RESEARCH ARTICLE

Pathogenicity of *E. coli* O78 *Riemerella anatipestifer* Chicken Isolate either alone or in combination in Ducks

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Abstract

The objective of this study was to find the relation between the pathogenicity of (*E. coli)* O78 chicken isolate alone or mixed with *Riemerella anatipestifer* infection by conventional methods and modern techniques. The results of this study revealed that the pathogenicity of all groups of *Riemerella anatipestifer* (RA) was 100% while it was 90% among the ducks inoculated with *E. coli* and was 100% in the injected with both strains of *Riemerella anatipestifer* and *E. coli* together and 0% in the control negative group. *RA* and *E. coli* strains were correctly identified using the VITEK 2 compact system with 99% and 94% probability, respectively. The results of polymerase chain reaction confirmed the identification results using specific primers either for *E. coli* O78 or *Riemerella anatipestifer* giving products 720bp and 665bp, respectively. Histopathological examination revealed that liver showed congestion in blood vessels and hepatic cell necrosis in the group inoculated within *Riemerella anatipestifer*, While liver showed only congested blood vessels in *E. coli* inoculated group. On the other hand, Liver showed severe vacuolar degeneration with focal amyloid infiltration in *E. coli* and *Riemerella* inoculated group.

Keywords: Duck, *E. coli*, Pathogenicity, *Riemerella anatipestifer*.

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Introduction

The poultry industry is one of the main agricultural industries in the world. In Egypt, ducks are from the largest waterfowl-breeding industry. In comparison to chickens, ducks are better adapted to varying environmental conditions and require less care, and are more resistant to several diseases (Adzitey and Adzitey, 2011). Duck morbidity and mortality are more commonly caused by bacteria than viruses (Wei *et al.*, 2013). Among the respiratory diseases of ducks, *R. anatipestifer* and *E. coli* infections are of primary economic importance to the duck industry. *R. anatipestifer* and *E. coli* have been reported to threaten duck health throughout the world (Wei *et al.,* 2013). Avian pathogenic *E. coli* (APEC) can infect ducks of different ages, especially in 4–9 week-old ducks started with respiratory disease, usually followed by systemic infection (Guabiraba and Schouler, 2015). Also, ducks like other farm animals are the primary reservoir for *E. coli,* including potential pathogenic types and the opportunity for cross-contamination, and consequently, food born poisoning or illness exists through the contaminated food (Adzitey *et al.,* 2012). Acute *R. anatipestifer* infection primarily affects ducks between 2-8 weeks causing mortality ranging from 1- 75%. Because both *E. coli* and *R. anatipestifer* infections occur at about the same age, this work was undertaken to study the effect of *E. coli* O78 chicken isolate alone and combined with *R. anatipestifer* on ducks for further production of bivalent inactivated RA-EC vaccine and studying its efficacy in prevention of both *E. coli* and *Riemerella* infections.

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Materials and Methods

Ethical Approval

Institutional Animal Care and Use Committee at Central Laboratory for Evaluation of Veterinary Biologics hereby acknowledge the research manuscript, and it has been reviewed under our research authority and is deemed compliance with bioethical standards in good faith.

Bacterial Strains

E. coli O78 chicken isolate and *R. anatipestifer* serotypes (RA3 and RA4) were isolated and identified in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt.

Experimental Ducks

Three hundred day-old healthy Pekin ducklings obtained from a commercial farm at El-Minufiya Governorate

were used for this study. The ducks were kept and reared in separate pens at the Central Laboratory for Evaluation Veterinary Biologics (CLEVB) until used for the experiment. Blood samples were collected directly before the experiment and serological examinations following ELISA test were done to ensure that the ducks have no maternal or acquired antibodies against *E. coli* and *Riemerella.*

Experimental Design

E. coli O78 chicken isolate and *R. anatipestifer* serotypes RA3 and RA4 were prepared in a concentration of 1×10^8 CFU/mL then 0.2 ml was inoculated intramuscularly in a total of 300 four- weeks-old ducklings which were divided into six groups G1 to G6 having 50 ducks each. For determination of pathogenicity, the first group was inoculated with *R. anatipestifer* serotype RA3, the second group G2 was inoculated with *R. anatipestifer* serotype RA4, while the third one was inoculated with combination of *R. anatipestifer* serotypes RA3 and RA4. The fourth group was inoculated with *E. coli* O78 strain; fifth group G5 was inoculated with a combination of *R. anatipestifer* serotype R3, R4, and *E. coli* O78. The last 6th group was kept as control. Inoculated ducks were observed daily for 2 weeks for morbidity and mortality. After the observation period, all survived ducks were sacrificed for postmortem examination and isolation of inoculated strains from internal organs (Soman *et al.,* 2014).

