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ISOLATION AND IDENTIFICATION OF BACTERIA IN THE RESPIRATORY TRACT OF THE RED JUNGLE FOWL

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ISOLATION AND IDENTIFICATION OF BACTERIA IN THE RESPIRATORY TRACT OF THE RED JUNGLE FOWL

FATIN BINTI OMAR

A project submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Isolation and Identification of Bacteria in Respiratory Tract of the Red Jungle Fowl", by Fatin Binti Omar and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

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DEDICATIONS

This project paper is dedicated to the Allah S.W.T, who had created me and made all things possible,

To my lovely mother (Kamariah Binti Ibrahim)

My family,

My classmate 2016,

My friends

And to all my teachers who have committed themselves towards the noble cause of education.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

PENGASINGAN DAN IDENTIFIKASI BAKTERIA DALAM SALURAN PERNAFASAN DI DALAM AYAM HUTAN MERAH

Oleh

Fatin Binti Omar

2016

Penyelia: Assoc. Prof. Dr. Siti Khairani Bejo

Penyelia bersama: Assoc. Prof. Dr. Shaikh Mohamed Amin Babjee

Penyakit pernafasan adalah penyakit paling penting yang memberi kesan pada ayam dan ini telah diperhatikan Ayam Hutan Merah peliharaan. Objektif kajian ini adalah untuk mengasing dan mengenal pasti bakteria daripada sinus dan paru-paru Ayam Hutan Merah dan untuk menentukan pertalian diantara bakteria yang diasingkan dengan masalah pernafasan. Enambelas ekor ayam hutan merah yang terdiri daripada 12 ekor ayam menunjukkan tanda klinikal tidak sihat dan empat ekor ayam yang kelihatan sihat telah disampel dari ladang persendirian. Ayam tersebut disembelih dan kemudian sampel sinus dan paru-paru telah diambil daripada setiap ayam., Semua sampel tersebut dikultur pada agar darah dan agar coklat untuk pengasingan bakteria. Bakteria yang telah diasingkan seterusnya dikenal pasti dengan menggunakan ujian biokimia. Sebanyak 19 bakteria Gram positif (43.2%) dan 25 bakteria Gram negatif (56.8%) bakteria diasingkan daripada Ayam Hutan

Merah yang menunjukkan tanda klinikal tidak sihat. Sebanyak lima bakteria Gram positif (41.7%) dan tujuh bakteria Gram negatif (58.3%) diasingkan pada Ayam Hutan Merah yang kelihatan sihat. Tiada perbezaan yang significan ($P > 0.05$) dalam kekerapan pengasingan bakteria diantara ayam yang menunjukkan tanda klinikal tidak sihat dan ayam yang kelihatan sihat. Spesies bakteria yang diasingkan daripada Ayam Hutan Merah yang menunjukkan tanda klinikal tidak sihat ialah *Staphylococcus species* (25%), *Corynebacterium species* (18.20%), *Avibacterium avium* (9.09%), *Neisseria species* (6.82%), *Gallibacterium anatis* bv. *Haemolyticatis* (6.82%), *Escherichia coli* (6.82%), *Avibacterium gallinarum* (6.82%), *Proteus mirabilis* (4.55%), *Pasteurella multocida* subspecies *multocida* (4.55%), *Pasteurella sp/A* (4.55%), *Streptococcus sp* (2.27%), *Pseudomonas aeruginosa* (2.27%), dan *Aeromonas species* (2.27%). Spesies bakteria yang diasingkan daripada ayam hutan yang kelihatan sihat ialah *Staphylococcus species* (16.67%), *Corynebacterium species* (16.67%), *Avibacterium avium* (16.67%), *Bacillus species* (8.33%), *Gallibacterium anatis* bv. *Haemolyticatis* (8.33%), *Escherichia coli* (8.33%), *Pseudomonas aeruginosa* (8.33%), *Pasteurella multocida* subspecies *multocida* (8.33%) dan *Pasteurella haemolytica* (8.33%). Dapat disimpulkan bahawa Ayam Hutan Merah yang diternak menyimpan berbagai bakteria opportunis yang mungkin menyumbang kepada masalah pernafasan pada ayam yang immunocompromi.

Kata kunci: Ayam hutan merah, masalah pernafasan, bakteria

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project

ISOLATION AND IDENTIFICATION OF BACTERIA IN THE RESPIRATORY TRACT OF THE RED JUNGLE FOWL

By

Fatin Binti Omar

2016

Supervisor: Assoc. Prof. Dr. Siti Khairani Bejo

Co-supervisors: Assoc. Prof. Dr Shaikh Mohamed Amin Babjee

Respiratory diseases are the most important diseases affecting poultry and this has been observed in domesticated Red Jungle Fowl. The objectives of this present study were to isolate and identify bacteria from sinus and lung of Red Jungle Fowl and to determine the relationship between bacteria isolated with respiratory tract problem. Sixteen Red Jungle Fowl consisting of 12 clinically unhealthy and four apparently healthy were sampled from privet farm. The chickens were slaughter and then sinus swabs and lung samples were collected from each chicken. All samples were cultured on the blood and chocolate agar for bacteria isolation. The isolated bacteria was then were identified using biochemical tests. Nineteen Gram positive (43.2%) and 25 Gram negative (56.8%) bacteria were isolated from clinically unhealthy Red Jungle Fowl. Five Gram positive (41.7%) and seven Gram negative

