Research Article

Detection of some Pathogenic Bacteria Causing Sinusitis in Turkeys by Using Multiplex PCR

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Abstract | In Egypt, turkey sinusitis is a major phenomenon that make the turkey farmers take far away from the turkey production as a result of difficult treatment, medication cost, difficult isolation of causative bacterial agents, weight loss, reduced fertility, and hatchability, so this study aimed to determine the bacterial causative agents using multiplex PCR as an accurate rapid diagnostic technique. Exudates of swollen infra-orbital sinus were aspirated and aseptically collected from affected turkeys of seventeen flocks and examined by multiplex PCR against *Escherichia coli* (*E. coli)*, *Mycoplasma gallisepticum* (*M. gallisepticum*), *Mycoplasma meleagridis* (*M. meleagradis*), *Ornithobacterium rhinotracheale* (ORT), *Pasteurella multocida* (*P. multocida*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) . The most prevalent detected bacteria were *E coli* (13) followed by *M. gallisepticum* (12), ORT (12) and *M. meleagridis* (7). Two farms reported single infection, one with *M. gallisepticum* and one with *E. coli*. Only one farm had no infection while mixed infection recorded in 14 farms. *P. multocida* and *P. aeruginosa* were not detected. It was concluded that Balady breed had a higher resistance than other breeds, *E. coli* and/or *M. gallisepticum* were the primary cause of turkey sinusitis, *M. meleagridis* recorded the lowest incidence and frequency in examined turkey flocks, while ORT had a higher incidence and frequency similar to *M. gallisepticum*. It was necessary to apply further molecular studies on these pathogens to control and avoid their spread in turkey and other poultry flocks.

Keywords | Bacteria, Multiplex PCR, Sinusitis, Turkey

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INTRODUCTION

Avian respiratory lesion including sinusitis, air saculitis, tracheitis and pneumonia was considered a complex problem occurred where different microorganisms coupled with the sublevel of hygienic measures and simultaneously interacted (OIE, 2008). In turkeys the respiratory lesions were restricted to swollen infra-orbital sinuses contained mucoid to caseous exudate (OIE, 2018) causing several economic losses with reduced feed intake, body weight, egg production and hatchability (Ali and Youssef, 2003; Alessandri et al., 2005; Bradbury, 2006, Büyüktanir et al., 2008; Nicholas et al., 2009).

Sometimes, infectious sinusitis in turkey accompanied by occasional movement disorders due to the inflammation of ankle and shoulder joints (Hafez 2002).

Table 1: History of collected samples from turkey sinusitis

Farm No.	Area	Governorates	species	Breed	date
1	Kilo28 North coast	Alexanderia	Turkey	White Nicholas	11/5/2020
2	Hawaria	Alexanderia	Turkey	balady	22/5/2020
3	Naseria-1	Alexanderia	Turkey	Bronze	27/8/2020
4	Amria-K	Alexanderia	Turkey	balady	13/6/2020
5	Abees-1	Alexanderia	Turkey	Balady	9/8/2020
6	Kilo21 North coast	Alexanderia	Turkey	Balady	9/6/2020
7	Amria-A	Alexanderia	Turkey	Bronze	25/7/2020
8	Nobaria	Alexanderia	Turkey	Balady	20/10/2020
9	Abees-2	Alexanderia	Turkey	Bronze	15/11/2020
10	Naseria-2	Alexanderia	Turkey	Bronze	6/12/2020
11	Nobaria	Alexanderia	Turkey	Bronze	23/12/2020
12	Kilo35Desert road	Alexanderia	Turkey	Bronze	28/12/2020
13	Kilo48North coast	Alexanderia	Turkey	Balady	3/1/2021
14	Kilo59Desert road	Alexanderia	Turkey	White Nicholas	12/1/2021
15	Maryout-1	Alexanderia	Turkey	Bronze	15/1/2021
16	Maryout-2	Alexanderia	Turkey	Bronze	15/1/2021
17	Maryout-3	Alexanderia	Turkey	Bronze	15/1/2021

Not only *Mycoplasma* spp. but also other pathogens as *E. coli*, *P. aeruginosa*, *Staphylococcus aureus* (*S. aureus*) and *Salmonella gallinarun pullorum* (*S. gallinarun pullorum*) were isolated from infectious sinusitis in chicken and turkey farms particularly in endemic areas (Abd El-Hameed, 2000, Nascimento et al., 2005; Abd El-Hameed, 2006).

ORT, firstly isolated in 1981 from the respiratory tract of turkeys affected with fibrinopurulent airsacculitis while its characterization was done in 1993 (Bordoloi et al., 2020).

Micro-organisms like *Mycoplasma* and *Ornithobacterium* are delicate fastidious ones and difficult to be cultivated with the ordinary methods so in this study, multiplex PCR was used as rapid diagnostic approach help in pathogens detection and control of turkey sinusitis.