Isolation of Inoculated Strains

Heart and liver swabs were taken aseptically from each duck of each experimental group. Swabs were streaked on blood agar and MacConkey agar, incubated at 37◦C for 24 to 48 hrs for isolation of *R. anantpetifer* and *E. coli,* respectively (Stoute *et al.,* 2016).

Histopathology

Tissue sections of Lung, liver, trachea, bone marrow and spleen were taken at necropsy from each duck in each experimental group, fixed in 10% buffered formalin, and transported to Animal Health Research Institute for histopathological examination (Stoute *et al.,* 2016).

Identification of isolated strains

Biochemical Identification

Isolated strains were identified and confirmed biochemically by VITEK2® COMPACT system following Funke and Funke-Kissling (2004) method. A bacterial suspension was adjusted to a McFarland standard of 0.5 in 2.5 ml of a 0.45% sodium chloride solution with an ATB1550 densitometer (VITEK 2 DensiChek instrument, bioMe´rieux). The time between preparation of the suspension and card filling was always less than 30 min.

Identification of Isolates by Polymerase Chain Reaction (PCR)

Isolated strains were identified and confirmed by PCR using *Riemerella* and *E. coli* specific primers (Table 1).

DNA Extraction

DNA templates of both strains were extracted from culture by using EasyPure Bacteria Genomic DNA Kit (Trans, Cat. No. EE161). The extracted genomic DNAs of both strains were fractionated on 0.7% agarose gel.

Polymerase Chain Reaction (PCR)

5 µl of genomic DNA, 12.5 µl of 2x EasyTaq PCR SuperMix (Trans AS111), 1 µl of each primer (50 pmole), and 5.5 µl of deionized water were added to microcentrifuge tubes. The amplification reactions were performed under the following conditions: 94°C for 4 min, then 29 cycles each at 94°C for

Table 1: Primers used for identification *of E. coli* and *Remerella*

Primer	*Primer Sequence*	*Product size*	*Reference*
phoA (E. coli)	F5′-CGATTCTGGAAATGGCAAAAG-3′ R5′-CGTGATCAGCGGTGACTATGAC-3′	720 bp	Hu *et al.* (2011)
16srRNA (RA)	F5′-CAGCTTAACTGTAGAACTGC-3′ R5′-TCGAGATTTGCATCA-3CTTCG-3′	665bp	Shancy *et al.* (2018)

Table 2: Pathogenicity percent in ducks inoculated with *E. coli* and *RA* virulent strains

Groups	*G1*	*G2*	*G3*	*G4*	*G5*	*G6*
Inoculated strain	R3	R4	R3+R4	O78	O78+R3+R4	Control
Total No.	50	50	50	50	50	50
Mortality	50/50	50/50	50/50	45/50	50/50	0/50
Mortality %	100%	100%	100%	90%	100%	0%
Clinical Signs	0/50	0/50	0/50	5/50	0/50	0/50
PM lesions	50/50	50/50	50/50	45/50	50/50	0/50
Re-isolation	50/50	50/50	50/50	45/50	50/50	0/50
Totally affected	50/50	50/50	50/50	45/50	50/50	0/50
Pathogenicity %	100%	100%	100%	90%	100%	0%

1.5 min, 62°C for 1.5 min and 72°C for 2 min; lastly 72°C for 10 min in the case of *E. coli* (Hu *et al.,* 2011), while in the case of *R. anatipestifer* it was 95°C for 5 min, then 25 cycles each at 94°C for 30 sec, 54°C for 50 sec and 72°C for 1 min; lastly 72°C for 7 min (Shancy *et al.,* 2018). The PCR products were analyzed on 1% agarose gel.

Results and Discussion

Pathogenicity and Mortality

As revealed from the data 100% pathogenicity was observed in G1, G2 and G3 which were inoculated with *R. anatipestifer* , whereas *E. coli* inoculated samples (G4) exhibited 90% pathogenicity and G-5, which was inoculated with *RA and E. coli* showed 100% pathogenicity; control group G-6 showed no pathogenicity as shown in Table 2.

Mortality percent and clinical signs were observed in five ducks of G-4 only with 90 % in 1st and 2nd week or otherwise 100 % mortality was observed in all other groups in 1st and 2nd week. Our results corroborate with the findings of Stoute *et al.* (2016) and Soliman *et al.* (2018) who reported 100 % mortality in 4 weeks-old ducks challenged with RA and which decreased to 50-70 % at 8 weeks ducks at the same time the authors reported 90 % mortality challenged with *E. coli* O78 in 4-week old ducks which decreased to 60 % in 8-week old ducks. From these findings, it is clear that age and strains affect mortality percent.

