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Insight into The prevalence of The planktonic and Biofilm-producing *Yersiniaa enterocolitica* in Poultry Meat in Egypt

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BACKGROUND: *Yersinia enterocolitica* (*Y. enterocolitica*) is a psychrotrophic food-borne pathogen that can cause gastrointestinal disease in humans. *Y. enterocolitica* is characterized by its capacity to grow at lower degrees and to form biofilm in the food chain. In our study, we aimed to assess the incidence of the planktonic *Yersinia enterocolitica* strains recovered from poultry meat sources (chicken, duck, geese, and pigeon) in addition to determining their ability to produce biofilm in Egypt. A total of 220 samples were gathered randomly from poultry meat and were subjected to conventional culture techniques in order to isolate *Y. enterocolitica* strains. All the suspected colonies were further examined via uniplex Polymerase Chain Reaction (PCR) using the16S rRNA-specific gene to confirm the *Y. enterocolitica* strains. All the confirmed isolates were diagnosed for their potential to form biofilm *in vitro* using the crystal violet glass tube method. A whole occurrence of *Y. enterocolitica* was 5.91% (13/220); *Y. enterocolitica* had been recovered from raw chicken meat, raw duck meat, and raw geese meat at rates of 6.67% (10/150), 5% (2/40), and 4.35% (1/23), respectively. Raw pigeon meat shows no contamination with *Y. enterocolitica*. A total of nine strains were found to be in biofilm form, while only four strains were a planktonic form. The incidence of *Y. enterocolitica* in food remains low. Despite this, the planktonic cells were found to have a recovery rate lower than the biofilms which have an industrial and public health concern and must be controlled.

Keywords: *Yersinia enterocolitica*, Poultry, Duck, Geese, Biofilm.

Introduction

Yersinia enterocolitica, a Gram-negative non-spore-forming rod belonging to the Enterobacteriaceae family, was not recognized as a human or veterinary microorganism until around the 1960s when it began to cause foodborne gastrointestinal illnesses [1]. *Y. enterocolitica* was discovered to be a psychrotrophic bacterium that can live and grow in low temperatures [2]. In 2015, yersiniosis, which is primarily caused by *Y. enterocolitica*, was reported among the first three most recovered foodborne illnesses in Europe [3].

The consumption of *Y. enterocolitica* contaminated food is the main cause of human illness that affects the host by causing severe enteritis accompanied by fever, inflammation of lymph nodes, and bloody diarrhea, which results in severe consequences as laparotomy due to pseudoapppendicitis in humans [4]. It usually affects more in young children and infants [5]. Extra-intestinal and post-infectious symptoms, such as reactive arthritis and erythema nodosum, have been identified. [6]. Previous reports found it difficult to determine the exact infection dose causing yersiniosis. Although, they agreed about

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the dose must exceed 4 log colony-forming units (CFU) and can reach 7- 9 log cells [7, 8].

Y. enterocolitica can be isolated from both aquatic or animal sources, including pigs, poultry, cows, and sheep [9]. Despite this diverse spectrum of animal reservoirs, swine colonization and transmission to related food products constitute a severe hazard to humans and are regarded as the primary reservoir [10]. Previous studies discussed poultry and ready-to-eat foods as a source for *Y. enterocolitica* infection [11, 12]. Poultry meat is often contaminated with *Y. enterocolitica* [13], specifically at abattoirs during the processing and loading of the poultry permitting the risk of transferring the microbe from the live bird to carcasses [14]. These improper slaughtering practices, as well as improper handling during the cooking process, are regarded as major vectors for *Y. enterocolitica* transfer to humans via poultry. Generally, reports found that the most frequently identified sources of *Y. enterocolitica* sickness in humans are animal-derived foods [15].