(58.3%) bacteria were isolated from apparently healthy Red Jungle Fowl. There was no significant difference between clinically unhealthy and apparently healthy Red Jungle Fowls in term of frequency of Gram positive and Gram negative bacteria isolated ($p > 0.05$). The bacteria species isolated from clinically unhealthy chickens were *Staphylococcus species* (25%), *Corynebacterium species* (18.20%), *Avibacterium avium* (9.09%), *Neisseria species* (6.82%), *Gallibacterium anatis* bv. *Haemolyticatis* (6.82%), *Escherichia coli* (6.82%), A*vibacterium gallinarum* (6.82%), *Proteus mirabilis* (4.55%), *Pasteurella multocida* subspecies *multocida* (4.55%), *Pasteurella sp/A* (4.55%), *Streptococcus species* (2.27%), *Pseudomonas aeruginosa* (2.27%), and *Aeromonas sp* (2.27%). The bacteria species isolated from apparently healthy chickens were *Staphylococcus sp* (16.67%), *Corynebacterium species* (16.67%), *Avibacterium avium* (16.67%), *Bacillus species* (8.33%), *Gallibacterium anatis* bv *haemolyticatis* (8.33%), *Escherichia coli* (8.33%), *Pseudomonas aeruginosa* (8.33%), *Pasteurella multocida* subspecies *multocida* (8.33%) and *Pasteurella haemolytica* (8.33%). It can be concluded that the domesticated Red Jungle Fowl harbour numerous opportunistic bacteria that might contribute to the respiratory problem in immunocompromised chickens.

Keywords: Red Jungle Fowl, respiratory problem, bacteria

1.0 INTRODUCTION

Respiratory tract diseases are a significant component of the overall disease incidence in poultry (Glisson, 1998). It is not just affecting the commercial chicken but also the Red Jungle Fowl which is the wild ancestor of all domestic chicken in the world. The Red Jungle Fowl is one of the most important species to mankind due to its economic and culturally significant to human civilization (Fumihito *et al.*, 1996). Respiratory disease also have been observed to be the main problem in a small Red Jungle Fowl farm.

Diseases of the respiratory tract are often complex, with anatomy, management, environment and nutrition (Nighot *et al.*, 2002) and they are caused by a wide range of pathogens include bacterial, viral, mycoplasma or fungal origins (Mohamed *et al*, 2007). It can be a component of a multisystemic disease or it may be the predominant disease with lesser involvement of other organ systems (Glisson, 1998). The disease has direct negative impact on the chicken such as reduced weight gain, reduced egg production and mortality. These negative impact will cause loss of billions of Malaysia Ringgit in the poultry industry.

Studies showed the respiratory tract was predominated with gram positive bacteria (Shah-Majid & Jah, 1987). In the presence of the respiratory infection, the Gram negative bacteria usually predominate in the organs of respiratory tract (Mohamed, 2007; Dashe *et al.*, 2013). Most of bacteria was an opportunists, capable of causing disease in individuals chicken that experiencing acute or chronic stress.

About 4.5 % of poultry disease which causes death in commercial chicken in Tanzania was due to bacterial diseases (Swai *et al.*, 2013). The common diseases were omphalitis (26.2%), collibacillosis (21.2%), salmonellosis (18.1%), pasteurellosis (18.1%) and infectious coryza (16.6%). These diseases are very common among the poultry farms all over the world.

Few studies have been done on the Red Jungle Fowl in Malaysia. The studies include ticks infestation on the Red Jungle Fowl (Konto *et al.*, 2015; Lee *et al.;* Lee & Amin-Babjee, 1993; Lee, Amin-Babjee & Krishnasamy, 1987). However, study on bacteria isolation and identification in the domesticated and farmed Red Jungle Fowl was not yet carried out. Therefore, this present study might provide information on the common bacteria present in respiratory tract of apparently healthy and clinically unhealthy domesticated and farmed Red Jungle Fowl.

Hence this study was undertaken to with the following objectives:

1) To isolate bacteria from sinus and lung of Red Jungle Fowl.
2) To identify the species of bacteria involve in the respiratory problem in Red Jungle Fowl.

2.0 LITERATURE REVIEW

2.1 Red Jungle Fowl

The Red Jungle Fowl (*Gallus gallus*) was originated from the Asian countries. There were two subspecies of *Gallus gallus* namely *Gallus gallus gallus* and *Gallus gallus spadicues.* The important distinguish features between these two subspecies of *Gallus gallus* is the presence of the white ear patches in *Gallus gallus gallus* while *Gallus gallus spadicues* has red ear patches (Fumihoto *et al.*, 1996).

