Article

Investigation of Serotype Prevalence of *Escherichia coli* Strains Isolated from Layer Poultry in Greece and Interactions with Other Infectious Agents

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Abstract: Colibacillosis is the most common bacterial disease in poultry and it is caused by avian pathogenic *Escherichia coli* (APEC), which is assigned to various O-serogroups. Previous studies have shown that APEC strains are more often related to certain O-serogroups such as O78, O2 and O1. *E. coli* has been reported to act either as a primary or secondary agent in complicating other infections. The aim of this study was to investigate the occurrence of and characterize the O-serogroups of *E. coli* strains isolated from commercial layer and layer breeder flocks showing macroscopic lesions of colibacillosis and increased or normal mortality in Greece. Furthermore, we attempted to assess the interaction between infectious agents such as *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), infectious bronchitis (IBV) and infectious laryngotracheitis (ILT) with *E. coli* infections in layer flocks with increased mortality. Our study revealed that in addition to the common serogroups (O78, O2), many other, and less common serogroups were identified, including O111. The O78, O111 and O2 serogroups were frequently detected in flocks with increased mortality whereas O2, O88 and O8 were reported more commonly in birds with colibacillosis lesions but normal mortality rates. These data provide important information for colibacillosis monitoring and define preventative measures, especially by using effective vaccination programs because *E. coli* vaccines are reported to mainly offer homologous protection. Finally, concerning the association of the four tested infectious agents with *E. coli* mortality, our study did not reveal a statistically significant effect of the above infectious agents tested with *E. coli* infection mortality.

Keywords: commercial layers; *Escherichia coli*; layer breeder; predisposing factor; O-serogroup

1. Introduction

*Escherichia coli* (*E. coli*) is a common member of the gut microbiome in birds. However, several strains—known as avian pathogenic *Escherichia coli* (APEC)—are implicated in avian colibacillosis, which has been described as the most common bacterial disease in poultry, having a serious economic impact on poultry production [1–4]. APEC is responsible for different systemic or localized infections [5] such as colisepticemia (characterized by the presence of fibrinous exudates in various organs) [6], respiratory infections and airsacculitis [7], swollen head syndrome [1], peritonitis/salpingitis/salpingoperitonitis [4,8], yolk sack infections in day-old chicks and skin infections (cellulitis) [6,9].
E. coli can be a primary pathogen, causing clinical disease; occasionally, other predisposing factors must be present to help E. coli express its pathogenic effect. Various infectious agents have been described to be complicated with E. coli. Many viruses, especially those with an effect on the respiratory system such as paramyxovirus (Newcastle disease) [10], coronavirus (infectious bronchitis) [11,12], metapneumovirus (avian rhinotracheitis or swollen head syndrome) [13,14], orthomyxovirus (influenza) [15,16] and laryngotracheitis virus (infectious laryngotracheitis) [17] can trigger avian colibacillosis. Viruses that can act in a similar way to immunosuppressive factors such as herpesviruses (Marek disease) or circovirus (chicken anemia virus) can stimulate colibacillosis just as many other viruses can also stimulate E. coli infections [1,18]. Bacteria such as Mycoplasma gallisepticum [1] or Mycoplasma synoviae [19], or parasites such as Eimeria or Ascaridia (or red mites) can also predispose to an E. coli infection [1,20].

The scope of this study was to investigate the serogroup prevalence of avian E. coli strains isolated from birds demonstrating colibacillosis lesions, characterizing the presence and frequency of different O-somatic antigens, in Greece. As other studies worldwide have already considered with the broiler production, our project was focused on flocks from layer production. Most of the studies related to broilers have revealed the prevalent role of O78, O2 and O1 serogroups as APEC strains [1,2,7,8]. Furthermore, a high diversity of O-serogroups have also been isolated from broilers suffering from colibacillosis and the presence of untypeable E. coli strains has also been established [1,2,7].

As it has been reported that the use of E. coli vaccines induces homologous immunity to the vaccine strain used, data on the E. coli serotype status of an area can support colibacillosis vaccination prevention schemes [21,22]. Furthermore, we evaluated the possibility that other infectious agents such as Mycoplasma gallisepticum, Mycoplasma synoviae, the infectious bronchitis virus and the infectious laryngotracheitis virus correlated with the presence of colibacillosis and increased mortality in hens [23].

2. Materials and Methods
2.1. Sampling
A total of 60 different farms and 140 flocks, including birds with colibacillosis lesions from diverse geographical areas in Greece were included in the present study. All samples were collected during the period 2016–2017. A mortality threshold of 0.1% per week was used to divide the flocks between ‘normal’ or ‘increased’ mortality (ISA management guide) [24]. A total of 231 E. coli isolates were recovered from organ samples, including the liver, pericardium, air sacks, yolk sacks and peritoneum/ovaries, which were collected from rearing pullets, commercial layers and layer breeders. During each farm visit, we collected 3–5 dead birds per flock according to farm availability. All birds were necropsied and in case there was a demonstration of colibacillosis macroscopic lesions such as perihepatitis, pericarditis, air sacculitis, omphalitis and peritonitis (Figure 1a–d), sampling was performed from the most apparent lesion.

2.2. Bacterial Isolation
Bacterial isolation was performed according to the following protocol. All samples were streaked on MacConkey agar (Bioprepare, Attica, Greece) and sheep blood agar (Bioprepare, Attica, Greece). Colonies that were phenotypically identified as Escherichia coli were further assessed for their biochemical properties by an analytical profile index (API) system. A total of 43 samples was evaluated by MICROGEN API GNA+B in a vet lab (Vet Analysis Lab, Athens, Greece) and, as a result, 24 samples were biochemically confirmed as E. coli and 19 needed further confirmation. Additionally, 207 samples, the 19 inconclusive and 188 new samples, were sent for an analysis to the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna ‘Bruno Ubertini’, Italy. Biochemical confirmation was conducted using the same protocol (MICROGEN API GNABatch 41362, expanalyr 2018) and, for all inconclusive samples, a further biochemical confirmation was
conducted using API 20E (BioMerieux, Batch 1004845000, exp. 10 February 2017). In total, 231 samples were confirmed as *E. coli*.

![Figure 1. Macroscopic lesions indicating colibacillosis: (a) perihepatitis/pericarditis; (b) air sacculitis; (c) omphalitis; (d) peritonitis.](image)

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### 2.3. Serogroup Characterization

A total of 207 samples biochemically identified as *E. coli* in Italy were also serotyped by using a slow agglutination procedure in microtiter plates according to the Guinee agglutination method [24]. This technique is based on the agglutination that occurs when an *E. coli* culture is mixed with its homologous O antiserum [25,26].