Biofilm formation protects the bacterial cells from all the exterior stress in addition to the majority of bacterial infections caused by biofilm. Foodborne pathogen as *Y. enterocolitica* were reported as biofilm producers' pathogens in the food chain [16]. Most microbial formation is biofilm with around 90% of the bacteria developing on biotic and abiotic surfaces [17]. The food safety concern against biofilm is growing especially due to their role in increasing the resistance of bacteria leading to difficulties in controlling food hygiene [18]. Biofilms are well-known for being a source of foodborne human diseases [19]. Nevertheless, in the instance of *Y. enterocolitica*, the significance of biofilm and the mechanisms that contribute to biofilm development are mainly unknown. Biofilm production defends microbial cells from antibacterial compounds, phages, phagocytes, and antibodies whilst in vivo. Similarly, the mechanism of biofilm production demonstrates an approach to protecting microbes from various environmental challenges. Moreover, the emergence of biofilms could end up in persistent infection due to microbial resistance to antibiotic therapy and host immune cells [20].

Limited reports have existed before from Egypt studying the prevalence of *Y. enterocolitica* from food sources (meat and poultry) [21, 22]. To the best of our knowledge, the differentiation between the planktonic and biofilm form of *Y. enterocolitica* in food sources such as ducks, geese, and pigeons other than chicken meat is not well characterized. We aimed to investigate the prevalence of *Y. enterocolitica* strains in chicken recovered from random places in addition to its prevalence in ducks, geese, and pigeons in Egypt. Furthermore, determine the biofilm formation rate between these isolates to illustrate the different prevalence of planktonic and biofilm forms.

Material and Methods

Ethical statement

The research strategy was approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt (Protocol code: M/65).

Sampling

Between September 2020 and May 2021 in Mansoura city, Egypt, a total of 220 samples of poultry meat were collected randomly from various major supermarkets, street markets, slaughterhouses, and smallholders, including raw chicken meat (n = 150), duck (n = 40), geese (n = 23), and pigeon (n =7). The samples were collected in sterile bags and immediately transported in an ice-filled container to the laboratory of Bacteriology, Mycology, and immunology Department at the Faculty of Veterinary Medicine, Mansoura University to be examined within 6 hours.

Identification of Y. enterocolitica

Isolation of Y. enterocolitica strains from samples

In the current study, *Y. enterocolitica* in poultry meat was identified using the International Organization for Standardization (ISO) 10273:2017 [23]. In brief, 10 g of each sample was cut with sterile scissors and tissue forceps and placed into sterile Stomacher bags containing 90 mL of Yersinia (PSB) broth (Biolife Italiana). The samples were homogenized for 2 minutes. The PSB-diluted samples were incubated at 25°C for 3- 5 days. A total of 0.5 ml of the incubated samples was mixed with 4.5 ml of 0.25% potassium hydroxide (KOH) and cultured onto selective CIN agar (Oxoid, UK) and MacConkey›s agar (Oxoid, UK) aerobically at 25 °C for 24-48 h. Colonies giving the appearance of the bull eye on CIN (small, and deep with red centres and surrounded by clear zones) while they appeared small and colorless on MacConkey's agar were presumptive as *Y. enterocolitica*. All the suspected to be *Y. enterocolitica* isolates were restreaked on tryptic soy agar (TSA, Oxoid, UK) plates for further examination.

The isolates were then morphologically and biochemically identified by Gram staining, catalase, oxidase, triple sugar iron, citrate utilization, esculin hydrolysis on Bile Esculin agar (Oxoid, UK), and urease activity testing. All biosafety and infection control were taken during the whole experiment according to Richmond and McKinney (Eds) [24].

Molecular confirmation of Y. enterocolitica

DNA sample extraction

All the biochemically confirmed isolates were extracted for DNA samples using the boiling method according to Alexopoulou et al. [25]. In brief, two or three colonies were picked up from 24 h *Y. enterocolitica* culture into 100 µl deionized free water, followed by using boiling for 10 minutes. Then all heated samples were centrifuged for a maximum speed of 3 to 5 min. All the supernatant was transferred for a new serial Eppendorf and stored at -20 °C to be used as a DNA sample.

