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Assessment of biofilm production in *E. coli* isolated from broilers farm water systems.

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ABSTRACT

An *Escherichia coli* biofilm consists of a bacterial colony surrounded by a protective extracellular polymeric substance (EPS) matrix, shielding the microorganisms from environmental threats and preventing infections.

The development of antibiotic resistance exacerbates the diversity in structural elements of biofilm, making it more difficult to eradicate.This study aimed at biofilm production evaluation in *E. coli* isolated from the broiler water system. one hundred swabs were collected from drinking water pipes and drinkers from different broiler farms in Giza and Dakahlia governorates in Egypt. The swabs and were examined for the presence of *E. coli and* their ability for biofilm production. Fifty swabs were positive for *E. coli* after biochemical and serological identification of isolates. The microtiter-plate test for determination of biofilm production showed that forty isolates produced biofilm at different degrees, 13 (26%) strong biofilms, 17 (34%) intermediate biofilms, and 10 (20%) weak biofilms. Ten isolates out of 50 (20%) exhibited no biofilm. Furthermore, PCR analysis targeting the adrA gene, associated with cellulose synthesis and biofilm formation in E. coli, confirmed the presence of this gene in all thirteen isolates categorized as strong biofilm producers. The study revealed diverse serotypes of *E. coli* in broiler farms, emphasized the prevalence of biofilm formation, particularly among strong biofilm producers carrying the *adr*A gene, highlighting the importance of stringent biosecurity measures and continuous monitoring to mitigate potential health risks in poultry farming environments.

1. INTRODUCTION

Escherichia coli, a Gram-negative, rod-shaped bacterium within the Enterobacteriaceae family, is a prevalent and pathogenic agent in avian populations, causing diseases in both fowls and mammals, with its primary and secondary presence collectively termed Avian Pathogenic *E. coli* (APEC) (Ewers et al., 2004).

Avian colibacillosis, caused by *E. coli*, is a major infectious disease in birds of all ages and is characterized by a diverse array of lesions such as perihepatitis, pericarditis and air sacculitis, egg peritonitis, salpingitis, omphalitis, coli granuloma, arthritis, osteomyelitis, cellulitis, septicemia, and death of the birds (Jordan et al., 2005).

The nutritional importance of managing the water that birds drink cannot be overstated because they drink twice as much water as they do foodEnsuring optimal growth and feed efficiency in poultry, facilitated by genetic selection, ideal growing conditions, and precise nutrition programs, becomes challenging in the absence of water quality assurance. (Maharjan et al., 2017).

The majority of bacteria in water distribution systems are found adhering (as a biofilm) to interior surfaces. A small amount of bacteria are also present in the water phase (Bagh et al., 2004).

The quality of the water can be affected by microbial activity and, in some situations, pose a major health concern to consumers when pathogenic bacteria proliferate in biofilms or when biofilms serve as a haven for invasive bacteria like enteropathogenic *E.coli*. (Silhan et al., 2006).

Drinker lines in poultry houses gradually develop microbial biofilms as a result of the accumulation of different minerals, dirt, rust, and algae over time. Accordingly, birds, especially chicks, are still susceptible to microbial biofilm threats (Zimmer et al., 2003).

Microorganism colonies and extracellular polymeric substances (EPS) that the organisms themselves manufacture form a biofilm, which is formed when microorganisms of the same or different species spontaneously encase themselves in a matrix of EPS that serves as both an environmental barrier and a surface for attachment to living or non-living carriers. Additionally, biofilms clog water filters and pipelines, restricting the flow of water, which can result in poorer flock performance (Maharjan et al., 2017)

Reversible fixation is made possible in the first stages of biofilm development by flagellar motility, which allows for the range to the surface. This low-intensity connection allows the bacterium to spread freely, but it also makes it easy to remove the germ using cleaning processes. (Haddock et al., 2010).

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The shocking development rate of disinfectant-resistant bacteria will significantly reduce the effectiveness of disinfectants (Zhu et al., 2021), and the often-found multidrug-resistant bacteria and multidrug-resistance genes constitute a serious threat to ecosystem health and human health (Cazares et al., 2020).

