Journal of Current Veterinary Research

ISSN: 2636-4026

Journal home page: http://www.jcvr.journals.ekb.eg

Avian disease

Molecular Detection of The *Ornithobacterium rhinotracheale* From Turkeys Flocks in Alexandria Governorate During 2020 – 2021

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ABSTRACT

Ornithobacterium rhinotracheale (ORT) bacterium in poultry causes respiratory disease with reduced body weight, and lowered hatchability. The disease has higher incidence in turkey species, rapidly developing antibiotic resistance, and difficulties in its diagnosis lead to higher economic losses, drug costs, misdiagnosis. Therefore, this work aimed to follow incidence of ORT bacterium in turkeys by using Polymerase Chain Reaction (PCR) in addition to sequencing, and phylogenetic analysis. Aspirated infected nasal fluids of seventeen turkey flocks affected with sinusitis were tested using PCR method for 16S rRNA genes of ORT then seven isolates were selected for sequencing, and phylogenetic analysis, followed by Gene Bank database submission. PCR results were positive for ORT bacterium at amplicon of 625 bp fragment in 12 out of 17 investigated turkey flocks with a percent of 70.59%.On sequencing of 16S rRNA genes, the selected isolates exhibited 100% similarity with each other, and Tur/France/94 strain and 99.8% with the Egyptian strains (MG773129-Egy-1, and MG773129-Egy-2), Tur/Hungary/2015, Ch/USA/91, Ch/South Africa/91, and Ch/France/95. All sequenced isolates were registered on the GenBank database and accession numbers were taken as follow, MW700375, MW700376, MW700377, MW700378, MW700379, MW700380, and MW700381. The higher incidence of ORT infection in turkey flocks was indicated that ORT alone could trigger turkey sinusitis with respiratory signs, also, their similarity of 99.8% with other Egyptian ORT strains means there were variation occurred in 16S rRNA gene which might play an important role in generation of new variant ORT strains.

Key words: ORT, PCR, 16S rRNA gene, Sequencing, Turkey.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is an economically important bacterial pathogen of turkeys and chickens worldwide, it was early isolated in Hungary, 1987 as *Pasteurella*-like organism from respiratory affected ducks, while in 1991-1992, Germany, it was identified in turkeys as bacteria-like *Riemerella anatipestifer*. Moreover, it was isolated from lungs and air sacs of broiler chickens in South Africa, 1991 and recorded as Gram-negative highly pleomorphic rods (van Beek *et al*., 1994).

In Egypt, Youssef and Ahmed (1996); El-Gohary (1998), El-Gohary and Awaad (1998); El-Gohary *et al*. (1998); El-Gohary and Sultan (1999); Abd El-

Ghany (2000) isolated ORT from broiler, turkey, and layer flocks either alone or concomitantly with other organism as well as other many studies were done on isolation, characterization, and treatment of ORT (Amal, 2002; Shihata and Ibraheem, 2004; Shahata *et al*., 2006; Attia, 2008; Elbestawy, 2010; Hegazy *et al*., 2015; Masoud *et al*., 2015; El-Abasy *et al*., 2016; Ellakany *et al*., 2019; Hassan *et al*., 2020).

Vandamme *et al*. (1994) described ORT bacterium as highly polymorphic Gram-negative rod, non-motile, non-spore former in family *Flavobacteriaceae*.

Ornithobacteriosis is a contagious disease affecting chickens and turkeys and characterized by yogurt-like fibrinous air sacculitis accompanied with consolidated lung (Hafez, 1996; Banani *et al*., 2001). Its virulence was increased with presence of infectious and non-infectious agents (van Empel and Hafez, 1999; Barbosa *et al*., 2019).

ORT was considered as a primary or secondary infection with other respiratory viral and bacterial pathogen (Welchman *et al*., 2013; Kursa *et al*., 2021).

Nearly about 18 serotypes of ORT were recovered from chickens, pigeons, turkeys, gooses, ducks, and rooks. Chicken isolates were homogeneous and belonged to serotype A with a percent, 95%, while turkey isolates were heterogeneous (Rubio and Salazar, 2010). Although the successful treatment of ORT, its antibiotic resistance may be rapidly developed (Devriese *et al*., 2001).