Molecular characterization of Y. enterocolitica strains using PCR

The extracted DNA samples were used as a template for PCR confirmation of *Y. enterocolitica* isolates. The suspected isolates were subjected to Applied Biosystem 2720 thermal cycler for amplification of the *16S rRNA* gene [26]. The primer sequence and the PCR condition were listed in Table 1. The cycle condition was performed as the following: initial denaturation for 5 min at 94°C, followed by 36 cycles at 94°C for 45 sec, 62°C for 45 sec, and 72°C for 45 sec, and a final extension of 72°C for 7 min. *Y. enterocolitica* isolates supplied from a previous study by Younis *et al.* [22] and water were used as positive and negative controls, respectively. The amplified DNA fragments were run in gel electrophoresis with 1% agarose containing ethidium bromide then gel documentation was used for visualization (Cleaver Scientific Ltd., UK).

Biofilm characterization of Y. enterocolitica

The *Y. enterocolitica* strains were examined for their capability to produce biofilm using the glass tube method [27]. In brief, all the strains were cultured on Tryptone Soya Broth (TSB, Oxoid) with NaCl 4% for 24 h. After 24h discard all the culture broth and stain the tubes with crystal violet 1% solution. All the tubes were left to stand with the stain for 15 minutes and a two- or three-times gentle washing was performed for these tubes using distilled water. The experiment was performed in triplicate. The tubes were diagnosed for the presence of visible film lining and results were interrupted as negative, weakly positive, positive, and strongly positive.

Results

Prevalence of Y. enterocolitica isolates in raw poultry meat

The overall incidence of *Y. enterocolitica* in raw poultry meat sold in different localities of Mansoura city revealed that of the 220 examined samples, 13/220 isolates (5.91%) were confirmed as *Y. enterocolitica* (Figure 1; Table 2). The isolation rates from chicken meat, ducks, and geese were 10/150 (6.67%), 2/40 (5%), and 1/23 (4.35%), respectively (Table 2). Pigeon meat samples were negative for *Y. enterocolitica* (Figure 1; Table 2). According to the sampling season, we found that the prevalence rate of *Y. enterocolitica* strains was at higher rates in winter (December, January, and February) than in spring (March, April, and May) and autumn (September, October, and November). The rate was 6/70 (8.57%), 4/70 (5.71%), and 3/80 (3.75%) in winter, spring, and autumn, respectively (Figure 2).

In vitro biofilm production of Y. enterocolitica strains

After air drying of the test tube, the occurrence of visible film lining the walls, and the bottom of the tube indicates biofilm production. In the current study, overall, 9 (69.23%) *Y. enterocolitica* strains were detected as biofilm producers (Figure 3; Figure 4). Five strains were counted to have a strong ability to produce biofilm; three strains were moderate, one strain was a weak biofilm producer, while 30.77% (4/13) were detected as non-biofilm producers (Figure 3; Figure 4).

TABLE 1. Primers and PCR cyclic conditions used in this study.

Target gene	Primer Nucleotide sequence (5'-3')	Target gene (bp)	Reference
Y. enterocolitica 16S rRNA	Y1: AATACCGCATAACGTCTTCG Y2: CTTCTTCTGCGAGTAACGTC	330	**Wannet et al. [26]**

