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Current Status of Multidrug Resistance of *Ornithobacterium rhinotracheale* from Avian Host

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ABSTRACT

Resistance of *Ornithobacterium rhinotracheale* (ORT) to antibiotics involves a decrease in antibiotic efficacy against the organisms. ORT causes an acute bacterial disease that affects the respiratory system of chickens. This disease results in severe issues of intensive poultry products and public health concern. Therefore, isolation, identification, investigation on antibacterial sensitivity and multidrug resistance patterns of ORT isolates were the main aims of this study. Tracheal samples (n=200) were collected from freshly dead birds with postmortem lesions of respiratory illness. ORT was isolated and identified by classical cultural and molecular methods. Antibacterial sensitivity testing of isolated organisms was carried out by disc diffusion method against seven antibacterial agents. The incidence values of ORT by cultural method were 20.0 and 3.0% from the tracheal samples of dead birds >18 and <18 days old, respectively. Out of 23 cultural positive samples, 15(65.21%) were found positive for the presence of 16S rRNA (625 bp) by PCR. The results of antibiotic sensitivity revealed that 66.6% of isolates were sensitive to amoxicillin, while 60.0 and 46.6% of isolates were sensitive to erythromycin and florfenicol, respectively. For difloxacine and doxycycline, the frequency of sensitive samples was 33.3 and 13.3%, respectively. The highest antimicrobial resistance of QRT isolates was seen against gentamycin and colistin (100%), followed by doxycycline (86.6%) and difloxacine (66.6%). In conclusion, it is very important to update the baseline resistance pattern data for this organism.

Key words: *Ornithobacterium rhinotracheale* (ORT), PCR, Antibacterial sensitivity, Multidrug resistance.

INTRODUCTION

The ORT infection is commonly known as ornithonbacteriosis. It mainly infects turkeys and chickens (broiler and commercial layers), causing respiratory discomfort, stunted growth and even death (Chin and Charlton 2008; Chin et al. 2008; Umali et al. 2018; Hassan et al. 2020; Messah and Pawitan 2020). ORT is a Gram-negative, rod-shaped bacterium belonging to the rRNA superfamily V. It shares taxonomic territory with the genera Cytophaga, Riemerella and Flavobacterium (Canal et al. 2005). ORT is generally regarded as a *Pasteurella*-like organism.

The cultural characteristics and fastidious requirements of QRT (i.e., small colony size, slow growth, enriched media and capnophilic incubation) may adversely affect bacterial isolations and reduce the detection rates. Therefore, molecular detection of *O. rhinotracheale* DNA from tissues or swabs, targeting the 16S rRNA gene with specific primers, is frequently used in routine diagnostics (Veiga et al. 2019).

Multidrug resistance of various organisms against different antibiotics is showing an increasing trend these days. Previous studies have revealed that multidrug resistance among the examined ORT strains increased from 89.5% in 2019 to 100% in 2020 (Nalvarte et al. 2019; Hassan et al. 2020). Several antimicrobial agents, including those most recently developed, are becoming ineffective against ORT, reinforcing the hypothesis of continuous development of drug resistance in these organisms (Watteyn et al. 2016). The present study was designed to investigate the current prevalence of ORT infection in broiler chickens in El- Sharkia Governorate, Egypt and to monitor the antibacterial sensitivity and multidrug resistance patterns of the isolates.

MATERIALS AND METHODS

Samples

Tracheal samples (n=200) were collected from freshly dead birds >18 and <18 days old with postmortem lesions of respiratory illness. These specimens were collected under aseptic condition and subjected to ORT isolation.

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Isolation and Identification

Isolation and identification of QRT was performed according to the procedure of Vandamme et al. (1994). Briefly, the samples were cultivated in brain heart infusion (BHI) broth, and then sub-cultured on 10% sheep blood agar, trypticase soy agar, MacConkey agar and nutrient agar media. Then the plates were incubated at 37°C for 48 hours under 7.5-10.0% CO_2 tension by gas bags (Oxoid) in a candle jar, and examined for suspicious ORT colonies.

Phenotypic Characterization

Pinpoint, circular, tiny, opaque to greyish, and non-hemolytic colonies were chosen. Bacteriological films were prepared, stained with Gram's stain and examined microscopically. Then catalase, triple sugar iron, nitrate reduction, indol, oxidase, Voges Praskauer and urease tests were performed, as described earlier (Cruickshank et al. 1975). The confirmed ORT isolates were kept at -80°C in brain heart infusion (BHI) broth containing 30% glycerol for further investigation.