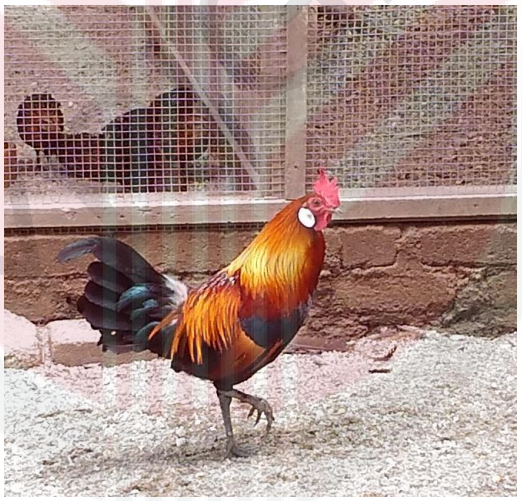

Figure 1: Male *Gallus gallus galus*

Figure 2: Female *Gallus gallus gallus*

Nowadays, the number domesticated Red Jungle Fowl farms were increasing in Malaysia. There are abundant of captive Red Jungle Fowl in the world but it is believed that they are endangered genetically due to hybridization with feral or domestic chicken (Peterson and Brisbin, 1998). In Malaysia, the domesticated Red Jungle Fowl was used as garden birds, for special meat production and as a decoy to trap wild birds (Amin-Babjee, personal communication February 2016).

2.2 Bacteria isolated from the respiratory tract of the domestic fowl

Shah-Majid & Jah (1987) studied the bacterial flora of the trachea, liver, spleen and heart blood of commercial chicken. The bacterial isolated from the trachea of healthy commercial chicken were *Staphylococcus species*, *Streptococcus species*, *Escherichia coli*, *Micrococcus species*, *Enterobacter species*, *Bacillus species*, *Citrobactor species* and *Pasteurella haemolytica*. The proportional occurrence of bacteria in the trachea (n=50) and nasal sinuses (n=50) were 6.0% *Klebsiella species*,

38.8% *Escherichia coli*, 8.6% *Pasteurella species*, 5.2% *Bacillus species* and 41.4% *Staphylococcus species* (Popy *et al.*, 2012). Even though tracheal flora of healthy chicken were predominated by gram positive microorganisms but respiratory disease was predominated by gram negative bacteria (Bisgaard, 1977).

A study was conducted by the *Dashe et al.*, (2013) to determine the distribution of aerobic bacteria in the visceral organs of the sick and apparently health chicken. A total of 2000 samples consisting of bone marrow, heart, liver, lung and spleen were collected from the clinically ill chicken while the oropharyngeal swabs were collected from the apparently healthy chicken. *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida*, *Proteus species*, *Klebsiella pneumoniae*, *Bacillus subtitis*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* were isolated from lung. Bacteria isolated from oropharyngeal swabs of apparently health chicken were *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pasteurella multocida*, *Streptococcus pneumoniae*, *Baccilus species* and *Proteus species*. There was no bacterial flora isolated from lower respiratory tract and sinuses of healthy chicken (Sorum and Sunde, 2001). Nasopharynx is normally populated by large number of bacterial species which are staphylococci, alpha-hemolytic streptococci and species within the *Pasteurellaceae* family.

2.3 Disease of respiratory tract of poultry caused by bacteria

2.3.1 Fowl cholera

Fowl cholera was a disease caused by *Pasteurella multocida* subspecies *multocida* and *Pasteurella multocida* subspecies *septica* and *gallicida* may also cause fowl cholera-like disease (Christensen & Bisgaard, 2000). The site of infection was respiratory tract route and sometimes oculo-nasal-oral route The clinical sign showed by affected chicken include depression, anorexia, mucoid discharge from the mouth, ruffled feathers, diarrhea, and increased respiratory rate (Christensen, 2013). It also will cause high mortality and morbidity in the chicken in the farm (Christensen & Bisgaard, 2000). *Pasteurella multocida* is commensal of the upper respiratory tract of the many avian species. Although it is commensal of the upper respiratory tract but it usually occurs as primary disease that require predisposing factors (Balakrishnan & Roy, 2012).

2.3.2 Infectious coryza

The causative agent of the infectious coryza is *Avibacterium paragallinarum* (preveiusly called *Haemophillus paragallinarum*), Gram negative bacteria, polar staining and non-motile (Akter, *et al.*, 2013). The clinical signs showed include nasal discharge, conjunctivitis with swelling of the sinuses, face and wattles, decreased feed and water intake with reduced egg production (10-40%) (Calnek *et al.*, 1991). Infectious coryza cause the upper respiratory tract disease and infection in the lower

respiratory tract may due to mixed infection with other disease such fowl cholera, pox, chronic respiratory disease and *Pseudomonas aeruginosa* infection (Giurov, 1984).

