

Phenotypic Characteristics, Antibiotic Susceptibility and Pathogenicity of *Ornithobacterium rhinotracheale*

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Abstract:- *Ornithobacterium rhinotracheale* (ORT) has been recently recognized as one of the respiratory pathogen in poultry. This bacterium produces clinical signs in chickens which are almost similar to the infection of *Avibacterium paragallinarum*, thus making diagnosis difficult. A collection of 18 isolates of *O. rhinotracheale* obtained from diseased chickens in Malaysia were characterized and their pathogenicity in embryonated eggs and chickens was investigated and compared to a reference strain. Antimicrobial susceptibility pattern of these isolates against seven antibiotics were determined using Kirby-Bauer diffusion test while minimal inhibition concentration (MIC) of tylosin and tilmicosin were performed using agar dilution method following the guidelines of Clinical and Laboratory Standards Institute. Biochemical and enzymatic tests results showed that the *O. rhinotracheale* isolates were made up of two variants that differ from the reference strain in their fermentation of arginine and glucose. Serotyping of these isolates revealed that all of the isolates were serotype A. Antimicrobial susceptibility test performed using antimicrobial disks showed that at least two of the isolates were resistant to more than three of the antibiotics tested. High (>128 µg/ml) minimal inhibition concentration (MIC) of tylosin and tilmicosin was observed in the isolates. Two representatives from these isolates were tested for their ability to cause mortality in egg embryos and their clinical manifestations in specific-pathogen-free (SPF) chickens. When compared to uninoculated embryos, these two isolates were found to cause significantly ($P < 0.01$) higher number of embryo mortality. Infection of these isolates in SPF chickens caused growth retardation but no respiratory symptoms were observed. The results obtained in this study have provided some basic information on the properties of *O. rhinotracheale* that would be useful in diagnosing the disease. The understanding of its role in poultry health provides some information that could be useful in implementing preventive measures against the disease.

Key-Words: - *Ornithobacterium rhinotracheale*, embryo mortality, growth retardation, chickens, antibiotic resistant, Malaysia

1 Introduction

Ornithobacterium rhinotracheale (ORT), a Gram-negative pleomorphic bacterium was isolated from commercial broiler chickens for the first time in Malaysia in 2000 [1]. Prior to 1994, this *Pasteurella*-like bacterium had been reported in

other countries to be able to cause highly contagious disease in poultry, particularly in turkeys and chickens [2, 3]. Severe necrotizing fibrino-purulent septicaemia is one of the clinical manifestations which resulted in economic loss to poultry industries due to increased in

condemnation [4]. In some cases, chickens infected with *O. rhinotracheale* did not present with clinical signs [5]. The variability in the severity that occurred in the chickens depends on factors such as strain virulence, adverse environmental and immune state of the flock, and is usually enhanced by co-infection with other pathogens [6, 7].

In making a diagnosis, the infection of *O. rhinotracheale* is usually mistaken for infectious coryza, which is caused by *Avibacterium paragallinarum* [8]. This bacterium which was previously known as *Haemophilus paragallinarum* causes acute respiratory disease in poultry, particularly chickens. The disease caused by *A. paragallinarum* occurs worldwide and causes economic losses mainly due to increased number of culls and marked reduction in egg production. Due to the importance of *A. paragallinarum* in poultry industries, infections due to *O. rhinotracheale* are usually overlooked or mistaken. Furthermore, the difficulty to isolate and identify the organism and its highly resistant to many antibiotics complicates the diagnosis of *O. rhinotracheale* [9].

The main purpose of this study is to understand the basic characteristics of a collection of *O. rhinotracheale* isolates which could be useful in diagnosing the disease. The knowledge on the role of this bacterium can also be useful as a platform for the planning of control and preventive measures against its infection.