PCR Assay

DNA was extracted from all ORT isolates. Genomic DNA was harvested using the QIAamp DNA mini extraction kit (Catalogue No. 51304, Qiagen, Germany) according to the instructions provided with the kit. The DNA concentration was measured using a UV spectrophotometer (Beckman DU640, CA, USA), which was set to 50ng/µL. Three microliters of each template were used in the PCR. The primers descried by Doosti et al. (2011) were applied in this investigation, according to Emerald Amp GT PCR master mix (Takara-Code No. RR310A) OR16S-F1 (TGGCATCGATTAAAATTG AAAG) and OR16S-R1 (CATCGTTTACTGCGTG GACTAC), which were copied at a 625bp fragment in the 16S rRNA. Their experiments were employed in a final volume of 25µL, which included 5µL of template DNA, 12.5µLof Emerald Amp GT PCR master mix (2x premix), 5.5µL of PCR grade water and 1µL of each ORT primer. Initial denaturation was applied at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes.

ORT Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of QRT isolates was performed against seven antibacterial drugs (Table 2), using standard disk diffusion method and trypticase soy agar (TSA) media. Procedure's outline and interpretation were the same as described by Murthy et al. (2008) and Clinical and Laboratory Standards Institute (CLSI 2018).

Statistical Analysis

The collected data were computerized and statistically analyzed using IBM crop SPSS program (Statistical Package for Social Sciences 2020) version 27.0. Qualitative data were represented as frequencies and relative percentages. Chi square test with Fisher exact correction was used to calculate difference between qualitative variables. The threshold of significance was fixed at 5% level (P-value). $P<0.05$ indicated significance effects; $P>0.05$ indicated non-significant effects, while $P<0.001$ indicates highly significant effects (Kirkwood and Sterne 2003).

RESULTS

Isolation and Phenotypic Identification of ORT

ORT colonies on blood agar were non hemolytic, small circular convex with round edges, opaque greyish white with a strong odor similar to butyric acid and showed poor adhesion to the agar surface. Colonies grew on BHI broth and trypticase soy agar, but not on MacConkey agar or nutrient agar media. ORT isolates appeared as Gram negative, pleomorphic, non-motile and non-sporulated, arranged in clusters or fat short rods. The results of biochemical tests for catalase, triple sugar iron, nitrate reduction, and indol were negative, while those for oxidase, Voges Praskauer and urease were positive.

Depending on morphological, cultural and biochemical tests, the incidence rates of ORT were 20.0 and 3.0% in tracheal samples from dead birds >18 and <18days old, respectively (Table 1). Statistical analysis showed that there was non-significant difference in frequencies of positive samples from >18day old birds and <18day old birds. But according to regions, there was a statistically significant increase in frequency of positive samples from birds >18 days old compared to samples from birds <18 days old in Belbis and private farm.

Molecular Identification using PCR

Out of 23 positive ORT isolates by conventional method, 15(65.21%) samples were positive for the presence of 16S rRNA (625bp) by PCR (Fig. 1 and 2).

ORT Antimicrobial Susceptibility Testing

Table 2 and 3 show that 66.6% of ORT isolates were sensitive to amoxicillin, while 60.0 and 46.6% of isolates were sensitive to erythromycin and florifinicol, respectively. For difloxacine and doxycycline, 33.3 and 13.3% samples were sensitive. The highest antimicrobial resistance was seen for gentamycin and colistin (100%), followed by doxycycline (86.6%) and difloxacine (66.6%). It was interesting to note that all ORT strains were resistance to 4-6 antimicrobial groups (multidrug resistance).

DISCUSSION

Due to ORT importance, precise diagnostic and characterization methods are necessary for its early control and production of a promising vaccine (Patel et al. 2018; Ellakany et al. 2019). ORT has been neglected in poultry farms, mainly due to the lack of appropriate diagnostic protocols and consequent treatment failure (Barbosa et al. 2020). Mayahi et al. (2016), Ellakany et al. (2019) and Al-Hasan et al. (2021) described initial isolation, typical morphology and biochemical investigations of ORT, as have been described in the present study.

Associations with age of birds, Our results relating to the incidence of QRT infection and age of birds are parallel with those of Xue et al. (2020), who found that by increasing the age, the rate of ORT also increased. ORT is a difficult bacterium to be cultured. It grows slowly and needs special growth conditions. Thus, attempts of isolation

Table 1: Incidence ORT in examined tracheal swaps from dead broilers less and above 18 days of age

Region	Total No of samples	Total positive samples		Birds <18 days of age			Birds >18 days of age			P value
				Total No of samples	Positive samples		Total No of samples	Positive samples		
		No	%		No	%		No	%	
Belbies	80	7	8.75	40	6	15.0	40	I	2.5	0.004*
Abo-hammad	40	5	12.5	20	4	20.0	20	I	5.0	0.34 NS
El-qurin	60	6	10.0	30	5	16.7	30	I	3.3	0.19 NS
Private farm (Gita)	20	5	25.0	10	5	50.0	10	0	00	0.03*
Total	200	23	11.5	100	20	20	100	3	3	<0.001**
			0.23 NS			0.09 NS			0.89 NS	

P: Fisher exact test: NS: Non significant (P>0.05) *: Significant (P<0.05) **: highly significant (P<0.001).

