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MOLECULAR IDENTIFICATION OF RESPIRATORY MICROBIOTA OF A YOUNG OSTRICH (*Struthio camelus*) USING TRACHEO BRONCHO ALVEOLAR LAVAGE

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ABSTRACT

Respiratory diseases caused by bacteria are common to all of ages of ostrich (*Struthio camelus*). Young chicks, however are more susceptible, and are considered a major hindrance in successful ostrich farming. Using culture dependent techniques, a diminutive prospect of bacterial pathogens and corresponding diseases associated with respiratory system has been described. Here we report the first culture independent analysis of a young chick died of respiratory disease using tracheo broncho alveolar lavage (T-BAL). The 16S rRNA sequences showed diversity corresponding to 2 phyla, 7 families and 8 genera. Both of the identified bacterial phyla were gram negative; *Proteobacteria* (97.3 %) and *Bacteroidetes* (2.7 %). Except for identification of *Myroides spp. MY15* that has been associated to human as opportunistic pathogen, lack of assignment of majority of reads to lowest taxa indicated the presence of yet uncharacterized organisms. Further molecular and epidemiological studies are needed for *Myroides spp. MY15* to understand its role in ostrich health care and differential diagnosis.

Key words: Culture independent analysis, Ostrich, *Myroides Spp. MY15*, Respiratory microbiota.

INTRODUCTION

Ostriches belong to a clan of flightless birds called the ratites that also include cassowaries, kiwis, moas and emus. There exist a long history of ostrich domestication and farming as a source of meat, leather, feathers and oil (Verwoerd, 2000; Huchzermeyer, 2002). With the increase in protein demand and subsequent commercial farming worldwide, there is likely to increase contact between ostrich and commercial poultry and subsequent diseases. Respiratory diseases are common to all ages of ostriches (Samson, 1997). However, high chick mortalities below three months of age are considered as one of the major obstacles in successful ostrich farming (Samson, 1997). Together, improper ventilation, cold stress, high ammonia level in indoor pens augments respiratory problems and subsequent mortalities due to poor prognosis Samson 1996; Samson 1997). Compared to isolation and identification of numerous bacterial infectious agents and consequent diseases in poultry industry worldwide (Byrum and Selmons, 1995; Glisson, 1998; Van Empel and Hafez, 1999), respiratory microbiota of ostriches is not well revealed (Shivaprasad, 1993; Samson, 1997; Knobl *et al*., 2001; Elfaki *et al.,* 2002). While concerns exist about bird's health in relation to globalized trade, reservoir of number of infectious organisms (Capua, 1998) and disease control, yet unknown culturing facilities for most of microbiota provides a diminutive prospect of respiratory microbiome and subsequent diseases in ostriches. Using 454-pyrosequencing, culture independent molecular analysis of respiratory microbiota of 37 day old ostrich died recently of symptoms clinically suggestive of respiratory disease has been described. Unbiased exploration of the respiratory system will be of help to improve ostrich's health care through understanding the susceptibility to wide range of environment load, disease diagnostics and future clinico-pathological studies.

MATERIALS AND METHODS

Of the total 90 ostrich in the farm located in Punjab (30°48'29"N 73°36'00"E), during winter, a 37 day old ostrich was presented to laboratory for necropsy examination. The bird died recently showing clinical symptoms suggestive of respiratory disease such as swollen head, nasal and lacrimal discharge and high body temperature. Within three days of clinical symptoms, a total of 11 birds were died. Hemorrhages on trachea mucosa were evident, whereas, rest of respiratory airways particularly lungs were devoid of any typical gross lesion.

As sterile as possible, tracheo-broncho alveolar lavage (T-BAL) was collected and processed for genome extraction (BiOstic® FFPE Tissue DNA Isolation Kit; Mobio, USA).