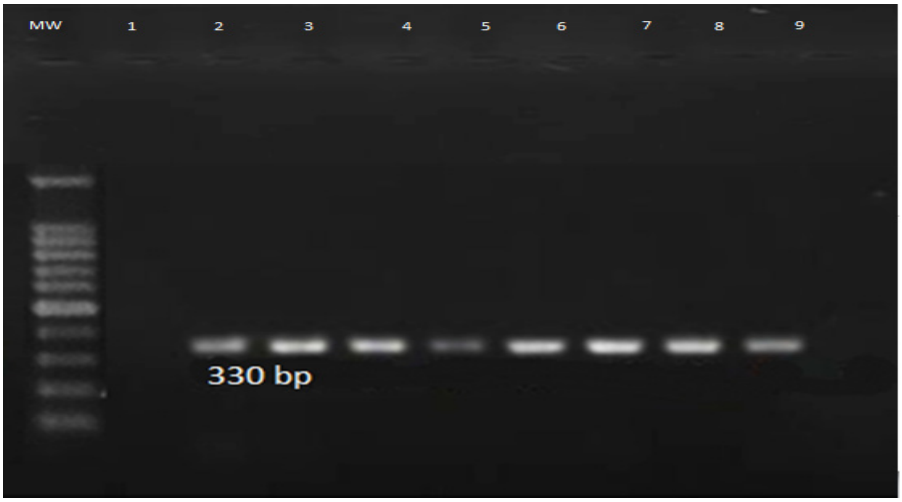

Fig. 1. Agarose gel electrophoresis PCR products of *Yersinia enterocolitica* 16SrRNA gene at 330 bp. Lanes: MW-DNA ladder: (100bp); lane 1: negative control; lane 2—positive control; lanes 3-9: *Yersinia enterocolitica* positive samples.

TABLE 2. Prevalence of *Yersinia enterocolitica* in poultry meat samples.

Meat category	No.	Sampling locations				No. (%) of Y. *enterocolitica*
		Major supermarkets	Street market	Slaughterhouses	Small holders	
Chicken	150	60	45	40	5	10 (6.67%)
Ducks	40	-	30	-	10	2 (5%)
Geese	23	-	20	-	3	1 (4.35%)
Pigeons	7	-	5	-	2	-
Total	220	60	100	40	20	13 (5.91%)

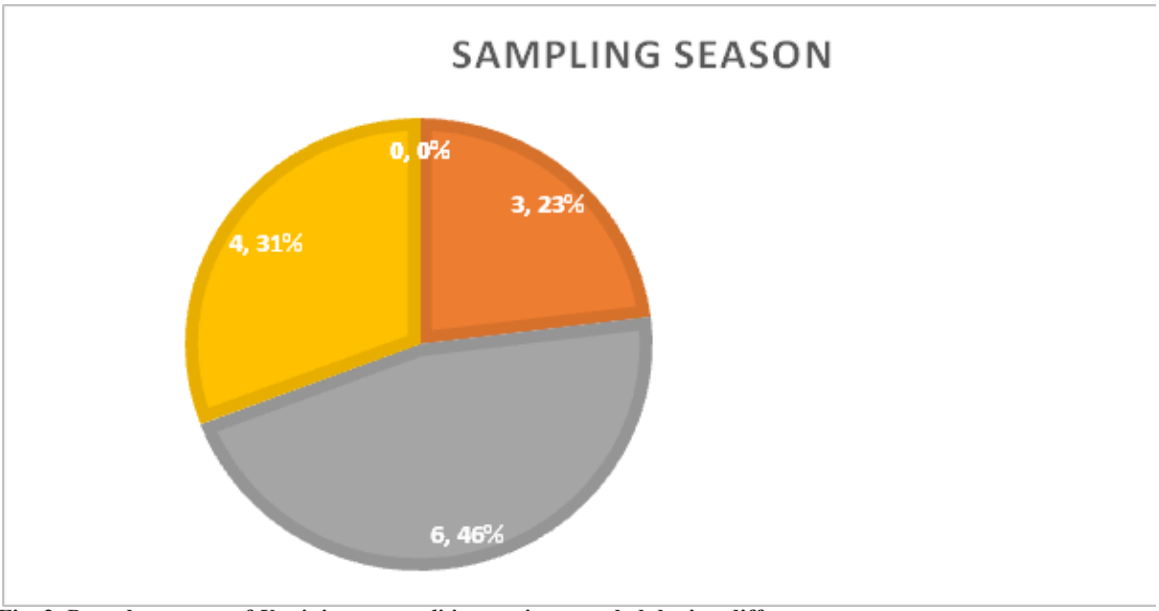

Fig. 2. Prevalence rate of *Yersinia enterocolitica* strains sampled during different seasons.