Conventional phenotypic methods of isolation and identification were based for definitive diagnosis of ORT (De la Rosa-Ramos *et al*., 2018; Hassan *et al*., 2020) but there were difficulties and misdiagnosis with other bacteria including *Bordetella*, *Haemophilus*, *Pasteurella*, *Riemerella* (Hafez *et al*., 1993; Bragg *et al*., 1997) and some viruses like *Pneumovirus* (Marien *et al*., 2005).

Therefore, this study used molecular techniques to identify ORT rapidly in specimens of infra-orbital sinus fluid taken from turkey flocks affected with sinusitis, and comparing ORT isolates with previous ones in phylogenetic tree, and submitting them to Gene Bank.

MATERIALS AND METHODS

Samples collection and processing

Samples of infra-orbital sinus fluid were collected under complete aseptic condition from seventeen turkey flocks of different breeds including Balady, Bronze, and/or White Nicholas during the period from 5/2020 up to 1/2021 at different localities of Alexandria governorate. Turkeys affected with infra-orbital sinusitis, high morbidity, no mortality, reduced feed intake, and lowered body weight. Affected age ranged from 50 days to 4 months. These samples were transmitted to Central laboratory for application of PCR against the 16S rRNA gene of ORT.

DNA extraction.

It performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), according to manufacturers' instructions. Briefly, DNA of samples was extracted as follow, 10 µl, proteinase K with 200 µl, lysis buffer were incubated with 200 µl, sample suspension at 56°C for 10 min. then after that 200 µl, ethanol(100%) added followed by sample washing, centrifugation, and DNA elution.

Nucleotide Primers

Metabion, Germany provided the required Primers (Table 1).

PCRreaction condition

1 µl from each primer (concentration = 20 pmol) was added to Master Mix, 12.5 µl, plus4.5 µl water, and 6 µl DNA template, Then placed in thermal cycler (Applied biosystem 2720).

Agarose gel electrophoresis

At room temperature, agarose gel (1.5%) was prepared to separate the PCR by electrophoresis, each gel slot was filled with 20 µl, PCR product of each sample as well as ladder, 100 bp (Fermentas, Germany). The gel documentation system (Alpha Innotech, Biometra) and the computer software were used for data analysis.

Sequence and phylogenetic tree

Gelextraction kit and Perkin-Elmer V3.1 cycle Terminator were obtained to purify PCR products of selecte identity (Altschul *et al*., 1990). Laser gene DNA Star version 12.1, Meg Align module created the phylogenetic tree (Thompson *et al*., 1994), and MEGA6, maximum likelihood, neighbor-joining, and maximum parsimony performed the phylogenetic analyses (Tamura *et al*., 2013).

Table (1). Target genes, Primers, and amplicon sizes**:**

Target genes	**Primers**	**Amplified segments (bp)**	**References**
ORT 16S rRNA	F: 5-TGGCATCGATTAAAATTGAAAG-3	625	Doosti *et al*., 2011
	R: 5-CATCGTTTACTGCGTGGACTAC-3		

RESULTS

PCR Results

ORT was diagnosed in twelve out of seventeen turkey flocks suffering from infraorbital sinusitis (Table 2). Positive samples for ORT give specific band at 625 bp size (fig. 1)

Sequencing and phylogenetic analysis of ORT isolates

Partial sequences of ORT16S rRNA genes of selected seven strains (Naseria-1, Amria-K, Abees-2, Naseria-2, Maryout-1, Maryout-2, and Maryout-3) were compared with those of Asian, European, and American ORT strains. All isolates branched in the same line with Tur/France/94, Tur/Hungary/2015, Ch/USA/91, Ch/South Africa/91, Ch/France/95, MG773129-Egy-1, and MG773129-Egy-2 (fig. 2). All selected ORT isolates were absolutely identical (100%) among themselves and with Tur/France/94 strain as well as they showed 99.8% similarity with the Egyptian strains (MG773129-Egy-1, and MG773129-Egy-2), Tur/Hungary/2015, Ch/USA/91, Ch/South Africa/91, and Ch/France/95 (fig. 3).