The following agglutination protocol was used: a pure culture of *E. coli* isolates was obtained and it was used to prepare a bacterial suspension with a final concentration corresponding with a McFarland standard of 6.0. The suspension was treated at 100 °C for 1 h to obtain the final antigenic suspension. The heat treatment inactivated the K antigen. The bacterial suspension was then tested with a range of different antisera. Thirty different antisera that included the most common APEC serogroups (O1, O2, O5, O8, O9, O15, O18, O20, O22, O26, O45, O49, O55, O64, O78, O86, O88, O101, O103, O111, O113, O118, O128, O138, O139, O141, O147, O149, O153 and O157) were initially used for serotyping the *E. coli* strains. Each antiserum was diluted 1:40 and then inoculated in microtiter plates (U-bottom); a quantity of 100 µL of the antigenic suspension was then added in each well. The two suspensions were mixed and the plates were incubated at 37 °C for 18 h. After the incubation, the presence of agglutination was checked for positivity. Additionally, 24 samples that were confirmed in a laboratory (Vet Analysis Lab, Athens, Greece) as
E. coli were sent for serotyping using agglutination testing to the Biovac laboratory (Biovac, Beaucouze, France).

2.4. Data Collection of Predisposing Factors

To assess the relation of other infectious agents with colibacillosis-increased mortality, the occurrence of other pathogens was investigated. Additionally, the following data were obtained: the results of serological testing through an ELISA, an applied vaccination program regarding these specific infectious agents and the presence of clinical symptoms indicating the disease. For the serological testing, 10 blood samples were collected from the flock at the same visit when the E. coli sampling took place. When possible, a second sampling after 3–4 weeks (pair samples) was performed. The flocks that were sampled for serological testing were randomly selected and organized according to the compliance of the farmer to allow for blood sampling. Blood samples from each flock were collected by awing branchial vein puncture. The samples were centrifuged at 4500 rpm for 10 min and the serum was collected.

2.5. Mycoplasma gallisepticum and Mycoplasma synoviae

None of the flocks sampled were vaccinated against either Mycoplasma gallisepticum or Mycoplasma synoviae. Therefore, the presence of positive antibody titers revealed an exposure to mycoplasma. The presence of 20% positive samples out of the 10 samples collected from each flock led to a characterization of an ‘infected’ flock (OIE terrestrial manual) [27]. The serum was tested by the ELISA method (Zoetis/Proflok MG kit, lot 1,501,808 and Zoetis/Proflok MS kit, lot 1500907). When the samples were negative, a second sampling was performed within 3–6 weeks to check for seroconversion. Therefore, we managed to characterize the infection status of 61 different flocks.

2.6. Infectious Bronchitis

Concerning infectious bronchitis, all sampled flocks were vaccinated against both mass and variant strains during the rearing period. To characterize the flock IBV infection status, we considered the flock antibody titers in combination with the flock vaccination program and the presence of clinical symptoms that indicated an IBV infection.

In order to assess the antibody titers against IBV with the ELISA method (Zoetis/Proflok IBV kit, lot 132880), we collected blood samples from 51 flocks. For 37 flocks, we managed to perform a paired sampling at an interval of 3–5 weeks whereas for 14 flocks, a single sampling procedure was performed. For flocks that were pair sampled, seroconversion was checked. In cases where a single sampling was performed, the mean titer was compared according to the Zoetis IBV guidelines for infection. Very high titers that were not in accordance with the vaccination program used were considered to be suggestive of an infection.

2.7. Infectious Laryngotracheitis

Regarding infectious laryngotracheitis (ILT) testing, both vaccinated and unvaccinated flocks were included in our study. In order to perform a characterization of the flock infection status for ILT, we used the serological profile of the flocks performed with the ELISA method (Zoetis/Proflok ILT kit, lot 123146) in combination with the flock vaccination program and the presence of clinical symptoms that indicated an ILV infection. The presence of antibody titers in cases where no vaccination was applied combined with the presence of clinical symptoms/lesions indicated an infection. When a vaccination had been applied, a rise in the mean antibody titer along with the clinical appearance were taken into consideration. The procedure for collecting the blood samples was the same as for IBV. Therefore, we collected blood samples from 51 flocks. For 37 flocks, we used pair sampling whereas for 14 flocks, a single sampling procedure was followed.
2.8. Statistics

The percentages of each serotype in birds with high and low mortality and among the types of birds as well as the percentages of the serogrouped samples from each geographic area tested were compared using Med Calc software. The association between specific pathogens and increased mortality due to an E. coli infection was tested using a chi-squared test. For all the analyses, the statistical significance was set at $p < 0.05$.

3. Results

3.1. O-Serogroup Characterization

In total, 231 samples were confirmed as E. coli. The total O-serogroup characterization associated with the flock mortality is shown in Table 1.

Table 1. Summary of E. coli strain results according to flock mortality trait.

| O-Serogroup | Total No. Of Strains | % of Total No. | Normal Mortality | % of Total No. | Increased Mortality | % of Total No. |
|-------------|----------------------|----------------|------------------|----------------|--------------------|----------------|
| O78         | 16                   | 6.9            | 0                | 9.1            | 16                 | 6.2            |
| O2          | 15                   | 6.5            | 4                | 9.1            | 11                 | 6.2            |
| O111        | 15                   | 6.5            | 0                | 8.5            | 15                 | 8.5            |
| O88         | 6                    | 2.5            | 2                | 3.6            | 4                  | 2.2            |
| O8          | 4                    | 1.7            | 1                | 1.8            | 3                  | 1.7            |
| O45         | 3                    | 1.2            | 1                | 1.8            | 2                  | 1.1            |
| O147        | 2                    | 0.8            | 2                | 3.6            | 0                  | 0              |
| O103        | 1                    | 0.4            | 0                | 0.5            | 1                  | 0.5            |
| O18         | 1                    | 0.4            | 0                | 0.5            | 1                  | 0.5            |
| O15         | 1                    | 0.4            | 1                | 1.8            | 0                  | 0              |
| O5          | 1                    | 0.4            | 1                | 1.8            | 0                  | 0              |
| O1          | 1                    | 0.4            | 0                | 0.5            | 1                  | 0.5            |
| Serogrouped | 66                   | 28.6           | 12               | 21.9           | 54                 | 30.7           |
| Untypeable  | 165                  | 71.4           | 43               | 78.1           | 122                | 69.3           |

Different superscript letters (a, b, c, d) within a column and different O-serogroups indicate a statistical significance ($p < 0.05$). * A mortality percentage of 0.1% per week was regarded as normal.

The majority of strains (71.4% in total) were untypeable by the tested range of monospecific antisera. In flocks with increased mortality, a lower percentage (69.3%) of untypeable strains was observed compared with 78.1% of flocks with a normal mortality.

For strains where typing was achieved, without taking into consideration the flock mortality trait, a total of 12 O-serogroups were found. The most prevalent serogroups were O78 (6.9%), O2 (6.5%) and O111 (6.5%); the remaining isolates were O88 (2.5%), O8 (1.7%), O45 (1.2%), O147 (0.8%), O103 (0.4%), O18 (0.4%), O15 (0.4%), O5 (0.4%) and O19 (0.4%).