2.3.3 Colibacillosis

Colibacillosis is caused by the *Escherichia coli* which is Gram negative, non acid fast, uniform staining, non-spore forming bacillus that grow both in aerobic and anaerobic condition. Intestinal tract of the animals is the reservoir for *E. coli* and it also commonly isolated from the upper respiratory tract of the animals. The clinical signs of the colibacillosis is non specific and vary with age, infected organ involved and concurrent disease among the animals (Nolan, 2013). In general colibacillosis shown air sacculitis, colisepticaemia, omphalitis, salpingitis, synovitis, arthritis and panopthalmitis (Vandekerchove *et al.*, 2004)

2.3.4 Bordetellosis

Bordetellosis is caused by the *Bordetella avium* which is Gram negative, small, rod bacteria. It is the disease of the upper respiratory tract and more common in the turkey as compared to the chicken. In the young chicken, *Bordetella avium* mainly play as secondary bacterial infection in the respiratory tract. The clinical signs showed in infected turkey include sneezing, oculo-nasal discharge, mouth breathing, tracheal collapse, and stunted growth (Glisson, 1998).

2.3.5 Mycoplasmosis

Mycoplasmosis is an important chronic respiratory disease in the chicken. The disease caused by *Mycoplasma gallisepticum.* The clinical signs show by chonic respiratory disease is conjuctivitis, sneezing, open mouth breathing and nasal discharge.

3.0 MATERIALS AND METHODS

3.1 Red Jungle Fowl Farm

Red Jungle Fowl was taken from a small farm at the Jenderam Hulu, Sepang, Selangor. This farm was mix poultry farm that consist of jungle fowl, village chicken, peacocks, quails and doves. The total population in this farm was about 500 birds. The farm was managed by free range and intensive system.

3.2 Sample collection

A total of 16 of domesticated Red Jungle Fowl were samples. The Red Jungle Fowl were divided into two group which were 12 of samples from clinically unhealthy and 4 of samples from apparently healthy Red Jungle Fowl. The clinically unhealthy Red Jungle Fowl was selected based on the observation and examination by the veterinarian. The chicken showed the clinical signs which include inactive, depressed, inappetance, ruffled feathers, lacrimation, conjunctivitis, nasal discharge, gasping symptoms and blind. All the symptoms shown were related to the respiratory problems.

These selected Red Jungle Fowl was euthanized and necropsy was performed at the Post Mortem Room, Faculty of Veterinary of Medicine, Universiti Putra Malaysia. Lung sample was collected aseptically to minimize the bacterial contamination. While the sterile cotton wool swabs was used for sampling the nasal sinus after the beak was removed by using the secateurs.

This research project was approved by the Institutional Animal Care and Use Committee (IACUC).

3.3 Bacterial isolation and identification

3.2.1 Primary culture:

All the samples were inoculated into the blood agar and chocolate agar. The interior portion of the lung sample was exposed by using the sterile scalpel blade and the inner surface of the lung was swabbed using the sterile cotton wool swab and then inoculated into these two agar. While for the nasal sinus swab, it was directly swabbed into these two agar mention earlier.

Inoculated blood agar and chocolate agar plates were incubated aerobically for 24 hours at 37ºC and anaerobically for 48 hours at 37ºC respectively. Samples in the blood agar with no visible growth or colony were re-incubated for another 24 hours.

3.2.2 Subculture methods:

The visible colony on the blood and chocolate agar after 24 hours and 48 hours incubation respectively was examined. The morphology of the each of the visible colonies were recorded based on their shape, size, color, surface texture, hemolytic activity (only on the blood agar) and smell.

Each of the visible colonies from the blood agar and chocolate agar was taken by using the sterile inoculating loop and streaking at the blood agar. Colonies taken

from the blood agar and chocolate agar were incubated aerobically and anaerobically respectively for 24 hours at 37^{o}C.

3.2.3 Gram staining:

Gram staining was done for each identical bacterial colony to identify the bacteria. The detail steps for Gram staining were described in Appendix 1. The stained smear was examined for Gram staining reaction, cell morphology and cell arrangement.

3.2.4 Biochemical test:

All the biochemical tests were performed to identify the types of bacteria. Colonies representing each bacterial species were identified and characterized using standard biochemical methods according to the methods described by Jang, Biberstein & Hirsh (2008). The detail steps for biochemical tests were described in Appendix 2.

3.3 Analysis of data

The presence or absence of the bacteria was recorded and tabulated into each of the samples. Frequency of the occurrence for each types of bacteria was then calculated according health status. The differences between the health status in the occurrence of each bacteria was tested by using the Chi-square test and Fisher's Exact test of significant at $\alpha = 0.05$.

4.0 RESULT

4.1 Distribution of Gram positive and Gram negative bacteria in apparently healthy and clinically unhealthy Red Jungle Fowl

A total of 15 bacterial species were isolated from lungs and sinuses of apparently healthy and clinically unhealthy Red Jungle Fowl. The distribution of the Gram positive and Gram negative bacteria isolates from both lung and sinus of clinically unhealthy chicken were 45.5% and 54.5% respectively (Table 1). While the distribution of the Gram positive and Gram negative in the apparently healthy chicken were 41.7% and 58.3% respectively (Table 1).