Using bar-coded fusion with 16S rRNA primers, 27F (5´AGAGTTTGATCMTGGCTCAG 3´) and 907R (5´TACGGGAGGCAGCAG 3´), one-way read amplicons (Lib-L) were prepared. The PCR reactions was carried out with ds DNA (5 to 10ng in total), forward and reverse primers (5 pmoles each), dNTPs (5 nmoles each), 0.25 uL of TAQ (Fast Start High Fidelity PCR system, Roche, Indianapolis, IN), and 10X buffer solution supplied with the enzyme (2.5uL). The samples were denatured at 94^{o}C for 3 minutes followed by 35 cycles of 94^{o}C for 15 sec, 55^{o}C for 45 seconds and 72^{o}C for 60 seconds each with a final extension at 72^{o}C for 8 min (Gene AMP PCR System 9700; Applied Biosystems, Foster City, CA). Product (~ 900bp) was separated on 1% agarose gel and purified (AgencourtAMPure technology, Beckman Coulter, Brea, CA). After clean-up (QIAquick PCR Purification kit, Qiagen,Valencia, CA), quality and quantity was assessed using a DNA 7500 LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit quantification. Using the 454/Roche GS FLX+ Titanium chemistry (Roche Diagnostics, Indianapolis, IN), pyrosequencing was carried out according to the manufacturer's instruction.

Primer sequences and barcodes were removed from GS-FLX-Titatium sequencer data (.sff file). Using MOTHUR, sequences were screened for read shorter than 100 nucleotides in length, have more than 1 mismatched barcodes and 8 bases in a row, and the average quality score lower than 25 (Schloss *et al*., 2009). High quality sequence reads were aligned to closest relative sequence, using BLASTn, against "Bacteria + Archeaea (isolates only)"database (www.microgator.org/taxcollector/). Finally, the aligned sequence data was analyzed through MetaGenomeANalyzer, MEGAN (Version 4.22, built February 22, 2011) using a default setting (MinSupport: 5, Min score: 35, Top percent: 10) (Huson *et al.*, 2011).

RESULTS

A total of 9,821 read were obtained from extracted gDNA (19.0 ng/uL) and were designated to bacteria domain and its descendents. Taxonomic diversity within T-BAL has been captured as indicated by rarefaction analysis between sequence retrieved and associated taxonomy leaves at the level of genera (Figure 1). Collapsing sequence file (.rma) to taxonomic node identified reads corresponding to 02 phyla, 06 order, 07 families and 08 genera. Of the total read, 8253 were found to be summarized within two phyla: *Proteobacteria* (8028, 97.3%) and *Bacteroidetes* (225, 2.7%). Based upon sequences summarized at family node (1263), the dominant family was *Shewanellaceae* (531, 42.0%) followed by *Morexallaceae* (196, 15.5%), *Aeromonadaceae* (179, 14.2%) and *Pseudomonacae* (153, 12.1%). Similarly, among the sequence read summarized at genera node (1231), abundance of read was found for *Shewanella* (531, 43.1%) followed by *Acinetobacter* (196, 15.9%), *Aeromonas* (179, 14.5%), *Pseudomonas* (153, 12.4%) and *Sphingobacterium* (89, 7.2%) (Figure 2). Because top BLAST hits have heterogeneous taxonomic lineages, among the reads assigned to species level, only one bacterial species named *Myroides spp. MY15* (44) belonging to phyla *Bacteroidetes* was identified (Figure 3.)