With reference to the division of samples according to the flock mortality trait, 9 serogroups were related to the increased mortality and 7 to the normal mortality group. The most prevalent serogroups between the isolates were: (a) O78 (9.1%), O111 (8.5%) and O2 (6.2%); and (b) O2 (7.2%), O88 (3.6%) and O147 (3.6%), originating from the increased and normal mortality flocks, respectively. Various other serogroups were confirmed for both groups, ranging from 0.5% to 2.2% (Table 1). The isolation frequency of O78 and O111 strains in flocks with increased mortality was significantly higher compared with the flocks with normal mortality ($p < 0.05$).

The serogroups classified based on the geographic distribution, type of birds and farm as well as per organ of origin are reported in Tables 2–5.
Table 2. Geographical distribution of serogrouped E. coli strains.

| Regions                        | O78 | O2  | O111 | O88 | O8  | O45 | O147 | O1  | O18 | O5  | O15 | O103 | % Grouped Strains | No. of Samples |
|--------------------------------|-----|-----|------|-----|-----|-----|------|-----|-----|-----|-----|------|-------------------|----------------|
| Attica                         | 10  | 4   | 3    | 1   | 2   | 1   | 1    | 1   | 2   | 1   | 1   | 1    | 31.82 a           | 66             |
| Sterea Ellada                  | 15  | 1   | 10   | 3   | 2   | 1   | 1    | 1   | 1   | 1   | 1   | 1    | 29.31 a           | 116            |
| Central and Western Macedonia  | 2   | 1   | 2    | 4   | 4   | 2   | 1    | 2   | 1   | 1   | 1   | 1    | 41.67 a           | 12             |
| Thrace and Eastern Macedonia   | 1   | 1   |      | 1   | 1   | 1   | 1    | 1   | 1   | 1   | 1   | 1    | 8.33 b            | 24             |
| Thessaly                       |     |     |      | 1   | 1   | 1   | 1    |     |     |     |     | 1    | 66.67 a           | 3              |
| Crete                          |     |     |      |     |     | 1   | 1    |     |     |     |     | 1    | 0.00 a            | 1              |
| Peloponnesus                   | 1   | 1   | 1    | 1   | 1   | 1   | 1    |     |     |     |     | 1    | 100.00 a          | 1              |
| Epirus                         |     |     |      |     |     |     |      | 1   | 1   | 1   | 1   | 1    | 20.00 a           | 5              |
| South Aegean Sea               | 1   | 1   | 1    | 1   | 1   | 1   | 1    |     |     |     |     | 1    | 0.00 a            | 1              |

Different superscript letters (a, b) within a column and different areas indicate a statistical significance ($p < 0.05$).

Table 3. Distribution of E. coli strains according to type of birds.

| Type of Birds                        | Rearing Farms: Pullets | Commercial Layers | Layer Breeders |
|--------------------------------------|------------------------|-------------------|----------------|
| Number of Samples                    | 65                     | 149               | 17             |
| Untypeable Samples                   | 39                     | 110               | 16             |
| Serogroups                           | No. | %   | No. | %   | No. | %   |
| O78                                  | 15  | 23.1 a | 1   | 0.7 b  | 0.0 b  | 0.0 b  |
| O2                                   | 0.0 b  | 15  | 10.1 c  | 0.0 b,c  | 0.0 b,c  |
| O111                                 | 4   | 6.2 c  | 11  | 7.4 c  | 0.0 b,c  | 0.0 b,c  |
| O88                                  | 1   | 1.5 b,c  | 4   | 2.7 b,c  | 1    | 5.9 b,c  |
| O8                                   | 3   | 4.6 b,c  | 1   | 0.7 b,c  | 0    | 0.0 b,c  |
| O45                                  | 0.0 b,c  | 3   | 2.0 b,c  | 0    | 0.0 b,c  |
| O147                                 | 0.0 b,c  | 2   | 1.3 b,c  | 0    | 0.0 b,c  |
| O103                                 | 1   | 1.5 b,c  | 0.0 b,c  | 0    | 0.0 b,c  |
| O18                                  | 0.0 b,c  | 1   | 0.7 b,c  | 0    | 0.0 b,c  |
| O15                                  | 0.0 b,c  | 1   | 0.7 b,c  | 0    | 0.0 b,c  |
| O1                                   | 1   | 1.5 b,c  | 0.0 b,c  | 0    | 0.0 b,c  |

Different superscript letters (a, b, c) within a column and different O-serogroups indicate a statistical significance ($p < 0.05$).

Table 3 reveals a differentiation in the serogroup prevalence according to age ($p < 0.05$). The O78 serogroup was predominant in layer chicks/pullet strains whereas the O2 serogroup was detected only in the laying flocks. Table 4 reveals the serogroup variation among the different farms/regions.

3.2. Interaction with Infectious Agents

The association of MG, MS, IBV and ILT infections with E. coli mortality is presented in Tables 6–9 while raw data are included in Supplementary files (Spreadsheets S1: MG, MS, IBV, ILT).

3.2.1. Mycoplasma gallisepticum and Mycoplasma synoviae

Mycoplasma serological monitoring revealed a high level of exposure to Mycoplasma gallisepticum and Mycoplasma synoviae. Regarding the Mycoplasma gallisepticum infection level, 78.94% of the flocks with normal E. coli mortality were positive against Mycoplasma gallisepticum whereas 73.81% of the flocks with increased E. coli mortality were positive. Most of the positive flocks, 42 out of 46, appeared to have all samples positive (10/10) whereas 2 flocks had 9/10 positive, 1 flock had 6/10 positive and 1 flock had 3/10 positive.

Concerning the Mycoplasma synoviae infection, the level of infection was even higher. A total of 94% of the flocks with normal mortality were positive whereas the percentage of flocks with increased mortality was 92.85%. Concerning the MS-positive flocks, 56 out
of 57 flocks had 10/10 positive serum samples whereas 1 flock was seroconverted from 0 positive samples to 2/10.

Table 4. Distribution of *E. coli* strains between farms with typeable serogroups.

| Farm | Type of Birds | Region | O-Serogroup (n) | Farm | Type of Birds | Region | O-Serogroup |
|------|---------------|--------|----------------|------|---------------|--------|-------------|
| No3  | LB            | A      | O88            | No24 | CL            | SE     | O2          |
| No4  | RFM           | SE     | O78 (7), O8 (2), O1 (1) | No27 | CL            | CWM    | O2          |
| No5  | RFM           | SE     | O78 (8), O111 (2) | No32 | CL            | CWM    | O88         |
| No6  | RFM           | TEM    | O88            | No34 | CL            | SE     | O2          |
| No7  | CL            | TEM    | O88            | No38 | CL            | SE     | O2          |
| No8  | CL            | O45    | No39           | No32 | CL            | SE     | O15         |
| No9  | CL            | A      | O111 (2)       | No42 | CL            | SE     | O2          |
| No10 | CL            | A      | O2 (5)         | No44 | CL            | A      | O147 (2)    |
| No11 | CL            | A      | O111 (4)       | No46 | CL            | A      | O88         |
| No12 | CL            | A      | O2 (2)         | No49 | RFM           | SE     | O111 (2)    |
| No13 | CL            | A      | O88            | No50 | RFM           | A      | O5, O8      |
| No14 | CL            | A      | O103           | No51 | RFM           | A      | O111 (2)    |
| No15 | CL            | SAS    | O2             | No53 | CL            | A      | O2          |
| No16 | CL            | CWM    | O111, O88      | No59 | CL            | SE     | O18, O8     |
| No17 | CL            | SE     | O111 (2)       | No60 | CL            | SE     | O15         |
| No18 | CL            | A      | O88            | No61 | CL            | SE     | O15         |