Isolation and Biochemical Identification of *E. coli* and *R. anatipestifer* from affected ducks

E. coli and *RA* were re-isolated from all dead and sacrificed carcasses in all groups where *E. coli* produced large-sized fermenting rounded, non-mucoid pink colonies on MacConkey agar, while *RA* produced non-hemolytic, small, circular, convex, transparent, dew drops like colonies on blood agar. *E. coli* and *RA* strains were correctly identified biochemically using VITEK 2 compact system with 99% and 94% probability respectively (Table 3). According to the identification levels and reporting of results of the VITEK 2 compact system, *E. coli* was identified by excellent confidence level with probability 99%, and RA was identified by very good confidence level with 94% probability. Funke *et al.* (1998) reported that 70 out of 73 *E. coli* samples tested with ID-GNB card, which contain 47 biochemical tests of the VITEK 2 system, were correctly identified as *E. coli,* while 2 samples were identified with low discrimination and 1 sample was misidentified. They concluded an accuracy rate of 88.5% identification to the species level after 3 hours. Funke and Funke-Kissling (2004) used new VITEK 2 cards (NGNC), which consist of 64 biochemical tests for identification of gram-negative rods where 58 out of 58 *E. coli* samples were correctly identified as *E. coli*. They correctly identified 637 out of 655-gram negative rods (97.3%) using the new gram-negative cards (NGNC).

Identification of Isolates by PCR

The genomic DNAs of both isolated strains were extracted, fractionated on 0.7% agarose and estimated the size of the genomic DNA found more than 23 kbp. The target antigen of *E. coli* O78 strain was confirmed as *E. coli* by its specific primer giving rise to a PCR product of 720 bp as shown in Fig. (1A). Also, the antigen of *RA* was confirmed as *Rimerella* by using its specific primer giving rise to a PCR product of 665 bp as shown in Fig. (1B).

Regarding the identification of Isolated strains using PCR, RA's target antigen was confirmed as *Rimerella anatipestifer* by using its specific primer giving rise to a PCR product of 665 bp (Fig 1B). The same RA PCR product was obtained by Soliman *et al.* (2018) who confirmed 4 out of 5 isolates as RA

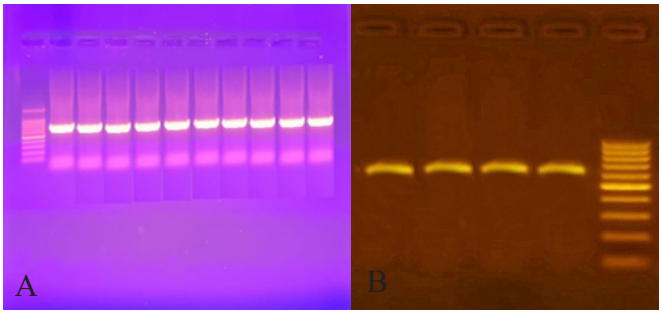

Fig. 1. (A): Results of PCR using a specific primer of *E. coli*. **Lane 1:** marker, Lane 2: positive control, lane 3 - 10: positive samples, **(B)** Results of PCR using a specific primer of *RA*. From right: Lane 1: marker, Lane 2: positive control, lane 3 - 4: positive samples.

Table 3: Biochemical identification of *E. coli* and *RA* using VITEK 2 compact system

Test	*E. coli*	*RA*	Test	*E. coli*	*RA*	Test	*E. coli*	*RA*	Test	*E. coli*	*RA*	Test	*E. coli*	*RA*	Test	*E. coli*	*RA*
APPA	-	-	ADO	-	+	PyrA	-	-	IARL	-	+	dCEL	-	+	BGAL	+	-
H2S	-	+	BNAG	-	-	AGLTp	-	-	dGLU	+	+	GGT	-	-	OFF	+	-
BGLU	-	+	DMAL	+	+	dMAN	+	+	dMNE	+	+	BXYL	-	-	BALap	-	-
ProA	-	-	LIP	-	-	PLE	-	+	TyrA	-	-	URE	-	+	dSOR	+	-
SAC	+	+	dTAG	-	-	dTRE	+	+	CIT	-	-	MNT	-	-	5KG	-	-
ILATk	-	-	AGLU	-	-	SUCT	-	-	NAGA	-	-	AGAL	+	-	PHOS	-	-
GlyA	-	-	ODC	+	-	LDC	+	-	IHISa	-	-	CMT	+	+	BGUR	+	-
O129R	+	-	GGAA	-	-	IMLTa	-	-	ELLM	+	-	ILATa	-	-			