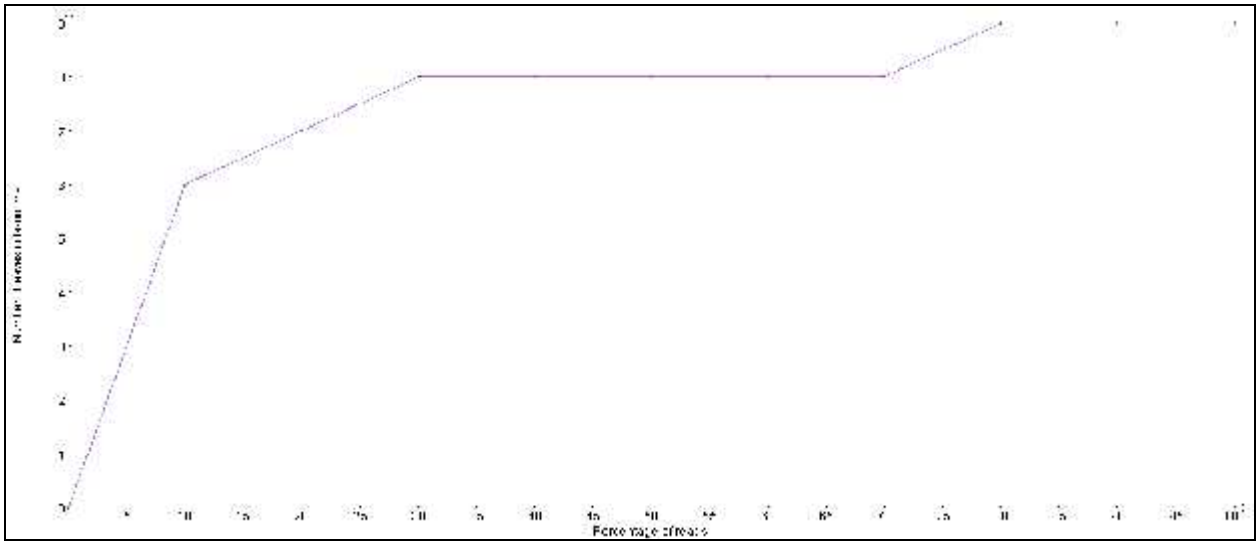

Figure 1: Taxonomy rarefaction plot for the T-BAL analyzed from Ostrich (*Struthio camelus*). The plot is made with percentage of reads present in a given sample to corresponding leaves in taxonomy database.