Type of birds: rearing farm pullets (RFM), commercial layers (CL), layer breeders (LB). Region: Attica (A), Sterea Ellada (SE), Central and Western Macedonia (CWM), Thrace and Eastern Macedonia (TEM), Thessaly (T), Crete (C), Peloponnesus (P), Epirus (E), South Aegean Sea (SAS), Northern Aegean Sea (NAS).

Table 5. Distribution of *E. coli* serogroups in relation to organ origin (number of strains).

| O-Serogroup | Pericardium/Liver Peritoneum | Air Sacks/Trachea | Yolk Sack |
|-------------|------------------------------|-------------------|----------|
| O78         | 14                           | 0                 | 2        | 0        |
| O2          | 7                            | 8                 | 0        | 0        |
| O111        | 12                           | 1                 | 2        | 0        |
| O88         | 4                            | 2                 | 0        | 0        |
| O8          | 0                            | 1                 | 2        | 1        |
| O45         | 0                            | 3                 | 0        | 0        |
| O147        | 0                            | 2                 | 0        | 0        |
| O103        | 1                            | 0                 | 0        | 0        |
| O18         | 0                            | 1                 | 0        | 0        |
| O15         | 0                            | 0                 | 0        | 0        |
| O5          | 0                            | 0                 | 0        | 1        |
| O1          | 0                            | 0                 | 1        | 0        |

Table 6. Status of MG infection among sampled flocks (MG: *Mycoplasma gallisepticum*).

| Flock Colibacillosis Mortality | MG Infection |
|--------------------------------|--------------|
|                                | No | Yes | Total |
| Normal                         | 4  | 15  | 19    |
| Increased                      | 11 | 31  | 42    |
| Total                          | 15 | 46  | 61    |

Table 7. Status of MS infection among sampled flocks (MS: *Mycoplasma synoviae*).

| Flock Colibacillosis Mortality | MS Infection |
|--------------------------------|--------------|
|                                | No | Yes | Total |
| Normal                         | 1  | 18  | 19    |
| Increased                      | 3  | 39  | 42    |
| Total                          | 4  | 57  | 61    |
Table 8. Status of IBV infection among sampled flocks (IBV: infectious bronchitis virus).

| Flock Colibacillosis Mortality | Suspect for IBV Infection |
|-------------------------------|---------------------------|
|                               | No | Yes | Total |
| Normal                        | 14 | 0   | 14    |
| Increased                     | 31 | 6   | 37    |
| Total                         | 45 | 6   | 51    |

Table 9. Status of ILT infection among sampled flocks (ILT: infectious laryngotracheitis).

| Flock Colibacillosis Mortality | Suspect for ILT Infection |
|-------------------------------|---------------------------|
|                               | No | Yes | Total |
| Normal                        | 13 | 1   | 14    |
| Increased                     | 27 | 10  | 37    |
| Total                         | 40 | 11  | 51    |

3.2.2. Infectious Bronchitis

The characterization of the flocks for the IBV infection status is shown in Table 8. The term ‘suspect for infection’ was used because no definitive diagnosis could be performed. Concerning the flocks tested, three flocks showed a remarkable increase of the mean titer in combination with respiratory symptoms and abnormal shell eggs whereas three other unvaccinated flocks showed very high antibody titers. Those high titers could indicate an IBV infection according to the Zoetis guidelines.

None of the 14 flocks with normal mortality that were checked for an IBV infection were characterized as suspect for infection. Concerning the 31 flocks with increased mortality, 6 flocks were characterized as suspect for infection (16.21%). Three of these flocks showed a high increase in the antibody titers in the second blood sampling (seroconversion), which could not be explained by the vaccination schedule; it was combined with the clinical symptoms of infectious bronchitis (respiratory and eggshell abnormalities). The other three flocks gave extremely high titers of antibodies and were considered to be suspect for infection according to Zoetis guidelines.

3.2.3. Infectious Laryngotracheitis

The characterization of the flocks for the ILT infection status is shown in Table 9 and the term ‘suspect for infection’ was used in order to indicate a possible infection.

From the ILT infection characterization results, it emerged that in flocks with normal mortality, only 1out of 14 flocks (7.14%) was suspected to have an ILT infection whereas 10 out of 37 flocks were suspected to have an ILT infection (27.02%) among the flocks where E. coli was isolated and showed increased mortality. All the flocks that were characterized as suspect for infection had clinical symptoms and tracheal macroscopic lesions suspected for ILT. Eight of these flocks had positive serological results without being vaccinated and three flocks showed a rise in the mean titer at a pair blood sampling.

4. Discussion

The presence of untypeable E. coli isolates has been reported in previous studies [28–36]. Approximately 180 O antigens are used today to characterize the strains in O-serogroups [1]. Therefore, the number of strains that remain untypeable is related to the number of applied monospecific antisera. In the present study, a series of 30 antisera was used in two different labs. The use of a higher number of antisera might reduce the presence of untypeable strains. Nevertheless, in studies where all (181) antisera were applied for E. coli characterization, several isolates still remained untyped [37–39]. The reason for unsuccessful typing could be the presence of surface antigens such as the K antigen in the bacterial cell of E. coli that inhibits the agglutination of the O antigen [26]. Furthermore, it is not possible to serotype rough strains that can autoagglutinate and the presence of new serotypes that have not yet been identified remains a possibility [1].
In our study, *E. coli* of serogroups O78 and O2 showed that these were the most prevalent serogroups identified among the flocks with increased mortality in both pullets and layers, which was in agreement with previous studies [29,32,33,35,36,40–48], confirming their predominance in many parts of the world and in both broilers and layers with colisepticemia. Furthermore, a variety of *E. coli* serogroups manifested between increased and normal mortality flocks, which has also been established in different research projects [34,41,42,49]. However, O78, O2 and O1 serogroups are not always the most prevalent serogroups of APEC strains in epidemiological studies. In contrast to our findings, various other serogroups have been reported to be predominant [30,39,50,51].