2 Materials and Methods

2.1 Bacteria and Media

Eighteen *O. rhinotracheale* strains involved in this study were isolated from chickens that were submitted for disease investigation to Veterinary Research Institute (VRI), Ipoh, Malaysia from 2000 to 2007. Swabs of the nasal cavity, trachea and air sac were cultured onto 5% sheep blood agar and incubated under microaerobic conditions (5-10% CO₂) [10]. Suspected colonies were stained by Gram's methods and subcultured onto chicken meat infusion (CMI) agar [11]. Colonies with characteristics of *O. rhinotracheale* were identified biochemically [10, 12] and their antigenicity was determined using agar gel

agglutination test against serotypes A to G according to the methods of van Empel *et al.* [10]. Each of the isolates was stored in brain heart infusion broth (BHI) with 30% glycerol at -80°C until use.

For comparative investigation, a reference strain B3263/91 of serotype A (obtained from Paul van Empel, Intervet-International, The Netherlands), used as reference strain, was originally isolated from diseased broiler chickens in South Africa. *Avibacterium paragallinarum* strain 6755/04 was isolated from a layer chicken with infectious coryza.

2.2 Biochemical and Enzymatic Reactions

The biochemical characterization was performed with nitrate reduction, catalase, urease, indole, MacConkey, arginine, lysine, ornithine, carbohydrate fermentation such as fructose, lactose, maltose, galactose and glucose. Carbohydrate fermentation test were carried using broth supplemented with 2% SPF inactivated chicken sera.

2.3 Polymerase Chain Reaction

Amplification of 16S rRNA genes of the *O. rhinotracheale* by polymerase chain reaction (PCR) technique was carried to aid in the identification of *O. rhinotracheale* [12].

2.4 Antimicrobial Susceptibility Tests and Resistant Plasmids

Kirby-Bauer disk-diffusion method was used to determine the susceptibility of the *O. rhinotracheale* isolates against amoxicillin (AMC30), ampicillin (AM10), chloramphenicol (C30), doxycycline (D30), enrofloxacin (ENR5), sulfonamide and trimethoprim (SXT25) and tetracycline (TE30); all of these disks are products of Oxoid, United Kingdom. The MICs of tylosin (Eagle Vet. Tech., China) and tilmicosin (Lilly, Austria) against these isolates were determined using agar dilution method due to unavailability of commercial disks. Stock solutions of antibiotics were prepared freshly on the day of the assay and filter sterilized. Both methods followed the guidelines of the Clinical and Laboratory Standards Institute.

2.5 Plasmid Isolation

The *O. rhinotracheale* cells were harvested by centrifuging overnight broth cultures at 10,000 rpm at 4°C for 30 minutes and the cell sediments were re-suspended for 5 minutes at room temperature. Plasmid extraction was performed to isolate resistance plasmid from all the isolates following the methods of Kado and Liu [13]. The amplified samples were electrophoresed through a horizontal 0.7% agarose gel at 100V for 30 minutes, using Tris-borate buffer. The gel was stained for 15 min in ethidium bromide, washed for 15 minutes in distilled water and viewed under ultraviolet illuminator.

2.6 Embryonated Eggs and Experimental Chickens

Seven day-old embryonated eggs and four week-old White-Leghorn chickens used in this study were obtained from SPF flock maintained in a filtered air, positive pressure house at VRI. These chickens were fed *ad libitum* with sterilized pellets free from contaminants.

2.7 Preparation of Inoculum for Egg and Chicken Inoculation

O. rhinotracheale and *A. paragallinarum* were grown on CMI agar in 8-10% CO₂ atmosphere for 48 hours. Colonies from these media were washed in phosphate buffered saline (PBS, pH7.2) and harvested by centrifuging at 12,000 g at 4°C for 30 minutes. The pellets were collected and washed twice with PBS at 24,000 g for 15 minutes and were re-suspended in PBS to contain at least 1 x 10⁸ CFU/ml of the organisms. They were diluted accordingly prior to inoculation into embryonated eggs and chickens.

