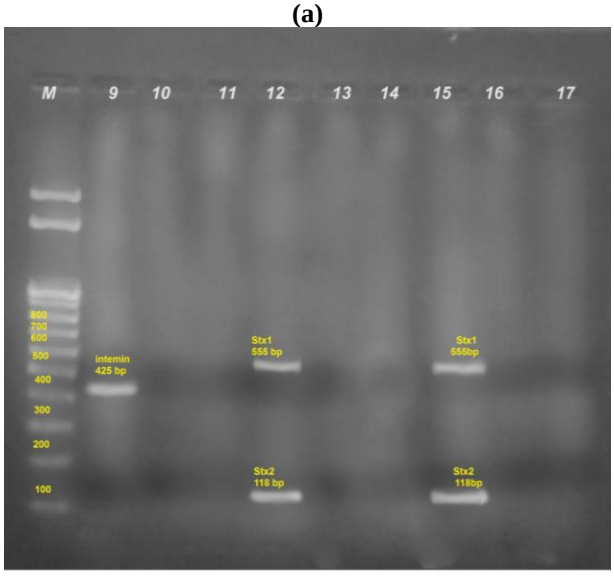
| *No. of randomly selected E. coli isolates* | *Source of isolates* | *Serotype* |
|---|---|---|
| 1 | Feed | $O_{124}$ |
| 2 | Feed | Untyped |
| 3 | Feed | Untyped |
| 4 | Water | $O_{26}$ |
| 5 | Water | $O_{128}$ |
| 6 | Water | Untyped |
| 7 | Water | Untyped |
| 8 | Litter | $O_{125}$ |
| 9 | Litter | Untyped |
| 10 | Litter | Untyped |
| 11 | Fresh heart blood | $O_{124}$ |
| 12 | Liver | $O_2$ |
| 13 | Liver | $O_{114}$ |
| 14 | Small intestine | $O_2$ |
| 15 | Small intestine | $O_{78}$ |
| 16 | Lung | $O_1$ |
| 17 | Lung | $O_{125}$ |
| 18 | Air sac | $O_{26}$ |
| 19 | Bone marrow | $O_{26}$ |
| 20 | Bone marrow | $O_{114}$ |

**Detection of virulence genes of isolated *E. coli* by using multiplex polymerase chain reaction (mPCR):**

Results of detection of $Stx_1$, $Stx_2$ and Intimin genes of 20 *E. coli* isolates by using multiplex PCR were presented in Figure (2).




**(a)**

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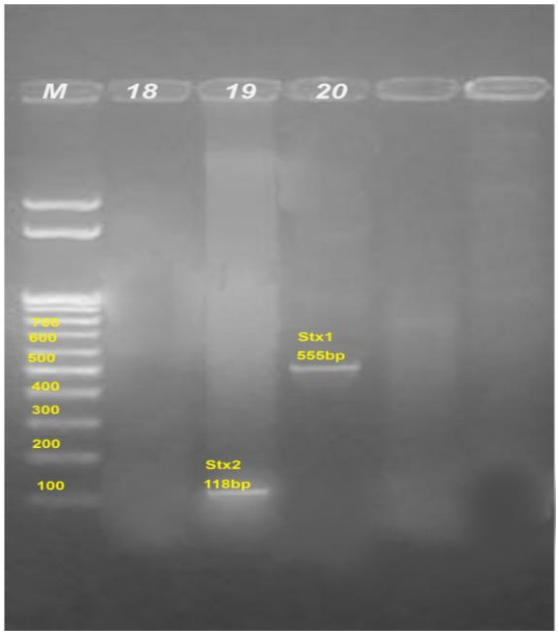


**(c)**
**Figure (2)**

Figure (2) Agarose gel electrophoresis of Stx1, Stx2 and Intimin genes from randomly selected E. coli (20 isolates). (a) Lane 1,2,4,7,8 represented negative amplification of Stx1, Stx2 and Intimin genes, while lane 3 positive amplification of Stx1 and Stx2 genes, Lane 5 positive Intimin gene and lane 6 is positive Stx1, Stx2 and Intimin genes (b) lane 10,11,13,14,16,17 represented the negative amplification of Stx1, Stx2 and Intimin genes while lane 9 positive amplification of Intimin gene and lane 12 show positive amplification of stx1 and stx2 genes.(c) Lane 18 represented he negative amplification of Stx1, Stx2 and Intimin genes while Lane 19 show positive amplification of Stx1 gene and lane 20 represented the positive amplification of Stx1 gene.