Bacteria create a variety of responses when they engage with disinfectants to prevent disinfectant penetration and subsequently lower their concentration of bacterial membrane surfaces through the biofilm (Zhang et al., 2019). The disinfectant cannot enter the bacteria because of the reduced permeability of their membranes (Vergalli et al., 2020).

A mechatronic sensor for a surface was developed by researchers (Pereira et al., 2008) that can differentiate between cleaning techniques (chemical or biological) and offer details on the existence of biofilms in their early phases and cleaning agents on the surface.

The first and most important step in improving the sanitization of equipment and facilities is cleaning. Surfactants or alkalis, which are widely used for cleaning, are utilized to suspend and dissolve food residues by reducing surface tension, denaturing proteins, and emulsifying fats.

The aim of this study is to investigate the prevalence of Escherichia coli in water pipes and drinkers on broiler farms, analyze the serotypes of the isolates, assess their biofilm formation capabilities, and detect the presence of the *adr*A gene, providing insights into the distribution and characteristics of *E. coli* strains in the context of broiler farm environments.

2. MATERIAL AND METHODS

2.1. Samples collection:

From 2020 to 2022 one hundred water swabs were collected from drinking water pipes and drinkers from different broiler farms in the Giza and Dakahlia governments. Swabs were collected aseptically from the pipe of the main water supply and drinkers then the swabs were transmitted through transport media to the laboratory in Animal Health Research Institute, Dokki for bacteriological examination (Cheesbrough, 1984).

2.2. Isolation of E. coli

Firstly, each swab was inoculated in nutrient broth then a loopful from the broth was streaked across the surfaces of Tryptone soya Agar (TSA), MacConkey agar, Eosin methylene blue agar (EMB), and Xylose Lysine Deoxycholate agar (XLD agar) and incubated at 37 °C for 24 hours on XLD agar medium (Cheesbrough, 1984).

2.3. Cultural and morphological identification

The Gram stain was applied to the suspected medium-sized colonies and examined under a microscope. Straight, non-sporulated Gram-negative rods that were either judged to be typical or suspicious were picked out and preserved in the semisolid agar medium for further identification according to Murray et al. (2003).

2.4. Biochemical identification

The suspected colonies characterized development of pink-red color indicated positive result with Voges Proskauer test. Development of brilliant red colored ring indicated positive indole test (Murray et al., 2003).

2.5. Serological identification of E. coli isolates

By using polyvalent and monovalent rapid diagnostic *E. coli* antisera (Denka Seiken Co., Ltd. Tokyo, Japan), and according to methods described by Lee et al. (2009), the confirmed fifty *E. coli* isolates were serotyped in the laboratories of Animal Health Research Institute, Dokki, Giza, Egypt.

2.6. Microtiter-plate test for determination of biofilm production.

According to Stepanović et al. (2000), fifty bacterial isolates were distributed into three wells of each sterile 96-well tissue culture plate, subjected to a 24-hour incubation, and assessed for biofilm formation using crystal violet staining and optical density measurement at 620 nm. The resulting optical density values, adjusted for background absorbance, served as indicators of the extent of bacterial adherence and biofilm development on the plate surfaces.

Following the equations below, the data were used to categorize the strains as non-, weak, moderate, or strong biofilm producers:

ODc < OD ≤ 2 × ODc weakly adherent to surface

2 × ODc < OD ≤ 4 × ODc moderately adherent to surface

4 × ODc < OD strongly adherent to the surface

2.7. PCR detection of adrA gene in E. coli isolates

2.7.1. Extraction of DNA

Isolates of strong biofilm production was selected for detetion of *adr*A gene using PCR. , extraction with done by the guidelines provided by the QIAamp DNA mini kit.