2.8 Embryo Mortality

Two isolates, 9018/03 and 4393/04 representing the *O. rhinotracheale* isolates were used in this study. A total of 160 embryonated SPF eggs were divided into four groups consisting of three tests and one control. In each test group, 10 eggs each were inoculated via yolk sac with 100 µl of the inoculum containing 10², 10³, 10⁴, 10⁵ and 10⁶ CFU of each of the isolates, 9018/03, 4393/04 and B3263/91. The control group was inoculated with the same volume of sterile PBS. The eggs were candled daily for embryo mortality for 12 days. Embryos that survived until day 12 post-infection

(p.i.) were sacrificed. All dead and live embryos were cultured onto CMI agar to re-isolate the inoculated organism.

2.9 Pathogenicity Study in Chickens

In order to distinguish the clinical manifestations produced by *O. rhinotracheale* and *A. paragallinarum* infections, *O. rhinotracheale* strain 4393/04 and *A. paragallinarum* strain 6755/04 were used in this experiment. A total of 40 chickens were weighed and were randomly divided into two test groups and one control group. The test groups consisted of 15 chickens and 10 chickens were placed in the control group. Ten of the chickens in the test groups were inoculated intranasally with either 10⁶ colony-forming unit (CFU) of *O. rhinotracheale* strain 4393/04 or *A. paragallinarum* strain 6755/04. Five remaining chickens in these groups were non-treated in-contact chickens. All the chickens in the control group were inoculated with 10 µl of sterile PBS via the same route.

Clinical manifestations due to the infection of *O. rhinotracheale* were observed by daily examination of the chickens in all the groups. Signs of watery eyes, swollen head, coughing, sneezing, nasal discharge, rales and dullness were recorded. At day 7 p.i., all the chickens were weighed before they were euthanized. Post mortem examination was carried out for observation of macroscopic lesions. Their sinuses, tracheas and air sacs were swabbed for culture to recover the inoculated organisms.

3 Results

3.1 Bacterial Identification and Serotyping

Small (1-3 mm) grey to grey-white colonies grew on sheep blood agar after 48 hours incubation, while on CMI agar, the colonies appeared tiny and colourless. Gram's stain showed Gram-negative, pleomorphic, rod-shaped bacteria.

The biochemical properties of the *O. rhinotracheale* isolates are shown in Table 1. It was observed that all of the isolates did not reduce nitrate, as compared to *A. paragallinarum*. Three variants were shown to exist amongst the 18 isolates but majority (10/18) of the isolates was phenotypically similar to the reference strain,

B3263/91. Eight of the isolates fermented glucose, of which three of them hydrolysed arginine.

Results of the specific DNA amplification for *O. rhinotracheale* by PCR revealed that all the 18 isolates produced an amplicon of 784 bp which confirmed them as *O. rhinotracheale*. Serotyping of the isolates revealed that all of them belonged to serotype A.

3.2 Antimicrobial Susceptibility Profile and Resistant Plasmids

The results of antimicrobial susceptibility test of the isolates are shown in Table 2. All the isolates were susceptible to chloramphenicol but were resistant to ampicillin, enrofloxacin and the combination of sulfanamide with trimethoprim. Majority (77.8%) of the isolates were also resistant to amoxicillin.

The agar dilution test showed that all of the isolates were found to require high concentration of tylosin and tilmicosin to inhibit growth of the isolates (Table 3). All of the isolates had MIC values of ≥ 64 $\mu\text{g/ml}$ for tylosin but for tilmicosin, MIC of >128 $\mu\text{g/ml}$ was observed in all of the isolates.

The attempt to isolate plasmid from all the isolates failed because none of the isolates were found to carry any plasmids.

3.3 Embryo Mortality

Embryo mortality was first observed on day 3 p.i. in all the test groups which continued throughout the experimental period (day 12 p.i). Inoculum dose as low as 10^2 CFU of isolates 9018/03 and 4393/04 was found to be able to kill the chicken embryos, comparable to the lethal effect of the reference strain, B3263/91 (Table 4). The highest number (26%) of mortality was recorded in the group inoculated with 4393/04 while the group inoculated with B3263/91 had the least number of mortalities. The control group did not have any embryo mortality. The number of embryo mortality between the test groups were not significantly different ($P>0.01$) but they were significantly higher ($P<0.01$) than the control group. The inoculated organisms were recovered

from dead as well as live embryos from both test groups but none from the control groups.

