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***Short Communication***

# Culture Independent Analysis of Respiratory Microbiome of Houbara Bustard (*Chlamydotis undulata*) Revealed Organisms of Public Health Significance

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## Abstract

This paper describes the first culture independent analysis of respiratory microbiota of the endangered houbara bustard (*Chlamydotis undulata*), a migratory bird with the potential to spread pathogens over wide geographic areas. The 16S rRNA sequences showed high diversity with reads corresponding to 5 phyla; *Proteobacteria* (47.1%), *Bacteroidetes* (27.9%), *Fusobacteria* (14.2%), *Firmicutes* (7.4%) and *Actinobacteria* (3.42%). Most read were not assigned to lower taxa, indicating the presence of yet uncharacterized organisms. However, several organisms, including *Myroides spp. MY15*, *Collinsella aerofaciens*, *Bacteroides fragillis*, *Enterococcus cecorum* and *Kurthia zopfii*, are known to be associated with various clinical outcomes in other animals, including humans, indicating the zoonotic potential of houbara bustard. Further molecular and epidemiological studies are needed, particularly for *Myroides spp. MY15*, to understand their role in disease or health of houbara bustard as well as to determine the public health significance of these findings. © 2014 Friends Science Publishers

**Keywords:** Culture independent analysis; Houbara bustards; *Myroides Spp. MY15*; Respiratory microbiota

## Introduction

A number of bacterial pathogens of public health significance, including *Anaplasma phygocytophilum*, *Borrelia burgdorferi*, *Pasteurella multocida*, *Compylobacter jejuni*, *Clostridium botulinum*, *Pseudomonas aeroginosa* and *Mycobacterium avium*, have been associated with the movement of free living birds either as biological and/or mechanical carriers (Sambyal and Baxi, 1980; Bailey *et al.,* 2000a; Hubalek, 2004; Abulreesh *et al.,* 2007; Khan, 2012; Dinev *et al*., 2013). In addition to the fact that only a small proportion (< 1%) of organisms is culturable (Schuster, 2008), highly variable and time consuming conventional microbiological procedures used for different known pathogens further complicate accurate and prompt diagnosis. Together, this provides a partial representation of airway microbiota, and diminutive prospect to determine or discover novel organism/pathogens and their association to clinical outcome. On the other hand, using hypervariable region (V1-V5) of 16S rRNA gene, metagenomics (i.e., pyrosequencing) is directly applicable to clinical samples and provides species specific sequences as an alternate to phenotypic identification of any known or novel pathogen in a given clinical sample with common procedure (Nakamura *et al.,* 2008). A number of novel organisms and/or their association to diseases similar to already known pathogens have been identified in recent years (Eckburg *et al.,* 2005; Wylie *et al.,* 2012).

Houbara bustard (*Chlamydotis undulata*) is included among "IUCN red list of threatened species (http://www.iucnredlist.org/)" largely due to over hunting and habitat loss to grazing. It is distributed across North Africa, the Middle East and western Asia (Combreau *et al.,* 2011). Pakistan is included among important wintering grounds where a large number of them are being trapped illegally for use in hunting (Bailey *et al.,* 2000b). Attempts have been made to conserve and rehabilitate them in recent years, placing humans in closer contact and raising the risk of zoonoses. Attempts to define the bacterial communities in upper respiratory tract of houbara bustard have been limited by the ability to culture these organisms (Bailey *et al.,* 2000a, b; Silvanose *et al.,* 2001). Here we describe the culture-independent analysis of the respiratory microbiota of a houbara bustard that died with signs of respiratory disease. In addition to identifying known and novel pathogens that might contribute to the death of this bird and the decline of the population, relating them to their closest Operational Taxonomic Unit (OTU), all of which have been cultured,

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can identify conditions under which these potential pathogens can be cultured and studied. These efforts will both contribute to saving this endangered bird as well as address the risk of zoonoses from such migratory birds.

## Materials and Methods

An adult houbara bustard (> 2 years of age) was presented to laboratory for necropsy examination. The bird was trapped to be exported; however, died with clinical symptoms suggestive of respiratory disease such as open mouth breathing, nasal discharge, sneezing and high body temperature. The necropsy showed hemorrhages on tracheal mucosa; however, no gross lesions were evident on lungs. Tracheo-broncho alveolar lavage was collected aseptically and processed for genome extraction (gDNA) using commercially available kit (BiOstic® FFPE Tissue DNA Isolation Kit; Mobio, USA).