The frequency of bacterial isolation was higher in unhealthy compared with apparently healthy chicken. The P value distribution of the Gram positive and Gram negative bacteria isolated from apparently healthy and clinically unhealthy Red Jungle Fowl was calculated by using the Chi-Square test. There was no significant difference between the clinically unhealthy and apparently healthy birds in term of the frequency of Gram positive and Gram negative bacteria isolated from these two group as the P value was higher than $\alpha=0.05$.

Table 1: Distribution of Gram positive and Gram negative bacteria of the apparently healthy and clinically unhealthy Red Jungle Fowl

	Apparently healthy		Clinically unhealthy		Chi -square test (α=0.05)
	No. of bacteria isolates	(%)	No. of bacteria isolates	(%)	
Gram positive	5	41.7	19	43.2	0.925
Gram negative	7	58.3	25	56.8	
Total	12	100	44	100	

4.2 Distribution of Gram positive and Gram negative bacteria in lung and sinus of apparently healthy and clinically unhealthy Red Jungle Fowl

The distribution of the Gram positive and Gram negative bacteria in the lungs of clinically unhealthy chicken were 35.3% and 64.7% respectively whilst in sinuses were 50% and 50% respectively. The distribution of the Gram positive and Gram negative bacteria in sinus of apparently healthy chicken were 48.1% and 59.1% respectively whilst in sinus were 40% and 60% respectively (Table 2). There was no significant difference between the clinically unhealthy and apparently healthy chicken in term of the frequency of Gram positive and Gram negative bacteria as the P value is higher than α=0.05. In lungs sample, Gram positive and Gram negative bacteria were highly isolated from are clinically unhealthy as compared to

apparently healthy chicken.

Table 2: Frequency of the bacteria isolated from lung and sinus of apparently healthy and clinically unhealthy Red Jungle Fowl

	Sinus					Lung				
	Apparently healthy		Clinically unhealthy		P value	Apparently healthy		Clinically unhealthy		P value
	No. of bacteria isolates	(%)	No. of bacteria isolates	(%)		No. of bacteria isolates	(%)	No. of bacteria isolates	(%)	
Gram positive	4	40	13	48.1	0.725	1	50	6	35.3	1.000
Gram negative	6	60	14	51.9		1	50	11	64.7	
Total	10	100	28	14		2	100	17	100	

4.3 Bacteria species isolated in lung and sinus of apparently healthy and clinically unhealthy Red Jungle Fowl

Three Gram positive bacteria species were isolated from lung samples which shown clinically unhealthy chicken (n=12). The bacteria species were *Staphylococcus species* (33.33%), *Streptococcus species* (8.33%), and *Corynebacterium species* (8.33%) (Table 3). Six Gram negative bacteria species were isolated from lung of the clinically unhealthy chicken namely *Neisseria species* (16.7%), *Gallibacterium anatis bv. haemolyticatis* biovar *haemolytica* (25%), *Avibacterium gallinarum* (8.33%), *Avibacterium avium* (8.33%), *Pasteurella*

multocida (8.33%) and *Pasteurella sp/A* (16.7%) (Table 4).

Two species of Gram positive bacteria namely *Staphylococcus species* (58.3%) and *Corynebacterium species* (58.3%) ; and eight species of Gram positive bacteria namely *Neisseria species* (8.33%), *Escherichia coli* (25%), *Avibacterium gallinarum* (16.7%), *Proteus mirabilis* (16.7%), *Avibacterium avium* (25%), *Pseudomonas aeruginosa* (8.33%), *Pasteurella multocida* subspecies *multocida* (8.33%) and *Aeromonas species* (8.33%) were isolated from sinus of chicken shown clinically unhealthy (Table 4). One species of Gram positive *(Bacillus species*) and Gram negative (*Avibacterium avium*) bacteria was isolated lung of apparently healthy chicken (Table 4). Gram positive bacteria isolated from sinus of apparently healthy chicken were *Staphylococcus species* (50%) and *Corynebacterium species* (50%) (Table 3). Gram negative bacteria: *Escherichia coli* (25%), *Gallibacterium anatis bv. haemolyticatis* biovar *haemolytica* (25%), *Avibacterium avium* (25%), *Pseudomonas aeruginosa* (25%), *Pasteurella multocida* subspecies *multocida* (25%) and *Pasteurella haemolytic* (25%) were isolated from sinus of apparently healthy chicken (Table 4).