The presence of O111 *E. coli* strains in birds with colibacillosis has been previously demonstrated. Zanella et al. [52] detected O111 isolates in layers with colibacillosis and polyserositis in Italy. Srinivasan et al. [53] found that the O111, O166 and O64 *E. coli* serogroups were the most common ones in layers with egg peritonitis in India whereas another study in the U.S. also found O111 and O78 strains in layers with peritonitis [54]. Furthermore, Giovanardi et al. [55] managed to detect an *E. coli* strain that was assigned to the O111 serogroup in a turkey suffering from colibacillosis and Khalifa et al. [56] reported the isolation of O111 *E. coli* strains in one-week-old broiler chicks with omphalitis. Finally, Mora et al. [57] revealed the presence of two emerging clonal serogroups of the O111 serogroup, emphasizing the increasing occurrence of this serogroup during recent years in Spain.

Other serogroups that were identified in our study have also been previously reported in studies of poultry suffering with colibacillosis such as O88 [33,46], O8 [46,50], O1 [40,43,44,47,48], O18 [31,43,47,48], O45 [46,48], O103 [31], O5 [30,42], O15 [30,35] and O147 [42]. Several serogroups were exclusively present in the increased mortality flocks (O78, O111, O18, O1 and O103) or within the normal mortality group (O147, O15 and O5) where as several serogroups were detected in both groups of birds (O2, O88, O8 and O45). These findings are in agreement with several research projects where common O-serogroups were isolated both from birds with normal mortality and birds with clinical colibacillosis [34,37,43]. Furthermore, Rodriguez-Siek et al. [31] attempted to characterize and compare APEC and fecal strains, reporting that several isolates from each category were assigned to unshared serogroups; however, common serogroups were detected in both the APEC and fecal strains. To summarize our findings with relevant studies conducted at broiler farms, the high prevalence of the O78 and O2 groups was in accordance with similar references from research projects performed on broiler poultry. The presence of less common serogroups such as O111 [56,57] has also been reported in broilers similar to various other serogroups [32,34,37,42] or untypeable strains [28,32,34].

The pathogenicity of *E. coli* strains is attributed to different virulence factors. There is a huge diversity in the virulence factors of APEC strains. Virulence factors are divided into adhesins, iron acquisition systems, invasions, toxins and protectins [58] and although it seems that there is no specific APEC genotype for all strains, it has been reported that certain virulence factor patterns are more likely to be detected in APEC strains compared with non-pathogenic *E. coli* strains. As a result, many different serogroups might include pathogenic strains [7,40,59] and strains that belong to the same serogroup could differ in pathogenicity [60]. Several serogroups such as O78 and O2 have been found to include strains that more often contain certain virulence patterns and thus have a pathogenic action. This explains why those serogroups are more prevalent in studies investigating APEC serogroups. The presence of an increased number of virulence factors or of certain virulence factor patterns that are responsible for *E. coli* pathogenicity in serogroups O78 and O2 has been reported in various research projects [31,40,43,61,62]. Similar findings have been reported for pathogenic *E. coli* strains that belong to the O111 serogroup. Yaguchi et al. [34] revealed that O111 isolated strains were homogenous regarding their virulence gene pattern and consistent with the important virulence factors of APEC strains. It seems that the virulence factor characterization might contribute to the identification of APEC strains.

Finally, in the present study, we observed a different serogroup occurrence trait of two serogroups (O78, O2) between the two age-dependent groups of birds (rearing and laying
period). The O78 serogroup was mainly found in young birds in comparison with the O2 serogroup that was detected only in the laying flocks. On the contrary, Dias da Silveira et al. [30] observed no differences in the presence of *E. coli* serogroups of isolates between day-old broiler chicks and adult broilers with colibacillosis. Similar findings of shared serogroups between different aged groups of birds were reported by Paudel et al. [63].

Our attempt to find an association between *Mycoplasma* and colibacillosis revealed a high prevalence of both *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections in the layers. However, no statistically significant difference was recorded between *Mycoplasma gallisepticum* infections and increased mortality \( (p = 0.66) \) due to colibacillosis. Similar findings were observed for *Mycoplasma synoviae* infections \( (p = 0.78) \).

The data for *mycoplasma* prevalence in poultry vary from country to country. Nevertheless, *Mycoplasma synoviae* infections in layers seem to be high in many countries (Germany: 95%; U.S.: 84%; UK: 78%) [64]. A Dutch survey reported that the surveillance program for *Mycoplasma synoviae* infections in commercial layer flocks revealed 73% positive flocks [65]. A Belgian study in commercial layers with *E. coli* infections revealed a high level of bird flock infections from *Mycoplasma synoviae* whereas none of the flocks were infected by *Mycoplasma gallisepticum* [41].

Our findings were in accordance with several other research projects that revealed no significant interaction between *Mycoplasma gallisepticum/synoviae* infections and colibacillosis outbreaks [41] or revealed that colibacillosis can occur in mycoplasma-free birds [66]. On the other hand, several studies have established the predisposing role of *Mycoplasma* [19,67,68]. *Mycoplasma gallisepticum* or *synoviae* infections may play a role in stimulating colibacillosis mortality. However, bird exposure to *mycoplasmas* is not necessarily followed by a colibacillosis outbreak because in our study, both MG and MS showed a high prevalence in the normal mortality flocks. This trait could be explained by the fact that *mycoplasma* can infect poultry and remain in a latent state, waiting for the appropriate combination of infectious agents or environmental factors to cause clinical disease [27]. Furthermore, the role of MS in respiratory infections is not always pronounced.

Concerning the role of IBV and ILT, no statistically significant difference was observed between the flocks with normal and increased *E. coli* mortality. However, it seemed that the flocks that were possibly infected with IBV tended to have a higher risk of mortality related to an *E. coli* infection because the six flocks suspected for an *E. coli* infection had increased *E. coli* mortality. Similar findings were reported for ILT where the occurrence of infection was higher in the increased *E. coli* mortality groups. This could be explained by the pathogenic action of IBV and ILT damaging the respiratory epithelium and thus facilitating the establishment of the *E. coli* infection.

In contrast to our findings, the relationship of IBV and *E. coli* has been reported in various research projects [11,69–71] as well as the association between ILT infections and colibacillosis [17,72]. However, our data about IBV and *E. coli* infections were in agreement with [41], who reported no significant relationship between those two agents.

5. Conclusions

To the best of our knowledge, this study provides, for the first time, an insight into the prevalent *E. coli* serogroups circulating in commercial layer and layer breeder flocks in Greece. Although several serogroups identified in our study such as O78 and O2 have frequently been reported to include APEC strains, less common serogroups such as O111 were also detected. This finding indicated that many different serogroups could be associated with colibacillosis and further molecular epidemiological studies should be executed to unravel specific APEC characteristics. Furthermore, these data can be used to develop more efficient intervention strategies against colibacillosis such as vaccination schemes because vaccination stimulates a mainly homologous protection against the used vaccine serotype.

Our investigation on the association between *E. coli* mortality and certain infectious agents such as MG, MS, IBV and ILT did not reveal a statistically significant effect of those
specific infectious agents on increased mortality related to an *E. coli* infection in layer poultry flocks. However, the triggering role of other infectious agents cannot be excluded. Therefore, further investigations should take place because a better understanding of the various predisposing factors can help in the effective prevention of *E. coli* infections and thus allow antimicrobial use to decrease in poultry production.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci9040152/s1, Spreadsheet S1: MG, MS, IBV, ILT.