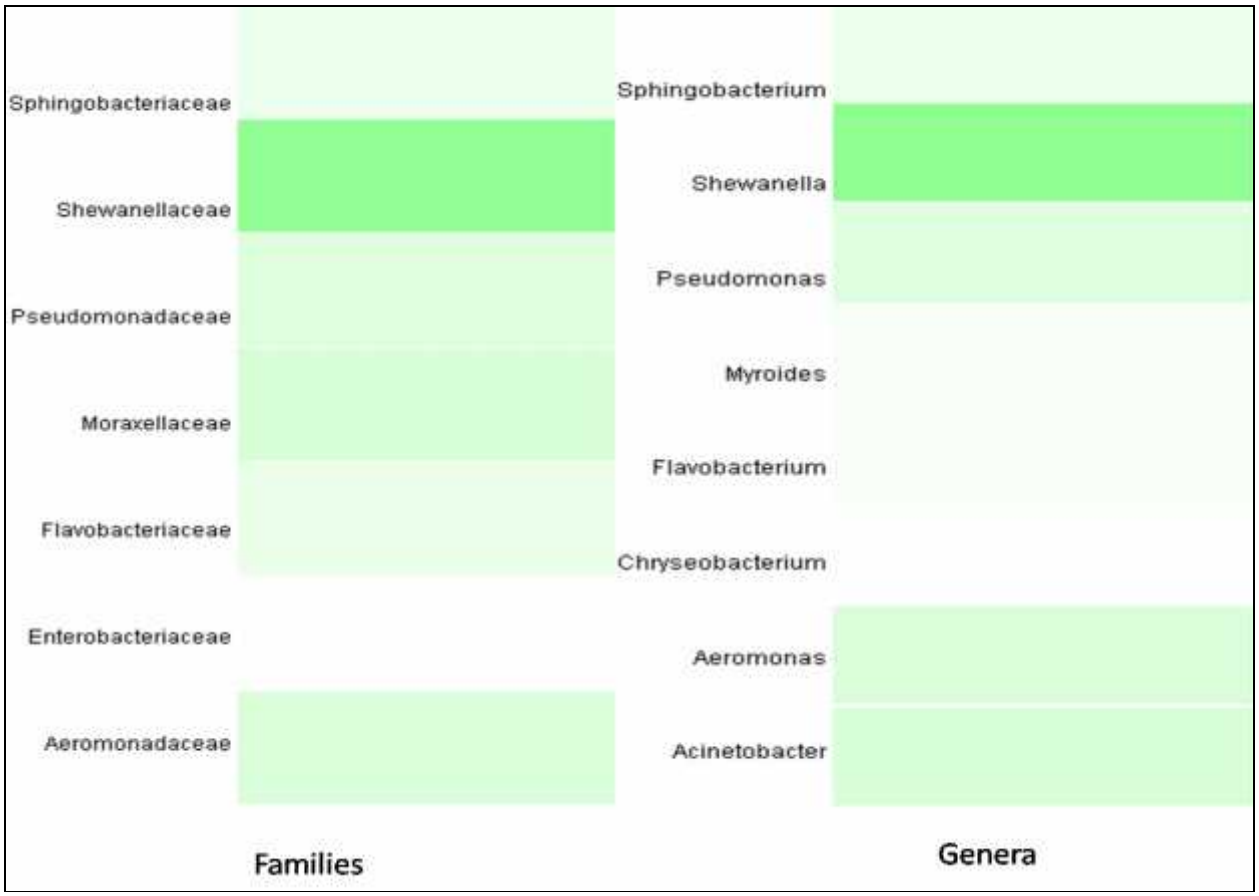

Figure 2: Relative abundance of phyla, families and genera identified in Ostrich (*Struthio camelus*)

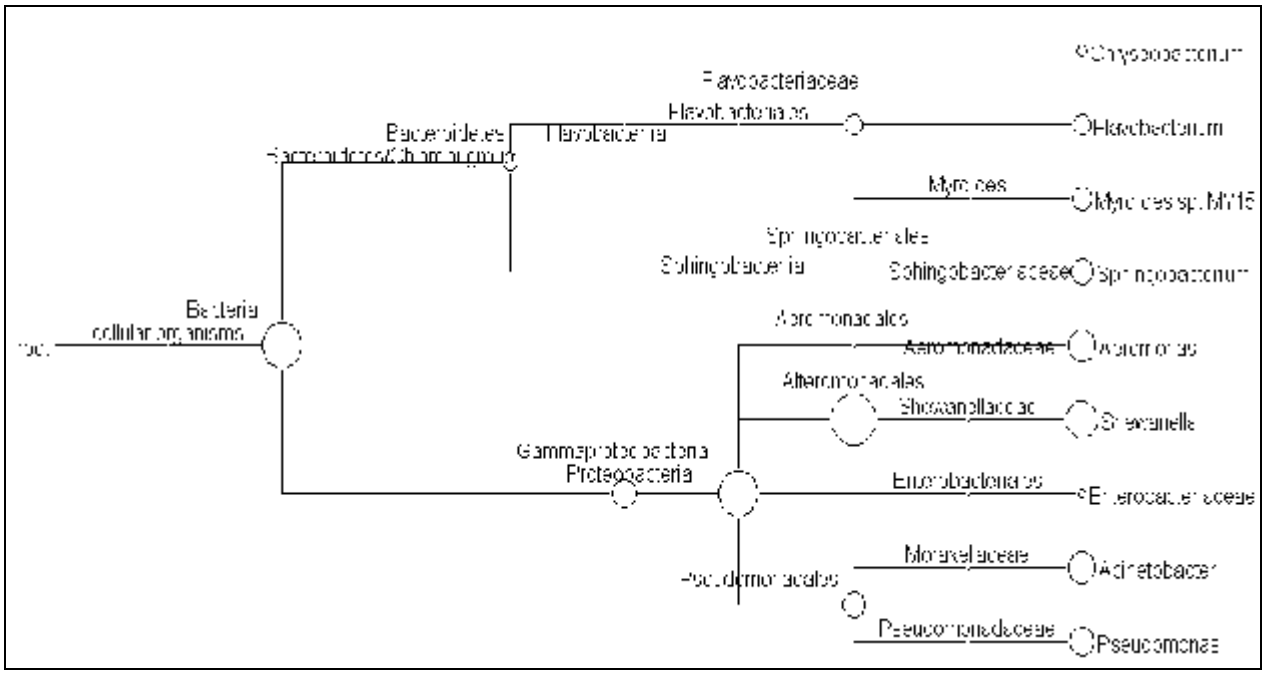

Figure 3: Phylogeny of 16S rRNA gene sequences derived from Ostrich (*Struthio camelus*). The number of reads assigned to each node in NCBI taxonomic tree is scaled logarithmically.