2.7.2. DNA extraction and purification

The High pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) was used to extract genomic DNA from heat-inactivated pure cultures by the manufacturer's instructions. The quantity and caliber of DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) Tables (1 and 2)

2.7.3. Agarose gel electrophoreses (Sambrook et al., 1989) DNA Purification and Extraction

Table 1 *adr*A gene sequence

Gene	Primer sequence	Amplified product	Reference
*adr*A	ATGTTCCCA AAAATAATG AA TCATGCCGC CACTTCGGT GC	1113 (bp)	Bhowmick et al. (2011)

Table 2 Cycling conditions of the *adr*A gene primer during PCR

Primary denaturation	94˚C	5 min.
Secondary denaturation	94˚C	30 sec.
Annealing	50˚C	1 min.
Extension	72˚C	1 min.
No. of cycles	35	
Final extension	72˚C	10 min.
Reference	Bhowmick *et al*. (2011)	

3. RESULTS

3.1. Isolation and identification of *E. coli*

Fifty *E. coli* isolates were isolated and identified by cultural characters cultivated on XLD agar medium. Typical *E. coli* colonies had a yellow color, pink colonies on MacConkey agar, and dark green metallic seen on EMB agar colonies. The Gram stain was applied to the suspected

colonies, which were then picked up and examined under a microscope. The colonies showed medium-sized, straight, non-sporulated Gram-negative.

3.2. Biochemical identification

Out of one hundred isolates, fifty swabs confirmed with biochemical test positive, citrate test negative, indole test positive, methyl red test positive, and Voges-Proskauer test negative.

3.3. Serotypes of *E. coli* isolates:

As shown in table (3), serotyping of fifty *E. coli* isolates recovered from broilers indicates that serotype O91 was the highest incidence , followed by serotypes O78 (18%), and serotypes O26 (8%).

Table 3 Serotyping of fifty *E. coli* isolates recovered from water pipes and drinkers in broiler farms.

Serotype	Number of isolates	%	Serotypes	Number of isolates	%
O166	3	6	O103	3	6
O26	4	8	O126	2	4
O91	10	20	O125	2	4
O144	2	4	O78	9	18
O158	2	4	O129	1	2
O6	1	2	O28	1	2
O27	2	4	O55	1	2
O159	3	6	O142	1	2
O128	2	4	O123	1	2

3.4. Biofilm detection of fifty *E. coli* isolates

Out of fifty *E. coli* isolates, forty isolates were positive for biofilm production by the Microtiter-plate test with different degrees, 13 (26%) strong biofilms, 17 (34%) intermediate biofilms and 10(20%) weak biofilms.

3.5. PCR for detection of *adr*A gene in *E. coli* isolates

All thirteen strong biofilm producer isolates were positive to the *adr*A gene at 1113 bp as in Fig. (1).

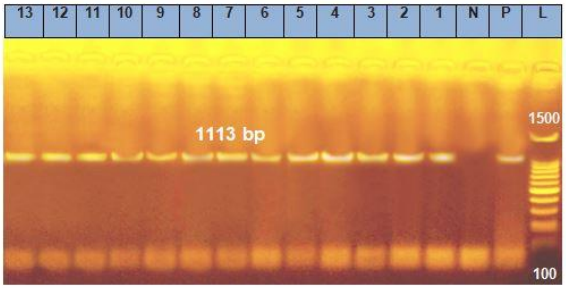

Fig 1 Lane1-13: Agarose gel electrophoresis of PCR amplicons after amplification of *adr*A gene bands at 1113bp, Lane p: positive control (*E. coli*: NCIMB 50034) Lane L: Negative control (*S. Entritidis:* ATCC 13076)

4. DISCUSSION

This study was conducted with one hundred samples collected from water pipes and drinkers on broiler farms, isolation and biochemical identification revealed that fifty swabs were positive for *E. coli* (50%). These results agreed with de Oliveira Branco et al. (2016**).** Eight of the fifteen *E. coli* strains found in the drinkers' swabs produced biofilm.

Regarding water quality, *E. coli* growth was found in 15 (45.45%) of the 33 water samples that were examined (de Oliveira Branco et al., 2016). Serotyping of 50 *E. coli* isolates recovered from broilers indicates that serotype O91 showed the highest incidence (20 %), followed by serotypes O78 (18%), and serotypes O26 (8%)**.** These results agreed with those In Jordan, a study by (Ibrahem et al., 2017)demonstrated a prevalence of 23.79, 14.86, and 12.63% for the common serotypes O78.