3.4 Pathogenicity Study in Chickens

It was observed that more number of chickens in the *A. paragallinarum*-infected group developed clinical signs of respiratory syndromes than those in the *O. rhinotracheale*-infected group (Table 5). Only one bird in the *O. rhinotracheale*-infected group had mild nasal discharge on day 2 p.i. which subsided by the next day. Six chickens, including three in-contacts in the *A. paragallinarum*-infected group presented with severe nasal discharge, watery eyes and swollen head which began on day 3 p.i. and persisted until day 7 p.i. None of the control bird developed any clinical signs. The in-contact birds in the *O. rhinotracheale* group did not show any clinical signs whereas the three in-contact birds in the *A. paragallinarum* group showed small amount of nasal discharge and slight swelling of the eyes.

At the time of necropsy (day 7 p.i), the chicken in the *O. rhinotracheale*-infected group that previously had nasal discharge was observed to retain the mucous but it was only limited in the nasal cavity, while the chickens (including in-contacts) in the *A. paragallinarum*-infected group had not only mucous in the nasal cavity but also cloudy air sacs. The tracheas of all the chickens in all the groups however, were normal.

Upon culture of swabs from the various sites of the upper respiratory tract, the inoculated organism was recovered from all of the chickens in the *A. paragallinarum*-infected group compared to only three chickens in *O. rhinotracheale*-infected group. The inoculated organisms were also recovered from in-contact chickens in both groups.

Between the test groups (excluding in-contacts), no significant difference ($P>0.01$) in the bodyweight gain was recorded, but when compared to the control groups, their bodyweight gain were significantly ($P<0.01$) lower. The in-contact chickens of the *O. rhinotracheale* group had a higher bodyweight than those in-contact groups of the *A. paragallinarum* group.

Table 1. Biochemical and enzymatic characteristics of *O. rhinotracheale* and *Avibacterium paragallinarum*

Test	A. <i>paragallinarum</i> 6755/04	Reference <i>O.</i> <i>rhinotracheale</i> B3263/91	<i>O. rhinotracheale</i> isolates		
			Variant 1	Variant 2	Variant 3
Nitrate reduction	+	-	-	-	-
Catalase	-	-	-	-	-
Urease	-	+	+	+	+
Indole	-	-	-	-	-
Growth on MacConkey	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	+
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-
Fermentation of					
fructose	+	+	+	+	+
lactose	-	+	+	+	+
maltose	-	+	+	+	+
galactose	-	+	+	+	+
glucose	-	+	+	-	-
No. of isolates			10	5	3

Table 2. Antimicrobial susceptibility of *O. rhinotracheale*

Antibiotic		No. of isolates					
		Resistant	%	Intermediate	%	Susceptible	%
Amoxicillin	AMC30	14	77.8	1	5.6	3	16.7
Ampicillin	AM10	18	100	0	0	0	0
Chloramphenicol	C30	0	0	0	0	18	100
Doxycycline	D030	1	5.6	5	27.8	12	66.7
Enrofloxacin	ENR5	18	100	0	0	0	0
Sulfonamide & Trimethoprim	SXT25	18	100	0	0	0	0
Tetracycline	TE30	5	27.8	13	72.2	0	0

Table 3. Minimal inhibition concentration of tylosin and tilmicosin to isolates of *O. rhinotracheale*

Antibiotic	No. of isolates with MIC ($\mu\text{g/ml}$)								Total
	>128	128	64	32	16	8	4	2	
Tylosin	10	6	2	0	0	0	0	0	18
Tilmicosin	18	0	0	0	0	0	0	0	18

Table 4. Number of embryo mortality in seven day-old chicken embryonated eggs artificially infected with *O. rhinotracheale*