MATERIALS AND METHODS

Samples collection and processing

Seventeen turkey farms (2 White Nicholas, 6 Balady and 9 Bronze) at Alexandria governorate were manifested by runny eyes, swelling of infra-orbital sinus either unilateral or bilateral with viscous, slimy or caseated exudates, loss of body weight, high morbidity, and low mortality (Table 1). Exudates of swollen infra-orbital sinus were aspirated and aseptically collected by syringes then transmitted into laboratory for application of multiplex PCR against *E. coli*, *M. gallisepticum*, *M. meleagridis*, ORT, *P. multocida*, and *P. aeruginosa*.

DNA extraction

QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used. 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. and mixed with 200 µl of 100% ethanol then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

Oligonucleotide Primers

Used Primers were supplied from Metabion (Germany) and listed in Table (2).

PCR amplification

Primers were utilized in a 25µl reaction containing 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20 pmol concentration, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products.

20 µl of the PCR products was loaded in each gel slot of 1.5% agarose gel (Applichem, Germany, GmbH). Generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Ethical approval

The present study was affirmed by the Ethics of Animal Health Committee, Desert Research Center, Egypt.

Table 2: Primers sequences, target genes, amplicon sizes and cycling conditions.

Multiplex	Target gene	Primers sequences	Amplified segment (bp)	References
A	*E. coli* phoA	CGATTCTGGAAATGGCAAAAG	720	Hu et al.,2011
		CGTGATCAGCGGTGACTATGAC		
	M. gallisepticum mgc2	CGCAATTTGGTCCTAATCCCCAACA	300	Lysnyansky et al., 2005
		TAAACCCACCTCCAGCTTTATTTCC		
	P. multocida Kmt1	ATCCGCTATTTACCCAGTGG	460	Oie, 2012
		GCTGTAAACGAACTCGCCAC		
	P. aeruginosa 16S rDNA	GGGGGATCTTCGGACCTCA	956	Spilker et al., 2004
		TCCTTAGAGTGCCCACCCG		
B	ORT *16S rRNA*	TGGCATCGATTAAAATTGAAAG	625	Doosti et al., 2011
		CATCGTTTACTGCGTGGACTAC		
	M. meleagridis 16S rRNA	CGA GCG AAG TTT TTC GGA AC	422	Lierz et al., 2008
		GGTACC GTC AGG ATA AAT GC		

RESULTS

Results of multiplex PCR

Regarding the results of multiplex PCR used for detection of causative agents of turkeys' sinusitis, Figure 1 exhibited all tested samples were negative for *P. aeruginosa* and *P. multocida* at 956, and 460 bp fragment respectively and positive for *E. coli*, ORT, *M. meleagridis*, and *M. gallisepticum* at 720, 625, 422, and 300 bp fragment respectively. Table 3 & 4 revealed out of 17 investigated turkey flocks, one Balady breed flock recorded no infection, two flocks of Balady breed had single infection (one affected with *E. coli* and the other affected with *M. gallisepticum*) while the remaining 14 flocks were affected with mixed infection as follow, Three flocks of Bronze breed were affected with the four investigated M.Os, *E. coli*, *M. gallisepticum*, *M. meleagridis*, and ORT, five flocks of 3 Bronze and 2 White Nicholas breed were affected with three M.Os, *E. coli*, *M. gallisepticum*, and ORT, two flocks of 1 Bronze and 1 Balady breed were affected with three M.Os, *E. coli*, *M. meleagridis*, and ORT, one flock of Balady breed was affected with three M.Os, *E. coli*, *M. gallisepticum*, and *M. meleagridis*, another one Balady breed flock was affected with *M. gallisepticum*, and ORT. Finally, two flocks of Bronze breed, one of them was affected with *E. coli*, and *M. gallisepticum* while the other was affected with *M. meleagridis*, and ORT.

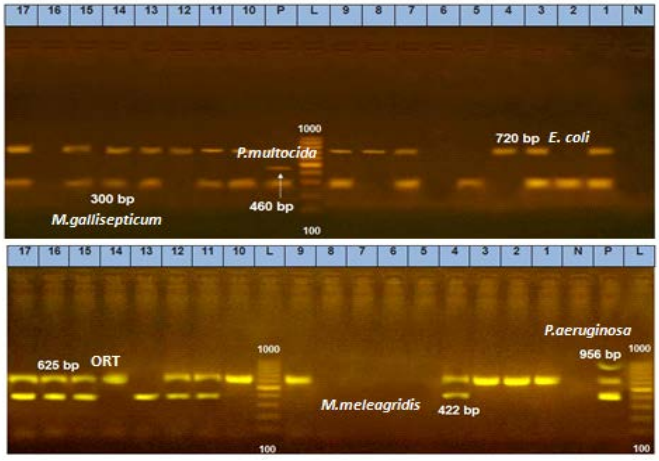

Figure 1: Multiplex PCR showed samples were positive for *E. coli*, ORT, *M. meleagradis*, and *M. gallisepticum* at the specific amplification of 720, 625, 422 and 300 bp fragment, respectively. All samples were negative for *P. aeruginosa* and *P. multocida* at the specific amplification of 956 and 460 bp fragment, respectively.