GenBank accession numbers of all selected isolates

The nucleotide and amino acid sequences of ORT 16s rRNA genes were submitted into GenBank under the accession numbers; MW700375 for isolate Naseria-1, MW700376 for isolate Amria-K, MW700377 for isolate Abees-2, MW700378 for isolate Naseria-2, MW700379 for isolateMaryout-1, MW700380 for isolate Maryout-2, MW700381for isolate Maryout-3 (Table3).

Table (2): Incidences of ORT isolated from turkeys

ORT negative	**%**	**ORT positive**	**%**	**Total**
5/17	29.41%	12/17	70.59%	17

Table (3): Data and GenBank accession numbers of sequenced ORT isolates

Serial number	Identified code	Governorate	Bird species	Breed	Date	GenBank accession
1	Naseria-1			Bronze	27/8/2020	MW700375
2	Amria-K			balady	13/6/2020	MW700376
3	Abees-2			Bronze	15/11/2020	MW700377
4	Naseria-2	Alexandria	Turkey	Bronze	6/12/2020	MW700378
5	Maryout-1			Bronze	15/1/2021	MW700379
6	Maryout-2			Bronze	15/1/2021	MW700380
7	Maryout-3			Bronze	15/1/2021	MW700381

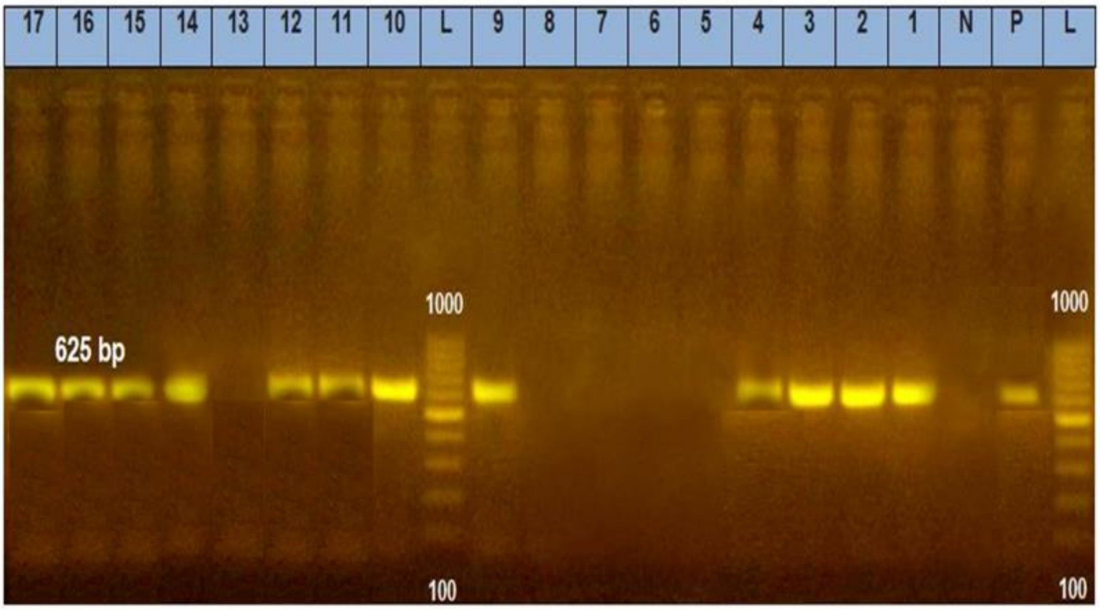

Figure (1). Showed lane L: Loader from 100-1000 bp lane P: Positive control. Lane N: negative control Lane 1-4, 9-12, 14-17: Positive Samples for ORT at amplicon of 625 bp. Lane 5-8, 13: Negative samples