Inoculated strain	No. of eggs inoculated	No. of embryo mortality						No. of <i>O. rhinotracheale</i> re-isolated (%)		P-value to control group
		Dose of inoculum (CFU)					Total	Dead	Survived	
		10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶				
Nil (control)	10	0	0	0	0	0	0	0 (0)	0 (0)	-
B3263/91	50	1	1	2	2	3	9	9 (18)	41 (82)	P<0.01
9018/03	50	1	1	2	2	5	11	11 (22)	39 (78)	P<0.01
4393/04	50	2	2	3	3	3	13	13 (26)	37 (74)	P<0.01

Table 5. Clinical manifestations, post mortem examination and recovery of organisms from four week-old SPF chickens experimentally infected with *O. rhinotracheale*

Inoculated organism		Clinical Signs ^a	Post mortem examination ^b	Recovery of organisms ^c	Average bodyweight gain (g)
<i>O. rhinotracheale</i>	Inoculated	1/10	1/10	3/10	37.5
4393/04	In-contact	0/5	0/5	3/5	44.0
<i>A. paragallinarum</i>	Inoculated	3/10	10/10	10/10	37.7
6755/05	In-contact	3/5	5/10	5/10	32.0
Nil (Control)		0/10	0/10	0/10	53.0

Numerator denotes the number of positive chickens and denominator denotes the total number of chickens used.

^aClinical signs include eye tearing, swollen head, coughing, sneezing, nasal discharge, rales and dullness.

^bGross lesions seen at post mortem examination included presence of mucous in nasal cavity and trachea, consolidated lung and cloudy air sac.

^cInoculated organisms re-isolated from nasal cavity, trachea, lung and air sac.

4. Conclusion

The importance of respiratory infection in poultry due to *O. rhinotracheale* has only been recognized in the 1990s [14]. This is because the manifestation of this bacterium resembles infectious coryza [8]. Besides the confusion, rapid-growing bacteria were shown to mask the growth of *O. rhinotracheale* colonies [6, 15]. In this study, some phenotypic characteristics of Malaysian *O. rhinotracheale* were revealed. Similar to other many other countries, serotype A seems to be the most prevalent serotype [3, 16, 17]. Although all the isolates were mainly isolated within the peninsular of Malaysia, variant strains that produce different biochemical reactions to typical strains of *O. rhinotracheale* would cause confusion during identification. Arginine dehydrolase negative produced by three of the isolates have also been similarly characterized by Hafez [15] and Hinz *et al* [18]. These isolates also failed to ferment glucose, similar to the characteristics of the isolates

reported by Vandamme *et al.* [19]. The 16s rRNA PCR technique was found to be useful to confirm the identification of *O. rhinotracheale*, which is in agreement to other published reports [20, 21].

The emergence of multi-drug resistance in *O. rhinotracheale* is now becoming one of the major veterinary concerns. Like the *O. rhinotracheale* strains isolated in other parts of the world, the *O. rhinotracheale* strains examined in this study have been shown to be resistant to many of the major antibiotics that are normally being used for the treatment of respiratory diseases of poultry [22, 23, 24]. Some antibiotics such as ampicillin, tilmicosin and tylosin have been reported to be effective against *O. rhinotracheale* infection [9, 25]. However in this study, it was found that the Malaysian *O. rhinotracheale* isolates are no longer effective against these drugs since evidences showed that they have now acquired resistance. It is a matter

to concern because the MICs of tilmicosin (>128 ug/ml) and tylosin (>128 ug/ml) for these isolates are considerably high compared to the MICs of tilmicosin (<1 ug/ml) and tylosin (<2 ug/ml) obtained by other investigators [25]. Perhaps this is the first report of *O. rhinotracheale* resistant to both tilmicosin and tylosin.

There have been reports that sensitivity of *O. rhinotracheale* to antibiotics varies depending on the strain from where it was isolated [26]. Devriese *et al.* [27] reported that 98% *O. rhinotracheale* strains isolated in Belgium were found to be sensitive to quinolones *in vitro*. Similar observations were seen in Turkey and Holland, whereby, most of their isolates were also resistant to quinolone [28, 29]. However in Germany, most of the *O. rhinotracheale* isolates have been found to be resistant to quinolone [23]. In this study, all of the Malaysian isolates were similarly found to be resistant to quinolone group. The prevalence of quinolone-resistant *O. rhinotracheale* strains could be attributed to point mutations that occurred at the GyrA subunit [24].