Incidence of different bacteria in turkey sinusitis

Table 5 showed each of *E. coli*, *M. gallisepticum*, and ORT affected White Nicholas turkey flocks with the same percent 100%. Simultaneously, each of *E. coli*, and *M. gallisepticum* affected Balady turkey flocks with the same percent 50% while each of *M. meleagridis*, and ORT simultaneously found in 33.3% of the same breed. Also with the equal percent, 88.9%, each of *E. coli*, and ORT affected Bronze breed, while 77.8%, and 55.6% of the same breed were affected with *M. gallisepticum*, and *M. meleagridis* respec

Table 3: PCR results of investigated turkey farms

Farm No.	Breed	*E. coli*	*M. gallisepticum*	*P. multocida*	*P. aeruginosa*	*ORT*	*M. meleagridis*
1	White Nicholas	+	+	-	-	+	-
2	Balady	-	+	-	-	+	-
3	Bronze	+	+	-	-	+	-
4	Balady	+	-	-	-	+	+
5	Balady	-	+	-	-	-	-
6	Balady	-	-	-	-	-	-
7	Bronze	+	+	-	-	-	-
8	Balady	+	-	-	-	-	-
9	Bronze	+	+	-	-	+	-
10	Bronze	+	+	-	-	+	-
11	Bronze	+	+	-	-	+	+
12	Bronze	+	-	-	-	+	+
13	Balady	+	+	-	-	-	+
14	White Nicholas	+	+	-	-	+	-
15	Bronze	+	+	-	-	+	+
16	Bronze	-	-	-	-	+	+
17	Bronze	+	+	-	-	+	+

Table 4: Different bacteria isolated from different turkey breeds affected with sinusitis

Number of affected flocks	Breed	No infection	Single infection	Mixed infection
1	Balady	▲		
1	Balady		▲ *E. coli*	
1	Balady		▲*M. gallisepticum*	
3	Bronze			▲ *E. coli*, *M. gallisepticum*, *M. meleagridis*, ORT
5	3 Bronze & 2 White Nicholas			▲ *E. coli*, *M. gallisepticum*, ORT
2	1 Balady & 1 Bronze			▲ *E. coli*, *M. meleagridis*, ORT
1	Balady			▲ *E. coli*, *M. gallisepticum*, *M. meleagridis*
1	Balady			▲ *M. gallisepticum*, ORT
1	Bronze			▲ *E. coli*, *M. gallisepticum*
1	Bronze			▲ *M. meleagridis*, ORT

Table 5: Incidence of different bacteria in turkey sinusitis of variable turkey breeds

Turkey flocks	Total number	*E. coli*		*M. gallisepticum*		*M. meleagridis*		*ORT*	
		NO.	%	NO.	%	NO.	%	NO.	%
White Nicholas	2	2	100	2	100	0	0	2	100
balady	6	3	50	3	50	2	33.3	2	33.3
Bronze	9	8	88.9	7	77.8	5	55.6	8	88.9
Total number	17	13	76.5	12	70.6	7	41.2	12	70.6

Table 6: Incidence of single and mixed infection

	Micro-organism	*E. coli*	*M. gallisepticum*	*M. meleagridis*	*ORT*	Total number
Single	-	1 (Balady)	1 (Balady)	-	-	2

Mixed	*E. coli*	-	10 (1 Balady, 2 White Nicholas, 7 Bronze)	6 (2 Balady, 4 Bronze)	10 (1 Balady, 2 White Nicholas, 7 Bronze)	26
	M. gallisepticum	10	-	4 (1 Balady, 3 Bronze)	9 (1 Balady, 2 White Nicholas, 6 Bronze)	23
	M. meleagridis	6	4	-	6 (1 Balady, 5 Bronze)	16
	ORT	10	9	6	-	25

tively. In general *E. coli* isolates were represented 76.5%, each of *M. gallisepticum*, and ORT represented the same percent, 70.6%, while *M. meleagridis* represented 41.2% of turkey flocks.