One-way read amplicons (Lib-L) were prepared using bar-coded fusion primers with 27F (5´AGAGTTTGATCMTGGCTCAG 3´) and 907R (5´TACGGGAGGCAGCAG 3´) 16S rRNA primers. The PCR reactions was carried out with 5 pmoles of forward and reverse primers each, ds DNA (5 to 10 ng in total), 5 nmoles of each dNTP, 0.25 uL of TAQ (Fast Start High Fidelity PCR system, Roche, Indianapolis, IN), and 2.5 uL of 10X buffer solution supplied with the enzyme. The samples were denatured at 94$^{o}$C for 3 min followed by 35 cycles of 94$^{o}$C for 15 sec, 55$^{o}$C for 45 sec and 72$^{o}$C for 60 sec each with a final extension at 72$^{o}$C for 8 min (Gene AMP PCR System 9700; Applied Biosystems, Foster City, CA). The PCR product was separated (approximately 1,000bp) on 1% agarose gel, extracted and purified using the AgencourtAMPure technology (Beckman Coulter, Brea, CA). After clean-up with a 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. Pyrrosequencing using the 454/Roche GS FLX+ Titanium chemistry (Roche Diagnostics, Indianapolis, IN) was carried out according to the manufacturer's instruction.

The GS-FLX-Titatium sequencer data (.sff file) was generated using the GS Amplicon software package (Roche, Branford, CT). Primer sequences and barcodes were removed from each raw sequence. Using mothur (Schloss *et al.*, 2009), sequence reads that were 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 were filtered out. BLASTn was used to align sequences against Ribosomal Database Project (RDP) "Bacteria + Archeaea (isolates only)"database (www.microgator.org/taxcollector/), containing about 164,517 almost full-length 16S rRNA sequence reads. The closest relative sequence was assigned to each corresponding best match in the database. The aligned sequence data was imported and analyzed further in MetaGenomeANalyzer (MEGAN V4.70.4, built February 22, 2011) using a default setting (MinSupport: 5, Min score: 35, top percent: 10) (Huson *et al.*, 2011).

**Sequence Submission:** The retrieved metagenomic sequence data (fastq) was submitted to NCBI database via equence read archives (SRA) with accession number SRR908060

## Results

DNA was extracted from Tracheo-broncho alveolar lavage of the recently deceased bird and 16S sequences amplified and sequenced, from which we identified 8,950 reads of high quality. Rarefaction analysis suggested that the taxonomic diversity was largely captured by this relatively modest sequencing depth (Fig. 1). Of the total read, 5442 matched five phyla: *Proteobacteria* (2563, 47.1%), *Bacteroidetes* (1519, 27.9%), *Fusobacteria* (772, 14.2%), *Firmicutes* (402, 7.4%) and *Actinobacteria* (186, 3.42%). *Proteobacteria* and *Bacteriodetes* predominated and together constituted 75% of sequence reads summarized at phyla node.

Reads that collapsed to lower taxonomic nodes identified 15 families and 14 genera. Of the 4972 reads that identified a family node, the dominant families were Flavobacteriaceae (1317, 26.5%) *Enterobacteriaceae* (1315, 26.4%), *Fusobacteriaceae* (771, 15.5%), *Aeromonadaceae* (555, 11.2%) and *Pseudomonadaceae* (294, 5.9%). Similarly, at genera node 3128 reads were identified as predominantly *Myroides* (1185, 37.9%), *Aeromonas* (555, 17.7%), *Fusobacterium* (434, 13.9%), *Pseudomonas* (294, 9.4%), *Bacteroides* (154, 4.9%) and *Proteus* (108, 3.5%) (Fig. 2). Many reads could not be assigned to species level because top BLAST hits have heterogeneous taxonomic lineages. Among the reads assigned to species level, most of them were *Myroides spp. MY15* (1054) followed by *Collinsella aerofaciens* (181), *Kurthia Zopfii* (55), *Enterococcus cecorum* (9) and *Bacteroides fargilis* (8) (Fig. 3).