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**References**

1. Barnes, H.J.; Nolan, L.K.; Vaillancourt, J.P. Colibacillosis in poultry. In *Diseases of Poultry*, 12th ed.; Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E., Eds.; Blackwell Publishing: Ames, IA, USA, 2008; pp. 691–732.

2. Luful Tabir, S.M. Avian colibacillosis and salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Inter. J. Environ. Res. Public Health* **2010**, *7*, 89–114. [CrossRef] [PubMed]

3. Zhuang, Q.Y.; Wang, A.S.C.; Li, A.J.P.; Liu, A.D.; Shuo Liu, A.B.; Ming Jiang, A.W.; Ming Chen, J.A. Clinical survey of common avian infectious diseases in China. *Avian Dis.* **2014**, *58*, 297–302. [CrossRef]

4. Landman, W.J.M.; van Eck, J.H.H. The incidence and economic impact of the *Escherichia coli* peritonitis syndrome in Dutch poultry farming. *Avian Pathol.* **2015**, *44*, 370–378. [CrossRef]

5. Koutsianos, D.; Athanasiou, L.; Spyropoulou, M.; Prentza, Z.; Dedousi, A.; Polizopoulou, Z.; Mossialos, D.; Koutoulis, K.C. Evaluation of hematological variables in layer pullets after vaccination and challenge with *E. coli*. *Comp. Clin. Pathol.* **2021**, *30*, 113–118. [CrossRef]

6. Dinev, I. *Escherichia coli* infections. In *Diseases of Poultry—A Colour Atlas*, 1st ed.; 2M Print House Ltd.: Stara Zagora, Bulgaria, 2007; pp. 8–19.

7. Guabiraba, R.; Schouler, C. Avian colibacillosis: Still many black holes. *FEMS Microbiol. Lett.* **2015**, *362*, fnv118. [CrossRef] [PubMed]

8. Brugère-Picoux, J.; Vaillancourt, J.P.; Bouzouaia, M.; Venne, D.; Shivaprasad, H.L. *Manual of Poultry Diseases*, 1st ed.; Association Française pour l’Avancement des Sciences: Paris, France, 2015; pp. 300–315.

9. Norton, R.A. Avian cellulitis. *World’s Poult. Sci. J.* **1997**, *53*, 337–349. [CrossRef]

10. El Tayeb, A.B.; Hanson, R.P. Interactions between *Escherichia coli* and Newcastle disease virus in chickens. *Avian. Dis.* **2002**, *46*, 660–667. [CrossRef]

11. Matthijs, M.G.; Van Eck, J.H.; Landman, W.J.; Stegeman, J.A. Ability of Massachusetts-type infectious bronchitis virus to increase colibacillosis susceptibility in commercial broilers: A comparison between vaccine and virulent field virus. *Avian Path.* **2003**, *32*, 473–481. [CrossRef]

12. Dwars, R.M.; Matthijs, M.G.; Daemen, A.J.; van Eck, J.H.; Verveelde, L.; Landman, W.J. Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus. *Vet. Immunol. Immunopathol.* **2009**, *27*, 65–76. [CrossRef]

13. Nakamura, K.; Mase, M.; Tanimura, N.; Yamaguchi, S.; Nakazawa, M.; Yuasa, N. Swollen head syndrome in broiler chickens in Japan: Its pathology, microbiology and biochemistry. *Avian Path.* **1997**, *26*, 139–154. [CrossRef]
14. Al-Ankari, A.R.; Bradbury, J.M.; Naylor, C.J.; Worthington, K.J.; Payne-Johnson, C.; Jones, R.C. Avian pneumovirus infection in broiler chicks inoculated with Escherichia coli at different time intervals. Avian. Pathol. 2001, 30, 257–267. [CrossRef]

15. Mosleh, N.; Dadras, H.; Asasti, K.; Taebipour, M.J.; Tohidifar, S.S.; Farjanikish, G. Evaluation of the timing of the Escherichia coli co-infection on pathogenicity of H9N2 avian influenza virus in broiler chickens. Ir. J. Vet. Res. 2017, 18, 86–91.

16. Samy, A.; Naguib, M.M. Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: Field and experimental findings. Vet. Sci. 2018, 5, 23. [CrossRef]

17. Nakamura, K.; Imai, K.; Tanimura, N. Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. J. Compar. Pathol. 1996, 114, 11–21. [CrossRef]

18. Umar, S.; Munir, M.T.; Alsan, U.; Raza, I.; Chowdhury, M.R.; Ahmed, Z.; Shah, M.A.A. Immunosuppressive interactions of viral diseases in poultry. World’s Poult. Sci. J. 2017, 73, 121–135. [CrossRef]

19. Raviv, Z.; Ferguson-Noel, N.; Laibinis, V.; Wooten, R.; Kleven, S.H. Role of Mycoplasma synoviae in commercial layer Escherichia coli peritonitis syndrome. Avian Dis. 2007, 51, 685–690. [CrossRef]

20. Flechlay, A.S.; Thomas, E.; Sparagano, O. Poultry red mite (Dermanyssus gallinae) infestation: A broad impact parasitological disease that still remains a significant challenge for the egg-laying industry in Europe. Parasit. Vectors 2017, 10, 357. [CrossRef]

21. Ghanaim, H.; Abu-Madi, A.M.; Kariyawasam, S. Advances in vaccination against avian pathogenic Escherichia coli respiratory disease: Potentials and limitations. Vet. Microbiol. 2014, 172, 13–22. [CrossRef]

22. Koutsianos, D.; Gantelet, H.; Franzo, G.; Lecoupeur, M.; Thibault, E.; Cecchinato, M.; Koutoulis, K.C. An assessment of the level of protection against colibacillosis conferred by several autogenous and/or commercial vaccination programs in conventional pullets upon experimental challenge. Vet. Sci. 2020, 7, 80. [CrossRef]

23. Andreopoulou, M.; Franco, G.; Tucciareone, C.M.; Pretzta, Z.; Koutoulis, K.C.; Cecchinato, M.; Chaligianni, I. Molecular epidemiology of infectious bronchitis virus and avian metapneumovirus in Greece. Poult. Sci. 2019, 98, 5374–5384. [CrossRef]

24. ISA Poultry Product Management Guide. Available online: https://www.isa-poultry.com/documents/597/ISA_Brown_CS_Cage_English_guide.pdf (accessed on 22 July 2020).

25. Guinee, P.A.M.; Agterberg, C.M.; Jansen, W.H. Escherichia coli 0 Antigen typing by means of a mechanized microtechnique. Appl. Microbiol. 1972, 24, 127–131. [CrossRef]

26. Orskov, I.; Orskov, F.; Jann, B.; Jann, K. Serology, chemistry, and genetics of O and K antigens of Escherichia coli. Rev. Proc. 1975, 9, 667–710. [CrossRef]

27. Office International des Epizooties (OIE). Terrestrial Manual—online edition—Avian Mycoplasmosis. 2018, pp. 844–859. Available online: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.05_AVIAN_MYCO.pdf (accessed on 26 January 2022).