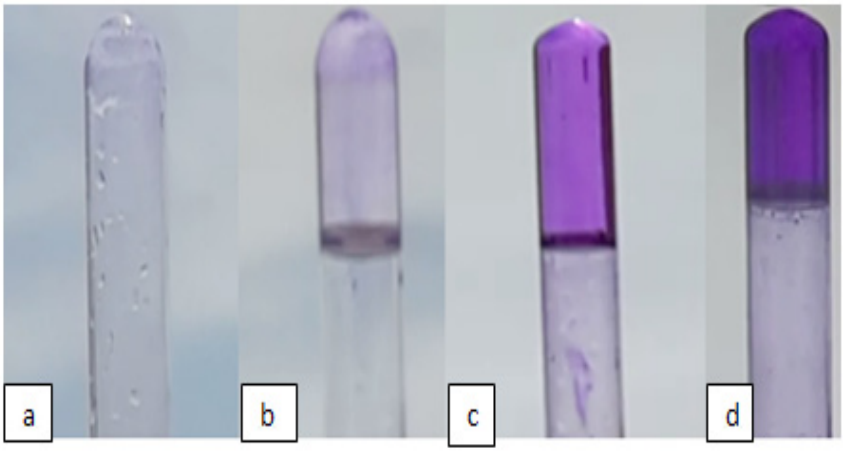

Fig. 3. Assessment of biofilm formation of *Yersinia enterocolitica* using tube test. (a) Non-biofilm producer, (b) Weak biofil

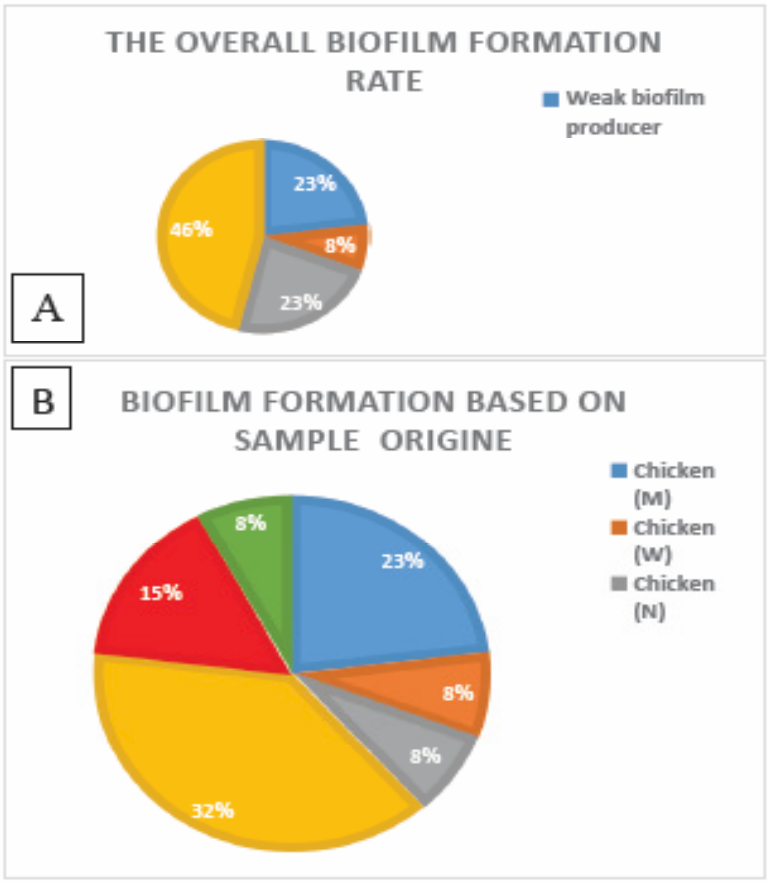

Fig. 4. Prevalence of biofilm-producing *Yersinia enterocolitica* within the different samples. The A figure shows the different rates of *Yersinia enterocolitica.* The positive biofilm producers represented about 54% of all the samples (23% weak biofilm producers, 23% strong biofilm producers, and 8% non-biofilm producers); and almost 46% of the poultry samples were non-biofilm producers. The B figure represents the ability of biofilm production based on the origin of the sample (Blue color, moderate biofilm production+ of chicken origin); (Orange color, weak biofilm production+ of chicken origin); (Grey color, no biofilm production+ of chicken origin); (Yellow color, strong biofilm production+ of chicken origin); (Red color, no biofilm production+ of duck meat origin); (Green color, strong biofilm production+ of chicken origin).