## Discussion

To best of our knowledge, this is the first molecular analysis of bacterial communities present in the respiratory tract of houbara bustard. Given the endangered status of houbara bustard, it was not possible to obtain additional birds for this analysis, and future studies will also be severely limited. Therefore, the information obtained in this study may be of extraordinary importance. We identified a diverse microbial community within the respiratory tract, indicating that houbara bustard can carry a large number of potential pathogens with it during its lengthy migrations. Whether any one of these organisms, or some combination of them, was responsible for the death of this bird is unknown.

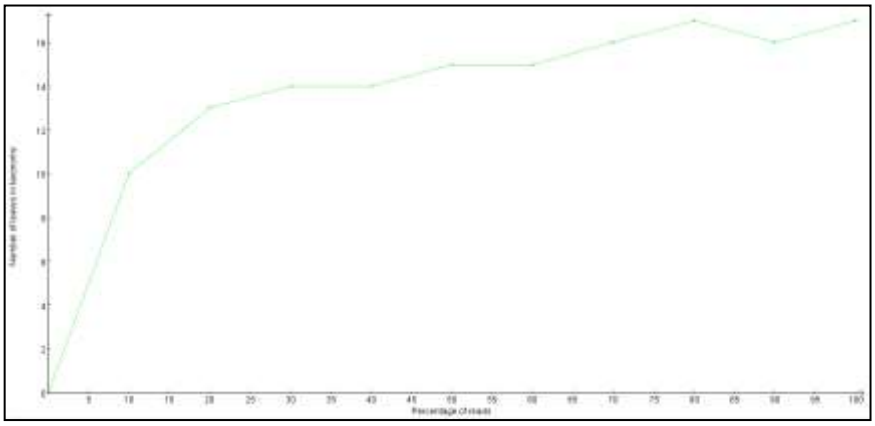

**Fig. 1:** Taxonomy rarefaction plot for the T-BAL analyzed from houbara bustard. The plot is made with percentage of reads present in a given sample to corresponding leaves in taxonomy database.