28. Allan, B.J.; van den Hurk, J.; Potter, A.A. Characterization of Escherichia coli isolated from cases of cloacibacillosis. Can. J. Vet. Res. 1993, 57, 146–151.

29. Ngeleka, M.; Kwaga, J.K.P.; White, D.G.; Whittam, T.S.; Riddell, C.; Goodphore, R.; Potter, A.A.; Allan, B. Escherichia coli cellulitis in broiler chickens: Clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect. Immun. 1996, 64, 3118–3126. [CrossRef] [PubMed]

30. Dias da Silveira, W.; Ferreira, A.; Brochi, M.; Maria de Hollanda, L.; Pestana de Castro, A.F.; Tatsumi Yamada, A.; Lancellotti, M. Biological characteristics and pathogenicity of avian Escherichia coli strains. Vet. Microbiol. 2002, 85, 47–53. [CrossRef]

31. Rodriguez-Siek, K.E.; Giddings, C.W.; Doetkott, C.; Johnson, T.J.; Nolan, L.K. Characterizing the APEC pathotype. Vet. Res. 2005, 36, 241–256. [CrossRef] [PubMed]

32. Zhao, S.; Maurer, J.J.; Hubert, S.; De Villena, J.F.; McDermott, P.F.; Meng, J.; Ayers, S.; English, L.; David, G.; White, D.G. Antimicrobial susceptibility and molecular characterization of avian pathogenic Escherichia coli isolates. Vet. Microbiol. 2005, 107, 215–224. [CrossRef]

33. D’Incau, M.; Pennelli, D.; Lavazza, A.; Tagliabue, S. Serotypes of E. coli isolated from avian species in Lombardia and Emilia Romagna (North Italy). Ital. J. Anim. Sci. 2006, 5, 298–301. [CrossRef]

34. Yaguchi, K.; Ogitani, T.; Osawa, R.; Kawano, M.; Kokumai, N.; Kaneshige, T.; Noro, T.; Masubuchi, K.; Shimizu, Y. Virulence factors of avian pathogenic Escherichia coli strains isolated from chickens with colisepticemia in Japan. Avian Dis. 2007, 51, 656–662. [CrossRef]

35. Camarda, A.; Circella, E.; Pennelli, D.; Battista, P.; Di Paola, G.; Madio, A.; Tagliabue, S. Occurrence of pathogenic and faecal Escherichia coli in layer hens. Ital. J. Anim. Sci. 2008, 7, 385–389. [CrossRef]

36. Heydel, C.; Leidner, U.; Fruth, A.; Weber, R.; Ewers, C. Phylogenetic diversity and serotype distribution among Avian Pathogenic Escherichia coli (APEC). In Proceedings of the World Veterinary Poultry Association Congress, Bangkok, Thailand, 16–20 September 2019; p. 365.

37. Blanco, J.E.; Blanco, M.; Mora, A.; Jansen, W.M.; Garcia, V.; Vazquez, M.L.; Blanco, J. Serotypes of Escherichia coli isolated from septicaemic chickens in Galicia (Northwest Spain). Vet. Microbiol. 1998, 61, 229–235. [CrossRef]

38. Wang, S.; Meng, Q.; Dai, J.; Han, X.; Han, Y.; Ding, C.; Liu, H.; Yu, S. Development of an allele-specific PCR assay for simultaneous sero-typing of avian pathogenic Escherichia coli predominant O1, O2, O18 and O78 strains. PLoS ONE 2014, 9, e96904. [CrossRef] [PubMed]

39. Knobl, T.; Moreno, A.M.; Paixao, R.; Tardelli Gomes, T.A.; Midollo Vieira, M.A.; da Silva Leite, D.; Blanco, J.E.; Piantino Ferreira, A.J. Prevalence of Avian Pathogenic Escherichia coli (APEC) Clone Harboring sfa Gene in Brazil. Sci. World J. 2012, 7, 437342.
40. Ewers, C.; Janßen, T.; Kießling, S.; Philipp, H.; Wieler, L.H. Molecular epidemiology of avian pathogenic *Escherichia coli* (APEC) isolated from colisepticaemia in poultry. *Vet. Microbiol.* 2004, 104, 91–101. [CrossRef]

41. Vandekerchove, D.; De Herdt, P.; Laevens, H.; Pasmans, F. Colibacillosis in caged layer hens: Characteristics of the disease and the aetiological agent. *Avian Pathol.* 2004, 33, 117–342. [CrossRef]

42. McPeake, S.J.W.; Smyth, J.A.; Ball, H.J. Characterization of avian pathogenic *Escherichia coli* (APEC) associated with colisepticaemia compared to faecal isolates from healthy birds. *Vet. Microbiol.* 2005, 110, 245–253. [CrossRef]

43. Schouler, C.; Scaheffer, B.; Bree, A.; Mora, A.; Dahbi, G.; Biet, F.; Oswald, E.; Mainil, J.; Blanco, J.; Moulin-Schouleur, M. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. *J. Clin. Microbiol.* 2012, 50, 1673–1678. [CrossRef]

44. Wun Jeong, Y.; Kim, T.E.; Kim, J.H.; Kwon, H.J. Pathotyping avian pathogenic *Escherichia coli* strains in Korea. *J. Vet. Med. Sci.* 2012, 13, 145–152.

45. Dou, X.; Gong, J.; Han, X.; Xu, M.; Shen, H.; Zhang, D.; Zhuang, L.; Liu, J.; Zou, J. Characterization of avian pathogenic *Escherichia coli* isolated in eastern China. *Genes* 2016, 576, 244–248. [CrossRef]

46. Hyun Kim, J.; Wan Kim, D.; Jin Lee, H.; Yeon Jeong, J.; Mi Jeong, O.; Kuk Kwon, Y.; Su Kang, M. Antimicrobial resistance and O serogroup distribution of avian pathogenic *Escherichia coli* in Korea. In Proceedings of the World Veterinary Poultry Association Congress, Bangkok, Thailand, 16–20 September 2019; p. 355.