Discussion

Yersiniosis is a foodborne disease that is typically transmitted through the consumption of raw or undercooked pork, poultry meat, fresh pasteurized milk, other dairy products, infected plants, seafood, and drinking water [28, 29]. Contact with an infected surface or equipment can contaminate food either directly or indirectly [28]. Contact with chicken feces and lack of hygiene in chicken slaughterhouses are the two most frequent reasons for chicken meat contamination with *Y. enterocolitica*, which could easily spread to and cause yersiniosis in humans [30]. Centres for Disease Control and Prevention (CDC) counted that nearly 90% of the yearly *Y. enterocolitica* infection is foodborne [31]. Gastrointestinal diseases accompanied by death cases can occur in developing countries [32-34]. Previous reports detected poultry meat can play a vital role in *Y. enterocolitica* transmission to humans. It can be attributed to the growth of chicken meat consumption which was 5%, more than beef, small ruminants, and pork (1.5%, 1.7%, and 3.1%, respectively) [35].

In the current study, the overall prevalence of *Y. enterocolitica* was 13/220 (5.91%) in raw poultry meat. *Y. enterocolitica* was recovered from raw chicken meat 10/150 (6.76%), raw duck meat 2/40 (5%), and raw geese meat 1/23 (4.35%). None of the seven raw pigeon meat samples was found to harbor *Y. enterocolitica*. Previous studies recovered *Y. enterocolitica* at similar prevalence rates of 4.3%, and 4.5% in Argentina and China, respectively [36, 37]. About seven samples (2.1%) were infected with *Y. enterocolitica in Poland [38]*. However, other investigations were found to have frequently higher prevalence rate which was 16.7% in Turkey, 25% in Iran, and 32.5% in Italy [39-41]. Meanwhile, in Spain, half of the samples were found to be *Y. enterocolitica* [42].

In Egypt, Younis et al. [21, 22] recovered *Y. enterocolitica* in chicken meat with a prevalence rate of 15.83% (19/120) and, 5.9% (41/700), respectively. This comes in accordance with Shabana et al. [43] which was able to recover 17.5% of *Y. enterocolitica* from raw chicken meat in Egypt. In China, the sum of 112 duck samples from four provinces was investigated and the contamination rate of *Y. enterocolitica among these samples* was 4.46% [41]. This was relevant to our study and a previous study by Peng *et al*. [42]. From our duck meat samples, only 5% of the samples were detected to be contaminated with *Y. enterocolitica* strains. However, in Egypt, a prevalence rate of 43.3 % was detected before [44]. In a study conducted in China, samples from geese were investigated; none of the samples were found to have *Yersinia spp.* strains [41], meanwhile, a previous study described 7.2 % were *Y. enterocolitica strains* [45]. The rare isolation rate in geese and pigeon meat samples may be attributed to the little sample size, which might be not representative. The different isolation rates can be attributed to the different hygiene levels practiced at the abattoir during slaughtering. Thus, scientists discussed hygienic considerations at the sampling place that must be taken [46]. In our study, samples were gathered from different places and at different seasons to compare the impact of the place on the contamination rate. We found that the contamination rate was not affected by different sampling areas, but by the sampling season which may be attributed to the prevalence of *Y. enterocolitica* growing at low temperatures.