The proportion of bacteria isolates (n=44) from both sinus and lung samples of clinically unhealthy Red Jungle Fowl were *Staphylococcus species* (25%), *Streptococcus species* (2.27%), *Corynebacterium species* (18.18%) and *Neisseria species* (6.82%), *Gallibacterium anatis bv. haemolyticatis* biovar *haemolytica* (6.82%), *Escherichia coli* (6.82%), *Avibacterium gallinarum* (6.82%), *Proteus mirabilis* (4.55%), *Avibacterium avium* (9.09%), *Pseudomonas aeruginosa* (4.55%),

Pasteurella multocida subspecies *multocida* (4.55%), *Pasteurella* sp/A (4.55%) and *Aeromonas species* (2.27%) (Table 5). The proportion of bacteria isolates (n=12) from both sinus and lung apparently healthy Red Jungle Fowl were *Staphylococcus species* (16.67%), *Bacillus cereus* (8.33%) and *Corynebacterium species* (16.67%), *Gallibacterium anatis bv. haemolyticatis* biovar *haemolytica* (8.33%), *Escherichia coli* (8.33%), *Avibacterium avium* (16.67%), *Pseudomonas aeruginosa* (8.33%), *Pasteurella multocida* subspecies *multocida* (8.33%), and *Pasteurella haemolytica* (8.33%) (Table 5).

In general the proportion of the bacteria isolates from both sinus and lung was higher in clinically unhealthy chicken compared to apparently healthy chicken. The P value also calculated by using the Fisher's exact test but the values was not consistent among the bacteria isolates because each cell have frequency less than five.

Table 3: Gram positive bacteria isolated from the sinus and lung of apparently healthy and clinically unhealthy Red Jungle Fowl.

Bacteria isolates	Lung						Sinus					
	Apparently healthy	%	Clinically unhealthy	%	Total	%	Apparently healthy	%	Clinically unhealthy	%	Total	%
	n=4	%	n=12	%	N=16		n=4	%	n=12	%	N=16	%
Staphylococcus species	0	0	4	33.3	4	25	2	50	7	58.3	9	56.3
Streptococcus species	0	0	1	8.33	1	6.25	0	0	0	0	0	0
Bacillus species	1	25	0	0	1	6.25	0	0	0	0	0	0
Corynebacterium species	0	0	1	8.33	1	6.25	2	50	7	58.3	9	56.3

Table 4: Gram negative bacteria isolated from sinus and lung of apparently healthy and clinically unhealthy Red Jungle Fowl.

Bacteria isolates	Lung						Sinus					
	Apparently healthy	%	Clinically unhealthy	%	Total	%	Apparently health	%	Clinically unhealthy	%	Total	%
	n=4	%	n=12	%	N= 16	%	n=4	%	n=12	%	N= 16	%
Neisseria species	0	0	2	16.7	2	12.5	0	0	1	8.33	1	6.25
Gallibacterium anatis bv. haemolyticatis biovar *Haemolytica*	0	0	3	25	3	18.75	1	25	0	0	1	6.25
Escherichia coli	0	0	0	0	0	0	1	25	3	25	4	25
Avibacterium gallinarum	0	0	1	8.33	1	6.25	0	0	2	16.7	2	12.5
Proteus mirabilis	0	0	0	0	0	0	0	0	2	16.7	2	12.5
Avibacterium avium	1	25	1	8.33	2	12.5	1	25	3	25	4	25
Pseudomonas aeroginosa	0	0	0	0	0	0	1	25	1	8.33	2	12.5
Pasteurella multocida subspecies *multocida*	0	0	1	8.33	1	6.25	1	25	1	8.33	2	12.5
Pasteurella sp/A	0	0	2	16.7	2	12.5	0	0	0	0	0	0
Aeromonas species	0	0	0	0	0	0	0	0	1	8.33	1	6.25
Pasteurella haemolytica	0	0	0	0	0	0	1	25	0	0	1	6.25

Table 5: Bacteria isolates from clinically unhealthy and apparently healthy of sinuses and lung of Red Jungle Fowl

Bacteria isolates	Clinically unhealthy		Apparently health		Fisher 's exact test
	Number of bacteria isolates (n=44)	%	Number of bacteria isolates (n= 12)	%	P value when α=0.05
Staphylococcus species	11	25	2	16.67	0.711
Streptococcus species	1	2.27	0	0	1.00
Bacillus cereus	0	0	1	8.33	0.386
Corynebacterium species	8	18.18	2	16.67	1.00
Neisseria species	3	6.82	0	0	1.00
Gallibacterium anatis bv. haemolyticatis biovar haemolytica	3	6.82	1	8.33	1.00
Pseudomonas aeruginosa	3	6.82	1	8.33	1.00
Avibacterium gallinarum	3	6.82	0	0	1.00
Proteus mirabilis	2	4.55	0	0	1.00
Avibacterium avium	4	9.09	2	16.67	0.599
Escherichia coli	1	2.27	1	8.33	0.386
Pasteurella multocida subspecies *multocida*	2	4.55	1	8.33	0.522
Pasteurella sp/A	2	4.55	0	0	1.00
Aeromonas species	1	2.27	0	0	1.00
Pasteurella haemolytica	0	0	1	8.33	0.214
Total	44	100	12	100	

5.0 DISCUSSION

Respiratory disease was a main problem in commercial chicken as well as in the domesticated Red Jungle Fowl. Bacterial infection in respiratory system can be primary or secondary causes of the mortality in chicken. Secondary bacterial infection usually occur when the immune system of chicken is being compromised or infected by other organisms especially viruses. In present study, Red Jungle Fowl selected have mild respiratory symptoms. However, post mortem finding did not show any significant lesion in the lung and sinus. Furthermore, there was no recent or previous outbreak of bacterial infection occurred in this farm.