Incidence of single and mixed infection

There was one out of 17 turkey flocks negative for multiplex PCR. Furthermore, two flocks were recorded with a single bacterial infection, one flock with *E. coli*, and the other flock with *M. gallisepticum*. Mixed infection with *E. coli* repeated 26 times (10 times mixed with *M. gallisepticum*, 10 with ORT, and 6 with *M. meleagridis*), *M. gallisepticum* repeated 23 times (10 times mixed with *E. coli*, 9 with ORT, and 4 with *M. meleagridis*), *M. meleagridis* repeated 16 times (6 times mixed with *E. coli*, 6 with ORT, and 4 with *M. gallisepticum*), and ORT repeated 25 times (10 times mixed with *E. coli*, 9 with *M. gallisepticum*, and 6 with *M. meleagridis*) (Table 6).

DISCUSSION

Turkeys were considered as competitive protein source in developing countries, so it was necessary to recognize their major pathogens and problems to control and never ignore them long-term.

Swollen infra-orbital sinus is a common respiratory disease which rapidly spread in turkey breeds. So that, in this study multiplex PCR were used to rapidly identify the causative bacteria particularly more delicate and fastidious ones like *Mycoplasma* and ORT.

There were no results appeared with *P. aeruginosa* and *P. multocida* primers, while on investigation of other bacteria, the most prevalent detected one were *E. coli* in 76.5% of affected turkeys followed by *M. gallisepticum* and ORT in 70.6% of cases simultaneously and *M. meleagridis* in 41.2% of cases.

One Balady flock had no infection (5.88%), two Balady flocks reported single infection (11.76%), one with *M. gallisepticum* and one with *E. coli* while mixed infection recorded in 14 farms, 82.35% (100% of each White Nicholas and Bronze and 50% of Balady flocks). from the field surveyed results, *E. coli* and *M. gallisepticum* were identified either alone or together with other bacteria, so they might be responsible for turkeys' sinusitis while *M. meleagridis* and ORT were not identified alone, also *E. coli*, *M. gallisepticum*, and ORT were more frequently mixed with each other than *M. meleagridis*, these results matched with those of Abd El-Hameed et al. (2009) who detected *M. gallisepticum*, either alone or coupled with other pathogens (*P. multocida* and *E. coli*) was the predominant etiologic agent responsible for the respiratory problems including infra-orbital sinusitis of the infected turkey flocks, Sokkar et al. (1986) identified *M. gallisepticum* and *M. meleagridis* either alone or mixed with *E. coli*, *P. aeruginosa*, *S. aureus* and *S. gallinarun pullorum* from turkeys located in different areas of Upper Egypt. Abd El-Rahman (1995) determined serologically *M. gallisepticum*, *M. meleagridis*, *M. synoviae*, and *M. Iowa* and concluded that *M. gallisepticum* was the predominant detected *mycoplasmas*, 90 % of the examined turkeys' flocks referring to the spread of *M. gallisepticum* rather than *M. meleagridis* among turkey's farms. Sokker et al. (1986); KeBin (2003) and Moustafa (2005) were naturally and experimentally reported synergistic action between *M. gallisepticum* and *E. coli* either alone or associated with other pathogens as a major etiologic agents responsible for swollen head syndrome in turkeys, chickens and ostriches. On the contrary, Eissa et al. (2000) detected *M. gallisepticum* and *M. synoviae* in 6.66 % and 13.33 % of examined turkey flocks, respectively indicating that *M. synoviae* was a prominent cause responsible for turkey's paranasal sinusitis rather than *M. gallisepticum*. Pan et al.. (2012) and Bordoloi et al. (2020) were reported that ORT had an important role in respiratory affections of broiler chicken and turkey flocks in association with *E. coli*, *M. gallisepticum*, *M. synoviae*, *Chlamydophila psittaci*, *Bordetella avium*, and *Streptococcus zooepidemicus*, also Welchman et al. (2013) and Kursa et al. (2021) mentioned that ORT was a part of the complex with other pathogens that syn-

ergize to induce the respiratory infection.

CONCLUSION

Turkey Balady breed had a higher resistance to infection than other breeds where it was recorded no or single infection. *E. coli* was the most predominant agents isolated from turkey sinusitis followed by *M. gallisepticum* and ORT with equal percent then *M. meleagridis* had the lowest incidence and frequency. *E. coli* or *M. gallisepticum* was the primary cause responsible for turkey sinusitis as each of them was present in single infection. In this study, a complex of bacterial respiratory pathogens including *E. coli*, *M. gallisepticum*, ORT, and *M. meleagridis* were identified in swollen infra-orbital sinus of turkeys, therefore, Further continuous molecular studies on these delicate pathogens were needed to help in their control.

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CONFLICT OF INTEREST

The authors declare that they do not have any Competing interests.

AUTHORS' CONTRIBUTION

Hanan El-Samahy designed, and revised the article; Amani Hafez helped laboratory analyses, and tabulation of experimental data; Mohamed Talaat helped in manuscript writing and Disouky Mourad helped field study, collected data, and conducted statistical analysis. All authors have read and approved the final manuscript.

ETHICAL CONSIDERATIONS

Ethical issues including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by the authors.

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