Biofilm formation has a severe impact considered one of the most growing concerns nowadays. This was attributed to two main factors; the biofilm producer pathogen will hinder the action of the immune system and the effect of antimicrobial agents. Meanwhile, the production of biofilm during the manufacturing process can affect the disinfection and cleaning processes [47]. *Y. enterocolitica* species have been illustrated to lose the ability to perform biofilms [48]. In some species (*Staphylococcus epidermidis,* and *Pseudomonas aeruginosa)*, biofilm production contributes to the expression of pathogenicity regarding its role as a virulence factor [49, 50]. Little knowledge concerning the role of biofilm production of *Y. enterocolitica* strains in the pathogenesis mechanism and infection was given. In our study, we focused on illustrating the prevalence of the planktonic and the biofilm producer *Y. enterocolitica* through different food sources specifically the most consumed poultry sources in Egypt. The current study detected 9 (69.23%) *Y. enterocolitica* strains as biofilm producers. Five strains were counted to have a strong ability to produce biofilm; three were moderate, one was a weak biofilm producer (Figure 5), while 30.77% (4/13) were detected as non-biofilm producers. In agreement with our study researcher found that two third of their isolates were in the biofilm form. [21].

In conclusion, *Y. enterocolitica* seriously threatens food quality and safety, thus adversely

affecting health [51]. Compared with planktonic cells, biofilms have significantly increased resistance to antimicrobials, therefore, foodborne bacteria can easily survive under commonly encountered stresses when the biofilm is formed [52, 53, 54]. Further studies must be considered to give us a brief view of the virulence of the planktonic and biofilm producer *Y. enterocolitica*in addition to their antimicrobial resistance, survival fitness, and ability to resist environmental stressors.

Authors' contributions

R.A.M performed experiments and analyzed the data; R.A.M wrote the first draft. A.S finalized the manuscript. G.Y and A.S designed the experiment and revised the manuscript. All authors approved the final version of the manuscript for publication.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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نظرة ثاقبة على انتشار سلالات اليرسينيا القولونية المنتجة للعوالق والغشاء الحيوي في لحوم الدواجن في مصر

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يعتبر ميكروب اليرسينيا القولونية (*Y. enterocolitica*) من الميكروبات التى تنتقل عن طريق الغذاء ويستطيع التسبب فى أمراض الجهاز الهضمي لدى البشر. يتميز *Y. enterocolitica* بقدرته على النمو فى درجات حرارة منخفضة وتكوين غشاء حيوي في السلسلة الغذائية. لقد هدفت دراستناإلى تقييم حدوث سلالات اليرسينيا المعوية القولونية فى لحوم الدواجن (الدجاج والبط والإوز والحمام) بالإضافة إلى تحديد قدرتها على إنتاج الأغشية الحيوية في مصر. لقد تم تجميع ٢٢٠ عينة بشكل عشوائي من لحوم الدواجن وتم فحصهاعن طريق تقنيات الاستزراع التقليدية من أجل عزل سلالات *Y. enterocolitica*. وكذلك تم فحص جميع العزلات المشتبه بها عبر تفاعل البوليميراز المتسلسل أحادي البلمرة (PCR) باستخدام الجين الخاص بـ *16S rRNA* لتأكيد عزل *Y. enterocolitica*.

ثم تم تشخيص جميع العزلات المؤكدة لقدرتها على تكوين غشاء حيوي في المختبر باستخدام طريقة الأنبوب الزجاجي باستخدام صبغة crystal violet.ولقد تم الكشف عن وجود *Y. enterocolitica* بنسبة ٥,٩١٪ (٢٢٠/١٣) وذلك كالتالى: لحم الدجاج ولحوم البط ولحوم الأوز بمعدلات ٦,٦٧٪ (١٥٠/١٠) و ٥٪ (٤٠/٢) و ٤,٣٥٪ (٢٣/١) على التوالي. وايضا تم العثور على تسعة سلالات تستطيع تكوين غشاء حيوي، بينما كانت أربع سلالات فقط في شكل بلانكتوني.ومع ذلك ، مازال معدل حدوث عترات اليرسينا القولونية قليل وعلى الرغم من ذلك لقد عزل نسبة اكتر من العترات القادرة على فرز الأغشية الحيوية والتي لها تأثير غير ايجابى بالمصانع وبين العامة ويجب السيطرة عليها.