Gram negative bacteria was predominant in lung and sinus of clinically unhealthy Red Jungle Fowl. This findings was consistent with Bisgaard (1977) who reported that the shifting Gram negative flora in the chicken with the respiratory infection. Gram negative was an opportunists, capable of causing disease in individuals that experiencing acute or chronic stress (Liau, 1997). Few species of bacteria were isolated from lung and sinus of apparently healthy chicken. Lower respiratory tract and sinuses was supposed to be free from any bacteria (Sorum and Sunde, 2000). However, the findings was inconsistent with this present study. This finding suggested that Red Jungle Fowl could possibly act as reservoirs for the bacterial infection or the infection was in subclinical stage where clinical signs was absent.

The most important bacteria isolated in this study were *E. coli* and *P. multocida*. *Escherichia coli* was isolated both in apparently healthy and clinically unhealthy and

both at the sinus samples. *Escherichia coli* can cause colibacillosis is a normal intestinal flora and upper respiratory tract of poultry. However, it might cause infection whenever the immune system of chickens is compromised. *Escherichia coli* can flare up in the immunosuppresion under stress condition such as weather change, transportation, malnutrition and overcrowding. Weather change maybe the one of factor cause the immunosuppresion in this clinically unhealthy Red jungle fowl as during sampling, there was changed from raining to hot season. *Escherichia coli* infection can become worsen when associated with other bacterial or viral infection. This bacterium also have been reported to complicate viral disease (Lewis, 1997). In this present study opportunistic bacteria was also isolated namely *Staphylococcus species, Avibacterium gallianarum, Proteus mirabilis, Pasteurella multocida* and *Pseudomonas aeruginosa*. All these bacteria may cooperated with *E. coli* that led to showing clinical signs and then mortality. While *E. coli* also was isolated in the apparently healthy chicken. It was suggested that, the individual immune system play important role in exhibit the clinical signs.

Pasteurella multocida subspecies *multocida* also was isolated once at lung and sinus of clinically unhealthy chickens. This bacterium is caused fowl cholera in the poultry. This bacterium is the most significant in the causing disease in the animals. The pathogenicity of this organisms is depend on the immune system status of animals itself. In the respiratory tract it can cause sub-acute form and manifested by rales and mucopurulent nasal discharges (Mcvey *et al.*, 2013). It is consistent with the clinical signs showed by a few chicken in the clinically unhealthy chicken group.

It also was isolated from sinus of apparently healthy chicken. This finding revealed that, the healthy chicken can become carrier and reservoir for this bacterium. This consistent with study done by Muhairwa *et al*., (2000), in their study on relationship of *Pasteurella* isolated from free ranging chickens and contact animals that healthy chicken can be carriers of *P. multocida* which cause clinical disease when the immune system is compromised. In similar study conducted by Christensen and Bisgaard, (2000) also stated that the carriers of *P. multocida* might exist within the poultry flock with no history of previous outbreak of fowl cholera and it could might act as transmission of the disease.

The other opportunistic bacteria isolated in the present study were *Staphylococcus species, Pseudomonas aeruginosa, A. gallianarum* and *Proteus mirabilis. Staphylococcus species* and *Pseudomonas aeruginosa* were isolated in the both group while *A. gallianarum and Proteus mirabilis* were only isolated in the clinically unhealthy. They can cause clinical case or infection when the immune system of hosts were compromised. These bacterium were ubiquitous except for *A. gallinarum,* it main habitat is upper respiratory tract of avian species. The *A. gallinarum* can cause mild respiratory tract infection and also act as secondary invader in the respiratory disease (Heddleston 1975). Co-infection of *A. gallinarum* with *Mycoplasma synovia* cause severe mortality in broiler chick (Droual *et al.*, (1992). While *Pseudomonas aeruginosa* have been reported to cause high mortality in chick with respiratory disease (Kebede, 2010). It can become more worth when combined with other bacteria infection especially *E. coli* and *Mycoplasma species.*

Proteus mirabilis also have been isolated from lung, trachea and kidney in birds with respiratory distress, diarrhoea, paralysis and high mortality (Barnes & Nolan (2008)).

The uncommon bacteria also have been isolated in this study which was *Neisseria species. Neisseria species w*as isolated only in the lung and sinus of the clinically unhealthy chickens. It have isolated from upper respiratory tract of cat and dog while in the avian it was isolated from lower intestinal tract (Liu *et al,*. 2015). *Neisseria weaveri* was isolated from the lung and trachea of chicken and turkey flock with respiratory disease (Chin, 2002).