## 4. Discussion

In these study, bacteriological examination of 678 organ samples, which were collected from recently dead broilers, revealed that *E. coli* isolates were recovered from 510 samples with overall prevalence of 75.2%.

Concerning fresh heart blood samples, 72 out of 95 samples of the examined fresh heart blood were *E. coli* positive with an incidence of 75.8%. These results were nearly similar to what were recorded by [22]. Meanwhile, [23] reported a lower prevalence of *E. coli* in a percentage ranged from 9.5 - 40.5%. Out of 95 liver samples,88 samples were positive for presences of *E.coli* with a percentage of 92.6%. These results were disagree with [24] and [25], who reported lower incidence of *E.coli* in liver samples.

Bacteriological examination of 95 examined lung samples, revealed 47 positive samples for presence of *E.coli*, with a prevalence rate of 49.5%. These results was coincide with those detected by [4] and [26].

Regarding to the occurrence of *E. coli* isolates in feed samples,72 out of 80 feed samples were positive for the presence of *E.coli*, these results were agree with that detected by [27].

Concerning the prevalence of *E. coli* isolates in drinking water samples, twenty two water samples out of 80 water samples were positive for *E.coli* with an incidence of 27.5%. These was close to that detected by [28]. On the other hand, lower occurrence of isolated *E. coli* from water samples was detected by [29]. However, higher prevalence of *E.coli* in water samples was reported by [30].
However , bacteriological examination of litter samples indicate the presence of *E.coli* in 64 litter samples, with a prevalence rate of 80%. These result was nearly similar to finding that detected by [31]. While lower prevalence rate was reported by [26], with a percentage ranged from 5.9 - 38 %.

In the present study API20E system was used for biochemical identification of isolated *E. coli* strains, which identified about 98% of the isolates and this result was closely related to that reported by [32], who used the API20E system for identification of isolated G-ve bacteria from isolated samples.

The most prevalent *E. coli* serotype isolates recovered from different sources of poultry broiler farms were Untypable *E. coli* serovare; followed by $O_{26}$; then $O_2$, $O_{124}$, $O_{125}$, and $O_{114}$; and finally the lowest prevalent serotypes were detected to $O_1$, $O_{78}$, and $O_{128}$. These results were agree with [33].

Analysis of cytotoxic effect which generated by isolating *E. coli* isolates on Vero cell culture are presented in Table (4) and Figure 1, these results are supported by [29] and [30].

Regarding to the occurrence of $Stx_1$ gene in *E. coli* isolates, 5 *E.coli* isolates were detected, containing $Stx_1$ gene with size of 555 bp PCR amplification. These result was nearly similar to findings which were recorded by [7], [31], [32] and [33].

Concerning to examination of *E. coli* isolates for the presence of *Intimin* gene, results detected 3 out of 20 *E.coli* isolates, yielded the expected size of 425 bp PCR amplification products for the *Intimin* gene (Figure 2). Meanwhile, other *E. coli* isolates were PCR negative. These findings were nearly agreed with those obtained from [34] and [35].

Finally, PCR amplification of $Stx_2$ gene of isolated *E. coli* strains showed 3 *E.coli* isolates out of 20 isolates were positive for $Stx_2$ gene yielded a consistent fragment of 118 bp as shown in Figure 2. These results ws substantiate what were reported by [36] and [31].

## 5. Conclusion

*E. coli* has a high cytopathic effect on Vero cells with an incidence of 90%. Examination of 20 *E. coli* isolates by using multiplex PCR analysis detected the presence of positive amplification of $Stx_1$ (555 bp),Intimin (425 bp) and $Stx_2$ (118 bp).

## 6. Acknowledgment

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