DISCUSSION

This is the first 16S rRNA based molecular examination of bacterial communities that involves trachea as well as lower respiratory tract of a young ostrich. Given the nature and cost of bird involved, farm owner did not agree to contribute additional birds, either healthy or diseased. Contrary to identification of both gram positive and gram negative bacterial species of phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* in domestic and wild birds (Byrum and Selmons, 1995; Poorniya and Upadhey, 1995; Glisson, 1998; Bailey *et al*., 2000; Hubalek *et al.*, 2004), the respiratory system of ostrich appear to harbor less diversity limited only to two gram negative bacterial phyla named *Proteobacteria* and *Bacteroidetes*. Less biodiversity retrieved may be ascribed to the fact that young ostrich has been kept in indoor pens and not exposed to environment outside. Though majority of sequence read were not assigned to species level, genera and species belonging to *Proteobacteria* such as *Klebsiella spp., Pseudomonas spp*., *Escherichia spp*. and *Pastuerella spp*. have been reported from respiratory tract of ostrich (Samson, 1997; Elfaki *et al*, 2002). To best of our knowledge, nothing is known of *Sphingobacterium*, *Shewanella*, *Myroides*, *Flavobacterium*, *Chrysobacterium*, *Aeromonas* and *Acinetobacter* and corresponding species in relation to health and disease in ostrich. Identification of number of phylotypes at different taxonomic node may be simply due to limitation of previous studies in conventional culturing procedures and part of respiratory tract explored. It is well recognized fact that known culturing procedures are limited to less than 1% organism and therefore, identification of large proportion of bacteria in a given clinical samples remained unknown (Schuster, 2008). Secondly, conventional culturing procedures though claimed lower respiratory tract as sterile compartment (Pecora, 1963); however, recent molecular studies have reported characteristic distribution of organism in human (Erb-Downward *et al*., 2011). For example, *Tropheryma whipplei* in recent years has been authenticated as lung inhabitant from BAL in patients with pneumonia (Bousbia *et al.*, 2010; Charlson *et al.*, 2011).

Identification of specie which is previously unknown in its association with respiratory system of birds either in health or disease, however well associated with infectious diseases in humans indicates potential to carry organism of public health significance. *Myroides spp.MY15* is a gram negative strain within family *Flavobacteriaceae* that has been recovered from clinical sources, soil, and seawater (Hugo *et al*., 2006; Yan *et al.*, 2012). It is considered as opportunistic pathogen that has been recently isolated as novel organism from human saliva (Yan *et al*., 2012). Though it needs infection vs. clinical outcome based studies, identification of *Myroides spp. MY15* in similar molecular examination studies of diseased birds using T-BAL (unpublished yet) highlight its importance. Further studies involving isolation, subsequent clinical trial and molecular epidemiological survey are therefore required to determine its role as a commensal or a potential bird pathogen.

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REFERENCES

Bailey, T. A., C. D. Silvanose, J. Naldo, O. Combreau, F. Launay, U. Wernery, J. Kinne, R. Gough and R. Manvell (2000). Health considerations of the rehabilitation of illegally traded houbara bustards *Chlamydotis undulata macqueenii* in the Middle East. Oryx. 34(4): 325 – 334.

Byrum, B. R. and R. D. Slemons (1995). Detection of Proteolytic Bacteria in the Upper Respiratory Tract Flora of Poultry. Avian Dis. 39(3): 622 – 626.

Capua, I. (1998). Crimean-congo hemorrhagic fever in ostriches: a public health risk for countries of European Union. Avian Pathol. 27: 117 – 120.