by detecting 16S rRNA gene giving the specific expected product at 662 bp. Shancy *et al.* (2018) and Tsai *et al.* (2005) also obtained the same product in the identification of RA isolates. Many workers (Qu *et al.,* 2006; James, 2010; Kuhn *et al.*, 2011; Shonima, 2012) studied on 16S rRNA gene for the detection of pathogenic bacteria and opined that it was used as a molecular marker.E. coli strain's target antigen was confirmed as *E. coli* by its specific primer giving rise to a PCR product of 720 bp (Fig. 1A). The same product was obtained by Elsayed *et al.* (2015) who identified 166 out of 200 *E. coli* samples collected from broiler chickens from different private farms in Dakahlia Governorate using the same *phoA* primer and concluded that *phoA* gene is a common gene specific to *E. coli* and can be used specifically to detect bacterial genes that code for cell envelop proteins. Eid *et al.* (2019) characterized *E. coli* isolated from meat and meat products in Port-Said markets by PCR using the same primer giving rise the same PCR product. Hu *et al.* (2011) developed a multiplex PCR for identification of both RA and *E. coli* using the same *phoA* primer for *E. coli* and obtained product of 720 bp. In the present study, it is confirmed that *E. coli* O78 chicken isolate is pathogenic for ducks which is also identified by VITEK 2 compact system and PCR with accuracy, since we have re-isolated and identified pathogenic RA and *E. coli* strains from ducks which showed significant importance in the duck industry so, we can recommend the production of bivalent inactivated RA-EC vaccine and studying its efficacy in the prevention of both *E. coli* and *R. anatipestifer* infections.

Histopathology:

The microscopical changes that occurred in different groups, the liver showed severe congested blood vessels, with congested sinusoids and microthrombus formation, and hepatocellular necrosis with vacuolar degeneration in *R. anatipestifer* inoculated groups (Fig 2A, 2B). Whereas in *E. coli* inoculated group the liver showed congested blood vessels (Fig 2C). On the other hand, the liver showed severe vacuolar degeneration (arrow) with focal amyloid infiltration (star) in *E. coli* and *R. anatipestifer* inoculated group (Fig 2D).

The microscopical changes in the *R. anatipestifer* group's trachea showed sloughed lining epithelium and mucous exudate and heterophils infiltration in the lumen and mucosal and submucosal congestion and edema, associated with a congested muscular layer (Fig 3A and 3B). While it showed a congested muscular layer in the *E. coli* group (Fig 3C) . On the other hand, the trachea showed edema and congested muscular layer in the *E. coli* and *R. anatipestifer* group as shown in Fig 3D.

The lung showed severe congested blood vessels and thrombus formation, in addition to edematous fluid in parabronchi and interstitium in *Riemerella* groups (Fig 4A) and bone marrow showed depletion of hematopoietic cells replaced by adipocytes in *Riemerella* groups and *Riemerella* combined with *E. coli* group (Fig 4B). The spleen showed thickening of the splenic capsule and the wall of blood vessels which characteristic of *E. coli* group and bone marrow showed depletion of hematopoietic cells replaced by adipocytes which were characteristic for *RA* groups (Fig 4C). Stoute *et al.* (2016) reported diffused lymphocytic meningitis as a significant finding in *RA* inoculated group. There were no other macroscopic or microscopic lesions that facilitated differentiation of RA's different serotypes from each other or *E. coli.*

The carcass is characterized by fibrinous pericarditis, hepatitis, air vasculitis, and pneumonia in both *RA* and *E. coli* groups but characterized by swollen mottled spleen only in the *E. coli* group. On the contrary Stoute *et al.* (2016) reported that the swollen mottled spleen was observed in both *RA* and *E. coli* groups and reported dark green discoloration of liver only in the *E. coli* group. Liu *et al.* (2013) reported severe gross lesions of fibrinous exudate in the pericardial cavity, over the surface of the liver and in the air sacs two days after inoculation with RA strains as well as histological changes, such as vesicular degeneration, cell necrosis, and fibrinous exudation in liver, spleen, and brain tissues.