47. Ibrahim, R.A.; Cryer, T.L.; Lafti, S.Q.; Basha, E.A.; Good, L.; Tarazi, Y.H. Identification of *Escherichia coli* from broiler chickens in Jordan, their antimicrobial resistance, gene characterization and the associated risk factors. *BMC Vet. Res.* 2019, 15, 159. [CrossRef]

48. Xu, X.; Sun, Q.; Zhao, L. Virulence factors and antibiotic resistance of Avian Pathogenic *Escherichia coli* in eastern China. *J. Vet. Res.* 2019, 63, 317–320. [CrossRef] [PubMed]

49. Solá-Ginés, M.; Cameron-Veas, K.; Badiola, I.; Dolz, R.; Majó, N.; Dahbi, G.; Viso, S.; Mora, A.; Blanco, J.; Piedra-Carrasco, N.; et al. Diversity of Multi-Drug Resistant Avian Pathogenic *Escherichiacoli* (APEC) Causing Outbreaks of Colibacillosis in Broilers during 2012 in Spain. *PloS ONE* 2015, 10, e0143191. [CrossRef] [PubMed]

50. Wang, X.M.; Liao, X.P.; Zhang, W.Z.; Jiang, H.X.; Sun, J.; Zhang, M.J.; He, X.F.; Lao, D.X.; Liu, Y.H. Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of Avian Pathogenic *Escherichia coli* in south of China. *Foodborne Pathog. Dis.* 2010, 7, 1099–1106. [CrossRef]

51. Ali, A.; Abd El-Mawgoud, A.I.; Dahshan Al-Hussien, M.; EL-Sawah, A.A.; Nasef, A. *Escherichia coli* in broiler chickens in Egypt, its virulence traits and vaccination as an intervention strategy. *Novel Res. Microbiol.* J. 2019, 3, 415–427. [CrossRef]

52. Zanella, A.; Alborali, G.L.; Bardotti, M.; Candotti, P.; Guadagnini, P.F.; Anna Martino, P.; Stoner, M. Severe *Escherichia coli* O111 septicemia and polyserositis in hens at the start of lay. *Avian Path.* 2000, 29, 311–317. [CrossRef]

53. Srinivasan, P.; Balasubramaniam, G.A.; Krishna Murthy, T.R.G.; Balachandran, P. Bacteriological and pathological studies of egg peritonitis in commercial layer chiken in Namakkal area. *Asian Pac. J. Trop. Biomed.* 2013, 3, 988–994. [CrossRef]

54. Trampel, D.W.; Wannemuehler, Y.; Nolan, L.K. Characterization of *Escherichia coli* isolates from peritonitis lesions in commercial laying hens. *Avian Dis.* 2007, 51, 840–844. [CrossRef]

55. Giovanardi, D.; Lupini, C.; Pesente, P.; Rossi, G.; Ortali, G.; Catelli, E. Characterization and antimicrobial resistance analysis of avian pathogenic *Escherichia coli* isolated from Italian turkey flocks. *Poult. Sci.* 2013, 92, 2661–2667. [CrossRef]

56. Khalifa, E.; Hamed, N.A.; El-Rahman, A. *Escherichia coli* as a causative agent in omphalitis in broiler chicks. *Animal Health Res. J.* 2013, 1, 26–35.

57. Mora, A.; López, C.; Herrera, A.; Viso, S.; Mamani, R.; Dahbi, G.; Alonso, M.P.; Blanco, M.; Blanco, J.E.; Blanco, J. Emerging avian pathogenic *Escherichia coli* strains belonging to clonal groups O111:H4-D-ST2085 and O111:H4-D-ST117 with high virulence-gene content and zoonotic potential. *Vet. Microbiol.* 2012, 156, 347–352. [CrossRef]

58. Koutsianos, D.; Athanasiou, L.; Mossialos, D.; Koutoulis, K.C. Colibacillosis in poultry: A disease overview and the new perspective for its control and prevention. *J. Hellenic Vet. Med. Soc.* 2020, 71, 69–75. [CrossRef]

59. La Ragione, R.M.; Woodward, M.J. Virulence factors of *Escherichia coli* serotypes associated with avian colisepticaemia. *Res. Vet. Sci.* 2002, 73, 27–35. [CrossRef]

60. Dziva, F.; Stevens, M.P. Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. *Avian Pathol.* 2008, 37, 355–366. [CrossRef]

61. Vandekerchove, D.; Vandemeule, D.; Adriaensen, C.; Zaleska, M.; Hernalsteens, J.P.; De Baets, L.; Butaye, P.; Van Immerseel, F.; Wattiau, P.; Laevens, H.; et al. Virulence-associated traits in avian *Escherichia coli*: Comparison between isolates from colibacillosis-affected and clinically healthy layer flocks. *Vet. Microbiol.* 2005, 108, 75–87. [CrossRef]

62. Johnson, T.J.; Wannemuehler, Y.; Doekkott, C.; Johnson, S.J.; Rosenberger, S.C.; Nolan, L.K. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *J. Clin. Microbiol.* 2008, 46, 3987–3996. [CrossRef]

63. Paudel, S.; Stessl, B.; Hess, C.; Zloch, A.; Hess, M. High genetic diversity among extraintestinal *Escherichia coli* isolates in pullets and layers revealed by a longitudinal study. *BMC Vet. Res.* 2016, 12, 221. [CrossRef]

64. Landman, W.J.M. Is *Mycoplasma synoviae* outrunning *Mycoplasma gallisepticum*? A viewpoint from the Netherlands. *Avian Pathol.* 2014, 43, 2–8. [CrossRef]

65. Feberwee, A.; De Vries, T.S.; Landman, W.J.M. Seroprevalence of *Mycoplasma synoviae* in Dutch commercial poultry farms. *Avian Pathol.* 2008, 37, 629–633. [CrossRef]
66. Landman, W.J.M.; Heuvelink, A.; Van Eck, J.H.H. Reproduction of the *Escherichia coli* peritonitis syndrome in laying hens. *Avian Pathol.* **2013**, *42*, 157–162. [CrossRef]

67. Bradbury, J.M. Poultry mycoplasmas: Sophisticated pathogens in simple guise. *Br. Poult. Sci.* **2005**, *46*, 125–136. [CrossRef]

68. El Aziz, N.K.; Eldesoky, I.E.; Ammar, A.M.; Eissa, S.I.; Mohamed, Y.H. Molecular studies on *Mycoplasma gallisepticum* and Avian Pathogenic *E. coli* induced infections in broilers. *Eur. J. Vet. Med.* **2014**, *2014*, 4.

69. Nakamura, K.; Cook, J.K.A.; Frazier, J.A.; Narita, M. *Escherichia coli* multiplication and lesions in the respiratory tract of chickens inoculated with Infectious Bronchitis Virus and/or *E. coli*. *Avian Dis.* **1992**, *36*, 881–890. [CrossRef] [PubMed]

70. Peighambari, S.M.; Julian, R.J.; Gyles, C.L. Experimental *Escherichia coli* respiratory infection in broilers. *Avian Dis.* **2000**, *44*, 759–769. [CrossRef] [PubMed]

71. Matthijs, M.G.; Ariaans, M.P.; Dwars, R.M.; van Eck, J.H.; Bouma, A.; Stegeman, A.; Vervelde, L. Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*. *Vet. Immunol. Immunopathol.* **2009**, *127*, 77–84. [CrossRef]

72. Gowthaman, V.; Singh, S.D.; Dhama, K.; Barathidasan, R.; Anjaneya Bhatt, P. Avian Pathogenic *E. coli* associated with respiratory disease complications in poultry. *Vet. Pract.* **2013**, *14*, 430–431.