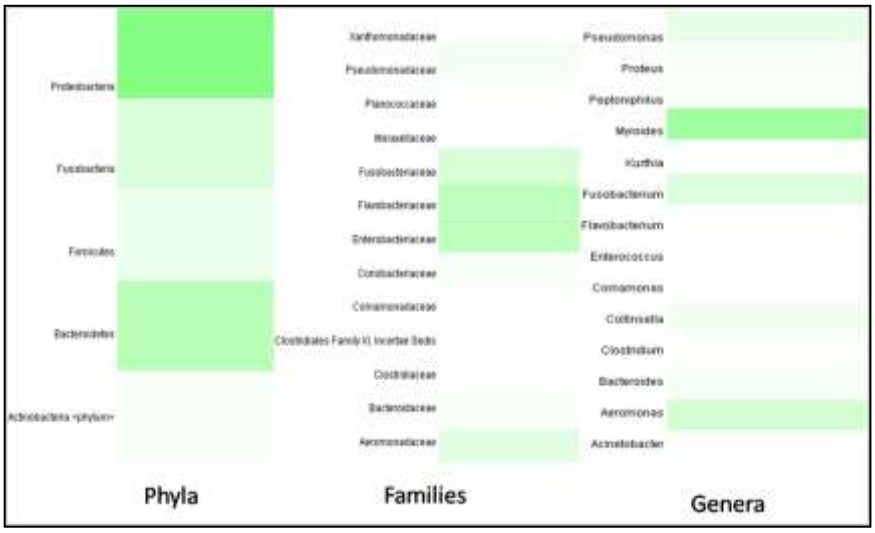


**Fig. 2:** Relative abundance of phyla, families and genera identified in houbara bustard

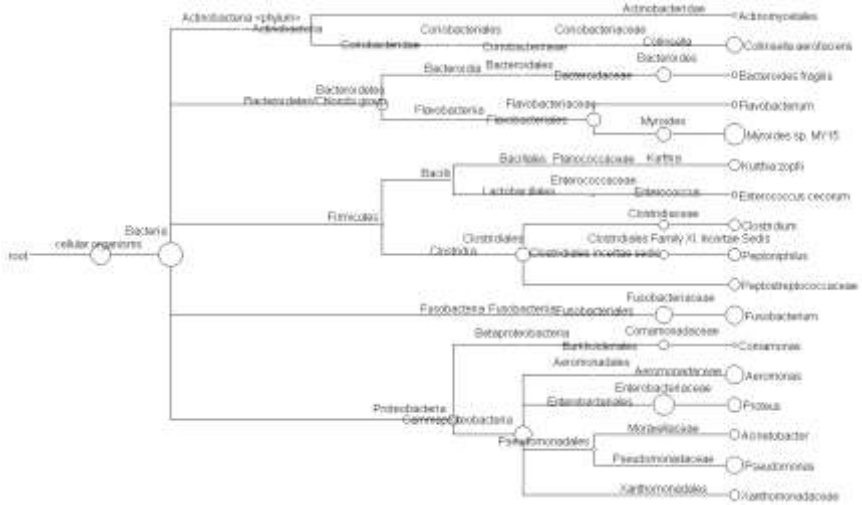

**Fig. 3:** Phylogeny of 16S rRNA gene sequences derived from houbara bustard. The number of reads assigned to each node in NCBI taxonomic tree is scaled logarithmically

A number of gram positive and gram negative bacterial species belonging predominantly to the phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* have been reported from respiratory system of diseased as well as healthy domestic and wild birds (Sambyal and Baxi, 1980; Byrum and Slemons, 1995; Glisson, 1998; Bailey *et al.,* 2000a, b; Hubalek, 2004; Abulreesh *et al.,* 2007). However, to best of our knowledge, *Fusobacteria* have not been previously associated with respiratory infection in birds.

Free living birds are considered an important potential vector for disease transmission from one geographical area to another (Bailey *et al.,* 2000b; Hubalek, 2004; Benskin *et al.,* 2009). Our detection of known pathogens that are previously not known to be associated with respiratory system of migratory birds, further increases this concern. For example, *Myroides spp. MY15* is a gram negative bacterium within the family *Flavobacteriaceae*, and has been recently isolated as novel organism from human saliva in a country that shares border with Pakistan, China (Yan *et al.,* 2012). Members of the genus *Myroides* have been isolated from clinical sources, soil, and seawater, and are considered opportunistic pathogen (Hugo *et al.,* 2006; Yan *et al.,* 2012). *Collinsella aerofaciens* is a gram positive bacilli that is abundant in human intestine (Kageyama *et al.,* 2000) and has been associated with human intestinal disorder (Swidsinski *et al.,* 2002). *Bacteroides fragillis*, penicillin resistant group associated with intestinal disorder, has been isolated from intestine of several mammals and birds (Avila-Campos *et al.,* 1990; Garcia *et al.,* 2012). *Enterococcus cecorum*, associated with arthritis and osteomyelitis, is another intestinal microbe isolated from birds (Boerlin *et al.,* 2012). Species of genera *Enterococcus* are gram positive that inhabit alimentary tract of human and have been associated to nosocomial pathogenecity and resistance to glycopeptide antibiotics (Fisher and Phillips, 2009). *Kurthia zopfii*, on the other hand, has been isolated from various sources, such as meat, wastewater, milk, feces and air from abattoirs as well as at an altitude exceeding 3,000 m (Stackebrandt *et al.,* 2006).

Compared to other species identified, the abundance of *Myroides spp. MY15* suggests it may have been involved in the respiratory symptoms of this bird, and perhaps other respiratory infections in houbara bustards. As *Myroides spp. My15* has not previously been isolated from the respiratory tract of avian species; and thus, has not been included in primary differential diagnosis, this information may contribute a candidate pathogen that can be readily detected via PCR in birds exhibiting respiratory signs. Since many sequence reads retrieved were assigned to relatively higher taxonomic level such as phylum, order and family, our results also indicate there are unknown organism, not closely related to any known OTU, that may be pathogens and should be further evaluated. A subsequent isolation and molecular epidemiological survey for these organisms may be necessary to ascertain their role either as commensals or pathogens within the respiratory system of birds or humans.

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