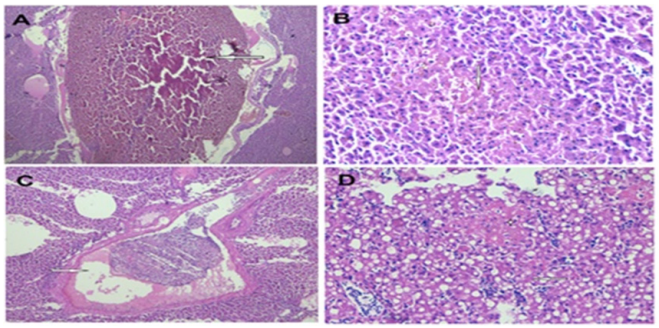

Figure 2: Microscopical changes in livers in different groups. **(A):** severely congested blood vessels, with congested sinusoids and microthrombus formation in *Riemerella* groups. **(B)** Hepatocellular necrosis with vacuolar degeneration in *Riemerella* groups. **(C):** congested blood vessels in *E. coli* group. **(D):** severe vacuolar degeneration with focal amyloid infiltration in *E. coli* and *Riemerella* inoculated group.

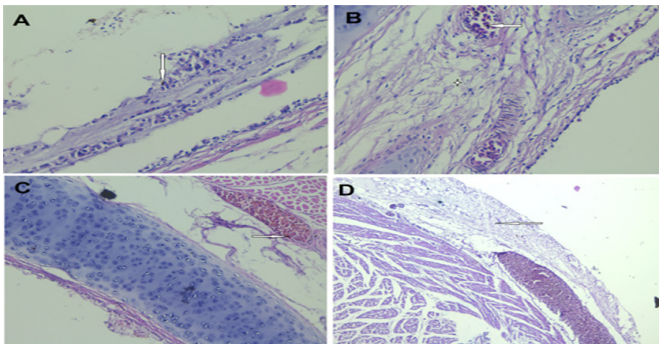

Fig. 3: Microscopical changes in the trachea in different groups. **(A):** sloughed lining epithelium in addition to mucous exudate and heterophils infiltration in the lumen in *Riemerella* groups. **(B)**: Mucosal and submucosal congestion and edema, associated with the congested muscular layer in *Riemerella* groups**. (C):** congested muscular layer in *E. coli* group. **(D):** edema andcongested muscular layer in *E. coli* and *Riemerella* inoculated group.

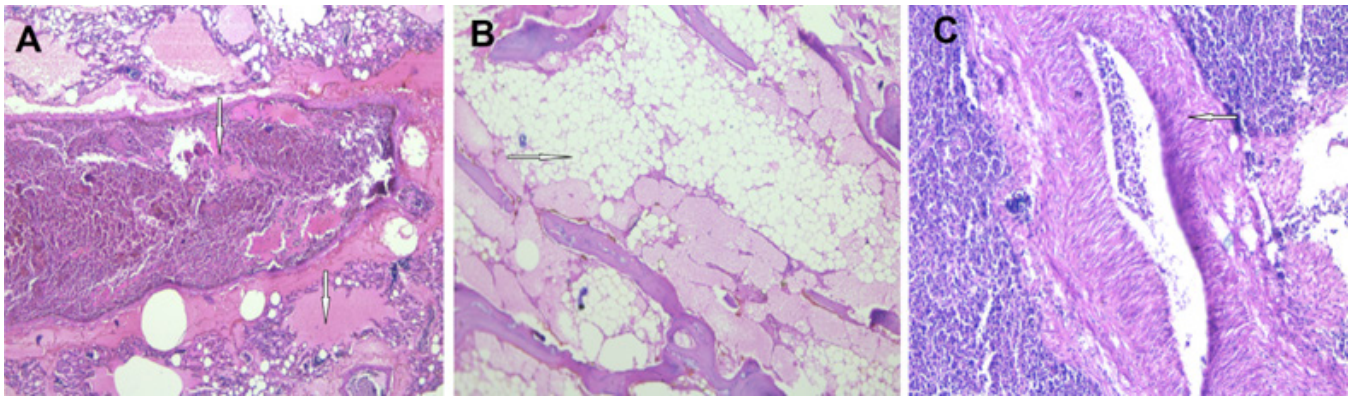

Fig. 4 (A): Microscopical changes of the lung. (B): Microscopical changes of bone marrow. **(C):** Microscopical changes of the spleen.

Conclusions

In the present study, it is confirmed that *E. coli* O78 chicken isolate is pathogenic for ducks which is also identified by VITEK 2 compact system and PCR with accuracy, since we have re-isolated and identified pathogenic RA and *E. coli* strains from ducks which showed significant importance in the duck industry so, we can recommend the production of bivalent inactivated RA-EC vaccine and studying its efficacy in the prevention of both *E. coli* and *Riemerella* infections.

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