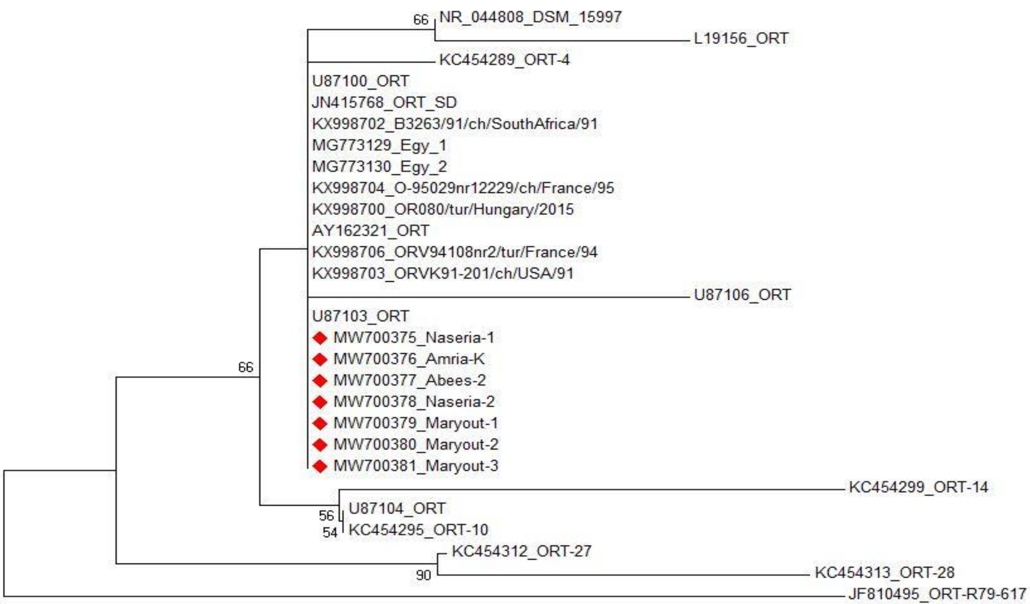

Figure (2). Phylogenetic tree and genetic relationships among the selected ORT isolates (indicated by red dots).

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	1	MG773130 Egy 2
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	2	MG773129 Egy 1
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	3	KX998704 O-95029nr12229/ch/France/95
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	4	KX998702 B3263/91/ch/SouthAfrica/91
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	5	KX998700 OR080/tur/Hungary/2015
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	6	JN415768 ORT SD
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	7	AY162321 ORT
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		99.8	99.8	99.8	99.7	99.7	99.7	99.5	99.5	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	8	U87100 ORT
9	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2		99.7	99.7	99.8	99.8	99.8	99.7	99.3	99.3	99.2	98.7	98.2	95.3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	9	KX998706 ORV94108nr2/tur/France/94
10	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3		99.7	99.5	99.5	99.5	99.3	99.3	99.0	98.8	98.3	97.8	95.2	99.7	99.7	99.7	99.7	99.7	99.7	99.7	10	KC454289 ORT-4
11	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3		99.5	99.5	99.5	99.3	99.3	99.0	98.8	98.3	97.8	95.5	99.7	99.7	99.7	99.7	99.7	99.7	99.7	11	NR_044808 DSM 15997
12	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.5	0.5		99.7	100.0	99.8	99.2	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	12	KX998703 ORVK91-201/ch/USA/91
13	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.5	0.5	0.3		99.7	99.8	99.2	99.2	99.3	98.5	98.0	95.2	99.8	99.8	99.8	99.8	99.8	99.8	99.8	13	U87104 ORT
14	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.5	0.5	0.0	0.3		99.8	99.2	99.2	99.0	98.5	98.0	95.3	99.8	99.8	99.8	99.8	99.8	99.8	99.8	14	U87103 ORT
15	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.3	0.7	0.7	0.2	0.2	0.2		99.0	99.0	99.2	98.3	97.8	95.2	99.7	99.7	99.7	99.7	99.7	99.7	99.7	15	KC454295 ORT-10
16	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.7	0.7	0.8	0.8	0.8	1.0		98.7	98.5	98.0	97.5	94.9	99.3	99.3	99.3	99.3	99.3	99.3	99.3	16	U87106 ORT
17	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	1.0	1.0	0.8	0.8	0.8	1.0	1.3		98.5	99.3	97.8	94.7	99.3	99.3	99.3	99.3	99.3	99.3	99.3	17	KC454312 ORT-27
18	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.2	1.2	1.0	0.7	1.0	0.8	1.5	1.5		98.2	97.7	94.5	99.2	99.2	99.2	99.2	99.2	99.2	99.2	18	KC454299 ORT-14
19	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.3	1.7	1.7	1.5	1.5	1.5	1.7	2.0	0.7	1.9		97.8	94.2	98.7	98.7	98.7	98.7	98.7	98.7	98.7	19	KC454313 ORT-28
20	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.7	2.0	2.0	1.9	1.9	1.9	2.0	2.4	2.0	2.2	2.0		93.7	98.2	98.2	98.2	98.2	98.2	98.2	98.2	20	JF810495 ORT-R79-617
21	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.3	0.5	0.7	0.5	0.7	1.0	1.2	1.4	1.8	2.1		95.3	95.3	95.3	95.3	95.3	95.3	95.3	21	L19156 ORT
22	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5		100.0	100.0	100.0	100.0	100.0	100.0	22	MW700375 Naseria-1
23	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0		100.0	100.0	100.0	100.0	100.0	23	MW700376 Amria-K
24	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0	0.0		100.0	100.0	100.0	100.0	24	MW700377 Abees-2
25	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0	0.0	0.0		100.0	100.0	100.0	25	MW700378 Naseria-2
26	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0	0.0	0.0	0.0		100.0	100.0	26	MW700379 Maryout-1
27	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0	0.0	0.0	0.0	0.0		100.0	27	MW700380 Maryout-2
28	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.2	0.2	0.2	0.3	0.7	0.7	0.8	1.3	1.7	0.5	0.0	0.0	0.0	0.0	0.0	0.0		28	MW700381 Maryout-3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		