Charlson, E. S., K. Bittinger, A. R. Haas, A. S. Fitzgerald, I. Frank, A. Yadaw, F. D. Bushman and R. G. Collman (2011). Topographical community of bacterial populations in the healthy human respiratory tract. Am. J. Respir. Crit. Care Med. 184: 957 – 963.

Elfaki, M. G., B. Abbas, O. M. Mahmoud, E. M. Haroun and E. M. Abdel-Magied (2002). Septicaemic pasteurellosis in ostriches (*Struthio camelus*) in central Saudi Arabia. Vet. J. 163: 218 – 221.

Erb-Downward, J. R., D. L. Thompson, M. K. Han, C. M. Freeman, L. McCloskey, L. A. Schmidt, V. B. Young, G. B. Toews, J. L. Curtis, B. Sundaram, F. J. Martinez and G. B. Huffnagle (2011). Analysis of the lung microbiome in the "Healthy" Smoker and in COPD. PLoS ONE, 6: e16384.

Glisson, J. R. (1998). Bacterial respiratory diseases of poultry. Poult. Sci. 77: 1139 – 1142.

Hubalek, Z. (2004). An annotated checklist of pathogenic microorganisms associated with migratory birds. J. Wildlife Dis. 40(4): 639 – 659.

Huchzermeyer, F. W. (2002). Diseased of farmed Crocodiles and Ostriches. Rev. Sci. Tech. Off. Int. Epi. 21(2): 265 – 276.

Hugo, C. J., B. Bruun and P. J. Jooste (2006). The genera *Empedobacter* and *Myroides*. *In*: M. Dworkin *et al*., eds., The Prokaryotes, a Handbook on the Biology of Bacteria, Third edition, vol. 7, 630 – 637.

Huson, D. H., S. Mitra, H. J. Ruscheweyh, N. Weber and S. C. Schuster (2011). Integrative analysis of environmental sequences using MEGAN 4. Genome Res. 21: 1552 – 1560.

Knobl, T., M. R. Baccaro, M. A. Moreno, T. A. T. Gomes, M. A. M. Vieira, C. S. A. Ferriera and A. J. P. Ferriera (2001). Virulence properties of Escherichia coli isolated from ostriches with respiratory disease. Vet. Microbiol. 83: 71 – 80.

Pecora, D. V. (1963). A comparison of transtracheal aspiration with other methods of determining the bacterial flora of the lower respiratory tract. N. Engl. J. Med. 269: 664 – 666.

Poornima, M. and S. Upadhye (1995). Bacterial flora of respiratory tract of poultry in health and disease. Mysore J. Agric. Sci. 29: 68 – 72.

Samson, J. (1996). Ostrich diseases and management in northern climate. Proc. Annu. Conf. Assoc. Avian Vet. 149-151.

Samson, J. (1997). Prevalent diseases of Ostrich chicks farmed in Canada. Can. Vet. J. 38: 425 – 428.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stress, G. G. Thallinger, D. J. Van Horn and C. F. Weber (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75(23): 7537 – 7541.

Schuster, S. C. (2008). Next-generation sequencing transforms today's biology. Nat. Methods. 5: 16 – 18.

Shivaprasad, H. L. (1993). Neonatal mortality in ostriches: an overview of possible causes, In Proc. Association of Avian Veterinarians (AAV), 31 August-4 September, Nashville, Tennessee. AAV, Boca Raton, Florida, 282-293.

Van Empel, P. C. M. and H. M. Hafez (1999). *Ornithobacterium rhinotracheale*: a review. Avian Pathol. 28: 217 – 227.

Verwoerd, D. J. (2000). Ostrich diseases. Rev. Sci. Tech. Off. Int. Epiz.19 (2): 638 – 661.

Yan, S., N. Zhao and X. H. Zhang (2012). *Myroides phaeus* sp. nov., isolated from human saliva, and emended descriptions of the genus *Myroides* and the species *Myroides profundi* Zhang *et al.* 2009 and *Myroides marinus* Cho *et al.*2011. Int. J. Syst. Evol. Microbiol. 62: 770 – 775.