Table 2: The antimicrobial sensitivity testing of 15 strains of ORT to different antimicrobial agents

Antimicrobial agents	Disc Concentration (µg)	Susceptible		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Amoxicillin	30	10	66.6	1	6.6	4	26.6
Flrofenicol	30	7	46.6	2	13.3	6	40.0
Erythromycin	15	9	60.0	2	13.3	4	26.6
Difloxacine	10	5	33.3	0	0	10	66.6
Doxycycline	30	2	13.3	0	0	13	86.6
Gentamycin	10	0	0	0	0	15	100
Colistin	15	0	0	0	0	15	100

Table 3: Multidrug resistance strains of ORT against different antimicrobial agents

AM groups	β-lactams	Phenicols	Macrolides	Floro-quinolones	Tetracyclines	Amino-glycosides	Lipopeptides		
AM agents	Amoxicillin (30µg)	Flrofenicol (30µg)	Erythromycin (15µg)	Difloxacine (10µg)	Doxycycline (30µg)	Gentamycin (10µg)	Colistin (15µg)	Total No of resistance	MDR
ORT 1	R	S	S	R	S	R	R	4	+
ORT 2	R	R	S	S	S	R	R	4	+
ORT 3	S	S	S	R	R	R	R	4	+
ORT 4	S	S	R	S	R	R	R	4	+
ORT 5	S	R	S	S	R	R	R	4	+
ORT 6	S	S	S	R	R	R	R	4	+
ORT 7	S	S	S	R	R	R	R	4	+
ORT 8	S	R	S	R	R	R	R	5	+
ORT 9	S	I	S	R	R	R	R	4	+
ORT 10	S	R	R	R	R	R	R	6	+
ORT 11	S	R	R	R	R	R	R	6	+
ORT 12	R	I	I	S	R	R	R	4	+
ORT 13	R	S	I	R	R	R	R	5	+
ORT 14	S	R	S	R	R	R	R	5	+
ORT 15	I	S	R	S	R	R	R	4	+

AM= antimicrobial; MDR = Multidrug resistant.

of ORT are often unsuccessful and plates are mostly overgrown by other bacteria. These findings imply that serological investigation (Zuo et al. 2018) and PCR can be used in the field to confirm ORT isolates as part of a general diagnosis (Doosti et al. 2011).

Many investigators (Hung and Alvarado 2001; Ozbey et al. 2004; Hassanzadeh et al. 2010) have applied the PCR method for verification of ORT strains, as it is a sensitive, quick, and specific approach. Al-Hasan et al. (2021) used reverse transcription-polymerase chain reaction (RT-PCR) in addition to PCR and a partial 16S rRNA gene was isolated.

Out of 23 positive ORT isolates by conventional method, 15(65.21%) samples were found positive for the presence of 16S rRNA (625bp) in the present study. On the other hand, Ozbey et al. (2004) reported that all samples positive for ORT by the culture were also found positive by the PCR. According to Ha et al. (2016), all ORT positive samples exhibited negative results by bacterial culture and conventional PCR. On the other hand, Hassanzadeh et al. (2010) found that the use of PCR raised the number of ORT that was negative by culture method. They added that the combination of culture, serology, and PCR maximized the diagnosis of ORT infections.

The current study was aimed to update the antimicrobial profile of ORT isolates to certain routinely used and newly developed antimicrobial drugs *in vitro*. The antibacterial sensitivity of ORT strains may vary depending on geographical area. Malik et al. (2003) and Türkyilmaz (2005) have hypothesized that this is due to underlying genetic differences between species and antibiotic efficacy of agents used.