Similar to the work done by other investigators, attempts to isolate resistant plasmids from *O. rhinotracheale* isolates failed [22, 31]. One of the possible reasons for the inability to detect plasmid in any of the *O. rhinotracheale* isolates is that the plasmid that carries antibiotic resistant genes may have integrated into the host chromosomes, as has been shown to occur in multi-drug resistant *Haemophilus influenzae* [32, 33]. Therefore, it is postulated that the ampicillin-resistant gene, *amp* which usually resides on plasmid is on the chromosome of *O. rhinotracheale*. This hypothesis however, requires experimental evidence. Further work on the mode of transmission of the antibiotic determinants would be useful to shed some light on the successful widespread of antibiotic resistance in strains of *O. rhinotracheale*.

The Malaysian *O. rhinotracheale* isolates examined in this study were shown to be able to cause mortality to chick embryos. These strains took three days to kill embryos whereas the strains that Varga *et al.* [26] used in their

experiment took nine days. The virulence of the isolates in this study was comparable to that of the reference strain, B3263/91. The differences in the observations confirm the report by van Empel *et al.* [7] that the pathogenicity of *O. rhinotracheale* isolates can be variable depending on the strains of the isolates.

Being a newly described respiratory pathogen in chickens and turkeys, very little information on its transmission has been documented. Varga *et al.* [26] suggested that *O. rhinotracheale* is not transmitted through egg during hatching based on their failure to re-isolate the inoculated *O. rhinotracheale* from experimentally infected chick embryos. The results of our experiment, however, have proven otherwise. The high percentage of *O. rhinotracheale* recovery from chick embryos at the end of the experiment suggests that *O. rhinotracheale* can be transmitted via eggs. This observation is in agreement to van Veen *et al.* who observed respiratory tract lesions in SPF chicks that were placed in hatching incubators during hatch [25]. Besides vertical transmission, *O. rhinotracheale* infection can also be spread horizontally as demonstrated by the recovery of inoculated *O. rhinotracheale* from in-contacts chickens in close proximity with infected chickens, thus confirming their mode of transmission.

Experimental infections into chickens have shown that severity of the disease produced by *O. rhinotracheale* varies between strains. A study from South Africa has demonstrated that *O. rhinotracheale* can cause respiratory tract lesions and growth retardation in both chickens and turkeys which is similar to infection by *A. paragallinarum* [34]. In the present study, respiratory symptoms shown by the chickens infected with *O. rhinotracheale* were less severe than those infected with *A. paragallinarum*, but both were equally able to retard growth in chickens. Although many reports have shown that *O. rhinotracheale* alone is able to cause severe respiratory symptoms in both turkeys and chickens [2, 10], the mild respiratory symptoms in the experimentally infected chickens signifies their low virulence. This is in accordance to other investigators that severity of clinical signs, duration of the disease and mortality in *O.*

rhinotracheale infection are extremely variable depending on the strains [8, 25]. Besides strains, the breed of the chickens also has an influence on the severity of clinical symptoms [6]. This possibly accounts for the mild respiratory symptoms that were observed in the experimentally infected chickens in our study. Therefore, it would be useful to investigate the larger numbers of *O. rhinotracheale* isolates to further understand its pathogenicity pattern.

In general, it can be concluded that growth retardation seems to be the most prominent clinical sign of *O. rhinotracheale* infection, as evident by the present study and other findings [2, 17, 34]. The observations obtained from the present study have shown that the Malaysian *O. rhinotracheale* strains produce mild clinical signs in chickens than *A. paragallinarum*. Besides raising awareness on the importance of *O. rhinotracheale* infection, the information obtained from this investigation would be useful in diagnosing the disease.

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