6.0 CONCLUSION

In conclusion, various bacteria species have been isolated from lung and sinus of Red Jungle Fowl. All bacterial isolates were identified as opportunistic bacteria which might cause respiratory problems in immunocompromised chicken.

7.0 RECOMMENDATION

1) Antibiotic sensitivity should be conducted for each species identified in this study.
2) The study should not only include sinus and lung but the whole organs of respiratory tracts.
3) Further study should be done on the role of pathogenicity of each species bacteria isolated from lung and sinus.

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APPENDICES

Appendix 1

Gram staining procedure:

1. A drop of normal saline was placed on the glass slide.
2. Using the wire loop, the bacterial colony was took and mixed with normal saline on the glass slide until it become thin uniform layer.
3. The slides was air dry and then heat fix by using bunsen burner.
4. The Crystal violet was flooded for one minutes and then rinse with tap water
5. After that, Lugol's iodine was flooded on the glass slide for one minute and rinse with tap water.
6. Then decolourized the smear with Acetone for three second and rinse with tap water.
7. Lastly, the Carbol Fusion for one minutes and then rinse with tap water

Appendix 2

Basic biochemical tests for Gram positive bacteria

Biochemical Tests	Description	Reaction
Catalase test	A drop of 3% H_2O_2 was placed on the clean slide and the colony of tested agar was picked with wire loop and added to the drop of H_2O_2.	Positive reaction was indicated by present of gas (air bubble)
Coagulase test	A drop of normal saline was placed on the clean slide and a few of colony that positive catalase test was picked with wire loop and mixed well with the drop of normal saline. Then a drop of rabbit plasma was added.	Positive reaction was indicated by present of clumping effect immediately.

Basic biochemical test for Gram negative bacteria

Biochemical Test	Description	Reaction
Oxidase test	A drop of oxidase reagents was placed on the filter paper. By using the wire loop, the tested colony was steak on the on the filter paper.	The positive reaction indicated by change into purple colour.
TSI test	The medium was inoculated by stabbing with straight wire and streaking at the slant area that containing bacteria colony. Then the tube was incubated aerobically or anaerobically for 24 hours at 37^{o}C.	The reaction of media was observed for any colour changes at the butt and slant of the test tube. The present of yellow and red colour indicates of acid and alkaline respectively. Present of black precipitate indicates of H_2O production and bubbles in test tube indicates of production of gas.

SIM test	The medium was inoculated by stabbing with straight wire that containing bacteria colony. Then the tube was incubated aerobically and anaerobically for 24 hours at 37°C. The reaction of media was observed. The one drop Kovac reaction was added to the test tube for the indole production test.	The positive reaction was indicated by present of pink colour in the reagent layer within one minute. The present of black precipitation in the test tube indicated positive for the H_2O production. If present of turbidity inside the test tube indicated positive for motility test.
Urease test	The tested colony was inoculated onto the slope of the urea agar medium, incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by pink colour.
Citrate test	The tested colony was inoculated onto citrate agar medium, then incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by blue colour.

Additional biochemical test

Biochemical test	Description	Reaction
Voges - Proskaure test	The tested culture was inoculated into the VP broth and incubated aerobically or anaerobically for 24 hours at 37°C. After incubated, six drop of 5% alpha-naphthol solution and two drops of 4% KOH aqueous solution were added into the test tube.	The positive reaction was indicated by red colour.
Mannitol and maltose test	The tested culture was inoculated into the solution and incubated aerobically or anaerobically for 24 hours at 37°C.	The positive raection was indicated by change into yellow colour.
Arginine dihydrolase (ADH) test	The tested culture was inoculated into the ADH solution and the glycerol oil was added to cover the layer of the solution. Then it was incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by change into turbid to purple.
6.5% NaCl	The tested culture was inoculated into the solution and incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by change into turbid.

Bile esculin test	The tested culture was streaked into the bile esculin agar and incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by present of black colour on the agar.
Lactose, Sorbitol, Arabinose, Maltose, Dulcitol and Trehalose test	The tested culture was inoculated in to the solution and incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by change into yellow colour.
Nitrate reduction test	The tested culture was inoculated into the solution and incubated aerobically or anaerobically for 24 hours at 37°C. After incubated, the nitrate reagent 1(sulfanilic acid) and 2 (naphthylamine) was added.	The positive reaction was indicated by change into red colour.
ONPG test	The tested culture was inoculated into the nutrient broth and the ONPG reagent was added. Then incubated aerobically or anaerobically for 24 hours at 37°C.	The positive reaction was indicated by change into yellow colour.
ODC test	The tested culture was inoculated into the ODC media and the glycerol oil was added to cover the layer of the solution.Then	The positive reaction was indicated by change into yellow colour.

	incubated aerobically or anaerobically for 24 hours at 37^{o}C.	