The findings highlight the diversity of *E. coli* serotypes present in the broiler population. This diversity is consistent with previous research, which has shown that *E. coli* is a highly adaptable bacterium capable of producing a wide array of serotypes (Ibrahem et al., 2017).

Rosario et al. (2004) revealed that several samples from a vertically integrated poultry operation in Mexico had various serotypes of *E. coli* bacteria. The most common serogroups among the 257 *E. coli* isolated from diverse samples were O19 (12%), O84 (9%), O8 (6%), and O78 (5%), and 85% of the organisms had a known serogroup these results agreed with our study.

Understanding the distribution of specific serotypes is crucial because some *E. coli* serotypes are known to be more pathogenic than others. For example, certain serotypes, such as O91, can be associated with severe diseases in poultry, including avian colibacillosis (Jordan et al., 2005). Consequently, the prevalence of these serotypes can have direct implications for the health and welfare of broiler flocks.

The prevalence of distinct serotypes holds epidemiological significance. It can aid in tracing the origin and transmission patterns of *E. coli* within and between broiler farms. This knowledge is essential for implementing targeted interventions and biosecurity measures to reduce the risk of disease spread (Ibrahem et al., 2017).

In the present study, we choose a Microtiter plate of 96 wells which is considered a qualitative method for the determination of biofilm formation this is in line with Abou Zeid et al. (2021), who stated that the Congo red agar and microtiter plate methods were employed to assess the production of biofilms. In Zagazig city, EL-Sharkia Governorate, Egypt, the biofilm formation of all the bacterial isolates was assessed using Congo Red Agar plates (CRA) and Tissue Cultures Plate (TCP) methods employing microtiter plates for quantification of biofilm formation after the crystal violet staining.

This study revealed that forty isolates from fifty produced biofilm with 96 microtiter plates. Which was divided into 10 samples out of 50 (20%) no biofilm, forty samples out of fifty produce biofilm degree classified into 13 samples out of fifty (26%) strong biofilms, 17 isolated out of 50 (34%) intermediate biofilms and 10 isolates out of 50 (20%) week biofilm. These findings agree with those published by (Kadhim Mohammed, 2022) that revealed 7 (14%), 28 (56%), and 15 (30%) isolates were weakly, moderately, and firmly adherent respectively, by using the microtiter plate method to measure biofilm formation.

The findings underscore the importance of implementing rigorous biosecurity measures on broiler farms. Effective biosecurity practices can help prevent the introduction and dissemination of pathogenic *E. coli* strains. Regular monitoring and control of water sources, sanitation, and bird health are essential components of biosecurity (Zimmer et al., 2003).

The presence of the *adr*A gene in all strong biofilm-forming isolates confirms its role as a key player in the biofilm-formation process. This gene likely contributes to the adhesive properties of *E. coli* and its ability to attach to surfaces and develop biofilms. (Zhang et al., 2019).

This study found that PCR detection of the *adr*A gene test revealed that the gene was present in every strain of the isolates that produced strong biofilms. This outcome was consistent with those of Yin et al. (2018), who estimated that the prevalence of the gene was close to 75% across all strains that produced strong biofilm.

The association between the *adr*A gene and strong biofilm formation is of particular concern due to the role of biofilms

in antibiotic resistance. Biofilm-embedded bacteria are often less susceptible to antibiotic treatments, and a better understanding of the genetic factors involved in biofilm formation can inform strategies to combat antibiotic resistance (Vergalli et al., 2020).

5. CONCLUSIONS

The study findings highlighted the significant prevalence of *E. coli* in drinkers and water pipe swabs from broiler farms, indicating a potential risk of waterborne *E. coli* infections in these environments. The identification of multiple *E. coli* serotypes, with serogroup O91 being the most widespread, underscores the diverse nature of the pathogen present. Furthermore, the study revealed the varying abilities of the isolated *E. coli* strains to produce biofilms, supported by the presence of the biofilm-related *adrA* gene.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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