Divergence

Figure (3). Genetic identity among the selected ORT isolates (blue text box).

DISCUSSION

ORT infection recorded in70.59% of investigated turkey flocks suffering from sinusitis. On sequencing of 16s rRNA genes for the selected seven ORT strains, all isolates were identical with each other and also Tur/France/94 strain while their identity with Egyptian strains (MG773129-Egy-1, and MG773129-Egy-2), Tur/Hungary/2015, Ch/USA/91, Ch/South Africa/91, and Ch/France/95 was similar in a percent of 99.8%. These results were close to those of Hauck *et al* (2015) that reported the higher ORT infection in turkeys (41%) when compared with chickens (6.9%), also, ORT infection in both chickens and turkeys was described by Karimi-Dehkordi *et al*. (2021) and Roussan *et al* (2011). Buys (1996) showed ornithobacteriosis susceptibility was higher in Turkey followed by chicken, duck, goose, pigeon, quail, ostrich, guinea fowl, gull, partridge, pheasant, and rook.ORT was recovered from 75 broiler chicken farms in Egypt (Abd El-Ghany, 2000). Elbestawy (2010) isolated ORT bacterium from broilers in Kafr El- Sheikh and El-Behera governorates with an incidence percent, 7.27%. Amal (2002) demonstrated 5.8% ORT incidence rate in broilers, Assuit governorate. Ellakany *et al*. (2019) revealed ORT isolates were detected in Broilers and layers with a percent of 11.66%and closely related to Asian, European, and American strains (98%-100%). Masoud *et al*. (2015) showed a similarity percent of 94%-98% among the selected five broiler's ORT strains and some Asian, and American ones. Thieme *et al.* (2016) when compared the results of 16S rRNA gene sequencing and multi-locus sequence typing (MLST) in 65 isolates of ORT, they demonstrated identity ranging 85.1%-100%.

CONCLUSION

In this work, the higher incidence of ORT detection was indicated ORT infection alone could trigger turkey sinusitis and respiratory diseases in turkey flocks. Their similarity of 99.8% with other Egyptian ORT strains means there were variation occurred in 16S rRNA gene which might play an important role in generation of new variant ORT strains. So that further continuous studies on molecular diagnosis and prevention/control strategy of ORT were urgently required to reduce economic losses in turkey flocks.

DECLARATIONS

Acknowledgement

Many grateful thanks to all the staffs and members of Animal and Poultry Health Department, and Maryout Research Station belonging to Desert Research Center, Ministry of Agriculture, Egypt.

Competition interest

There is no any Competing interests among the authors.

Authors' contribution

All authors have read and approved the final manuscript; Disouky Mourad collected the samples and tabulated the data; Amani Hafez performed the laboratory analyses; Mohamed Talaat helped in sample collection; Heba El-Sebaie shared in data and laboratory analyses; Hanan El-Samahy participated in sample collection, manuscript design, writing, and revision.

Funding

This research received no external funding.

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