The antibiogram analysis has shown variable results regarding the susceptibility of ORT to amoxicillin, flrofenicol, erythromycin, difloxacine, doxycycline, gentamycin and colistin in previous studies. Shahata et al. (2006) found that 100% of ORT isolates were sensitive to amoxicillin. Furthermore, Mohd-Zain et al. (2008) and Hegazy et al. (2015) discovered three distinct isolates which were amoxicillin and doxycycline sensitive. In addition, Mayahi et al. (2016), Hassan et al. (2020) and Al-Hasan et al. (2021) recorded that ORT strains were sensitive to florfenicol (100%), colistin (100%) and doxycycline (71.4%), respectively. On the other hand,

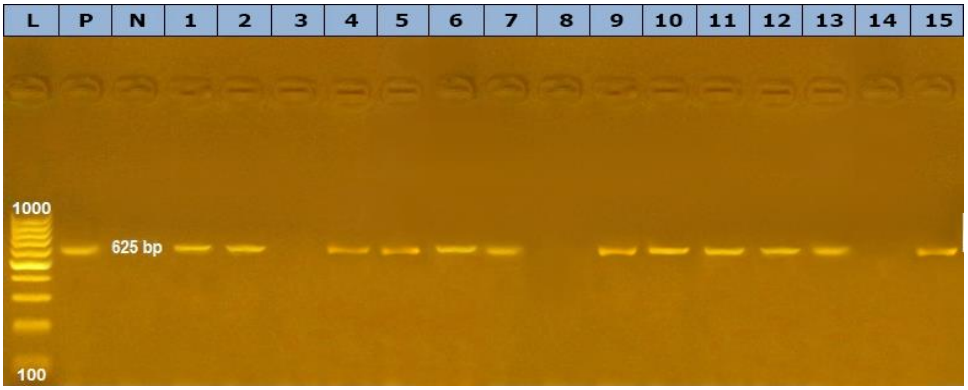

Fig. 1: PCR analysis of ORT isolates for the presence of 16S rRNA (625bp): (L) DNA ladder, (P) Positive control, (N) Negative control, Lanes 3, 8 & 14 are negative samples and Lanes 1, 2, 4, 5, 6, 7, 9, 10, 11, 12, 13 & 15 are positive samples (625bp).

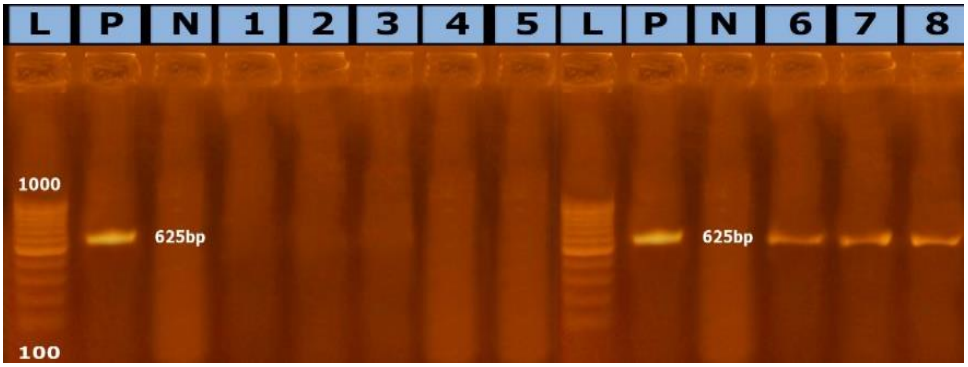

Fig. 2: An agarose gel electrophoresis for PCR products of ORT isolates stained with ethidium bromide. (L) DNA ladder, (P) Positive control, (N) Negative control, Lanes 1, 2, 3, 4 and 5 are negative samples and Lanes 6, 7 and 8 are positive samples for the presence of 16S rRNA (625bp).

Nhung et al. (2017) found ORT strains with median levels of antimicrobial resistance (AMR) against gentamicin and amoxicillin, all exceeding 50%. Keeping in view the increased resistance of different bacteria for different classes of drugs, Mayahi et al. (2016) mentioned that the sensitivity pattern of ORT strains to different drugs depends on the source of the strain and nature of routinely used antibiotics in the area.

As shown in Table 2, local ORT isolates were found to be multidrug resistant (100%). These results are supported by the findings of Hassan et al. (2020), Soriano et al. (2003) and Nalvarte et al. (2019), who found 94.4 and 89.5% multidrug resistance among the examined ORT strains, respectively. Such results were expected due to over and misuse of antibiotics in the local poultry industry that is followed by treatment failure, leading to huge economic losses (Churria et al. 2016; Nhung et al. 2017; Sharif et al. 2021). This contributes to the generation of new drug-resistant variant strains that will contaminate the soil and streams, spreading to other animals and humans (Barbosa et al. 2020).

Conclusion

Simultaneous use of both cultural and molecular techniques is more comprehensive in the isolation and identification of the ORT bacterium. Multidrug resistance against new antimicrobial drugs shows an increasing trend. These results emphasize the emergency need for continued monitoring of *O. rhinotracheale* isolates for antibiotic resistance.

Author's Contribution

Eman S Mohamed, Ahmed M Hamoud and Mona I El Enbaawy designed the plan of work, supervised the experiment and revised the manuscript writing. Mona I El Enbaawy carried out language editing and formatting the manuscript.

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