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ORIGINAL ARTICLE

The tracheal virome of broiler chickens with respiratory disease complex in Iran: the metagenomics study

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ABSTRACT

Background and Objectives: Avian respiratory disease complex (RDC) is one of the most detrimental economic diseases that affected different parts of the world. Various pathogens cause the disease, but the most significant viral pathogens include avian influenza virus (AIV), infectious bronchitis virus (IBV), and Newcastle disease virus (NDV) are the most prevalent. To detect these pathogens, various methods have been discovered in the last decades. Detection and characterization of viruses by metagenomics methods have improved our knowledge about the role of virome in the avian complex respiratory disease.
Materials and Methods: This research investigates the viral pathogen populations that mostly participate in emerging these diseases using the NGS method RNA-sequencing. In surveillance of ten broiler farms from different cities with respiratory symptoms, trachea samples were collected to determine the pathogenic virome causing the disease.
Results: In this metagenomics analysis, nine viral families were identified, comprising 72.82% of RNA viruses, 24.32% of RT viruses, and 2.86% of DNA viruses. RNA viruses had the highest contribution to the respiratory disease complex instead of disease, including paramyxoviridae, orthomyxoviridae, coronaviridae, and picornaviridae viruses. Other viruses from the RNA viruses and DNA virus families were also identified in addition to these results.
Conclusion: This research suggests that studies of pathogenic viromes in different diseases can help monitor different diseases and predict their future occurrence.

Keywords: Metagenomics; Chickens; Iran; Virome; Trachea

INTRODUCTION

In the poultry industry, the respiratory system is one of the most critical targets in a vast of devastating economic diseases. The tracheal mucosa is highly sensitive and responsive to infections. Avian respiratory disease complex (RDC) considers as a multifactorial disease. Viral pathogens concerning animal health welfare in farms are the most agents

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that account for RDC deaths. Subsequently, secondary respiratory diseases occur mainly by bacterial agents. The prevalent viral pathogens are members of the Riboviria realm, such as the Avian influenza virus (AI), Newcastle disease virus (NDV) from *Negarnaviricota* phylum, and Infectious bronchitis virus (IBV) from Nidovirales order (1-3). The realm contains a wide range of genetically diverse RNA viruses. There is a wide genetic and structural diversity in viruses. There are differences in the affinity of viruses to bind to host receptors. Therefore, different viral attachments (PVA) patterns are based on the diversity of receptor expression and distribution in different poultry species. These differences in the viral binding patterns and the distribution and expression of host receptors in avian species lead to the emergence of new viral strains and specific tissue tropisms (4-9). In the study of viral populations, the virome of target organs is also surveyed. Virome refers to collecting nucleic acids, both RNA and DNA, that make up the viral community associated with a particular ecosystem. The word is derived from viruses and genome and was first used by Forest Rohwer and colleagues to describe viral shotgun metagenomes. All macro-organisms have viromes that include bacteriophage and viruses.

Viromes are essential in the nutrient and energy cycling, development of immunity, and a significant source of genes through lysogenic conversion (10). There have been numerous studies of the virome in various diseases (11-15). In the surveillance of polymicrobial diseases, conventional methods for detection of viruses and other microbes are time-consuming to ascertain the viral or microbial populations in complex diseases. The most traditional method used to study virome in chickens with respiratory diseases is various polymerase chain reactions (PCR) that are limited in detecting all viruses. In PCR-based diagnosis, not all viruses in the target organ or disease can be detected. This method uses primers designed for specific genes from the genomes of identified viruses. Thus, viruses that do not have specific primers remain unknown. This study aims to detect the tracheal virome of broiler chickens with respiratory disease complex in Iran using a metagenomics study.

MATERIALS AND METHODS

Sample collection. Ten-broiler chicken farms (each farm comprising more than 10000 birds) with clinical signs of complex respiratory disease were evaluated. From each farm, collected ten tracheal samples from chickens with clinical signs randomly. The most frequent infectious signs of the respiratory tract were respiratory rales, excessive lacrimation, and conjunctivitis with the nasal and oral clear mucosal secretions. All farms located in Gilan province from different cities (Fig. 1). All samples were stored in 0.5 ml RNA later stabilization reagent (QIAGEN) at -20°C.

Viral RNA extraction and NGS. Tracheal samples (10 samples) from each farm were crushed and homogenized, and then their contents were pooled. The total RNA of pooled samples from each farm was extracted using the TRIzolPlus RNA Purification Kit (Thermo Fisher Scientific, USA). It was performed following the manufacturer's instructions. After extraction, 30 µl of total RNA from each farm (10 farms) were pooled. It was sent to Beijing Genomics Institute (BGI, China) and sequenced using the IlluminaHiSeq 4000 platform in two separate runs, generating paired-end 150 bp reads.

NGS bioinformatics analysis. Next-generation sequencing of pooled RNA extraction samples was performed by Illumine Hiseq4000 (BGI, China). The next-generation sequencing data (reads) were analyzed with high accuracy using the web-based tool genome detective. The whole known viral genomes were assembled (16). Sequence results were assessed for the reads quality and bioinformatics analysis. The reads' quality was evaluated using FastQC (17). The low-quality reads were filtered, and adapters trimmed with Trimmomatic (18). After the data were analyzed for quality control (QC), the data aligned with the virus genomic database by the protein-based alignment method DIAMOND, and virus-related reads were identified, and non-viral reads were removed (19). The viral reads de novo assembled using metaSPAdes, and viral contigs ascertained (16, 20). Taxonomy of viral reads was detected and represented by the Krona chart (21).

RESULTS

The NGS data included 23606490 paired-end reads (3540973500 base pairs) with an average 150

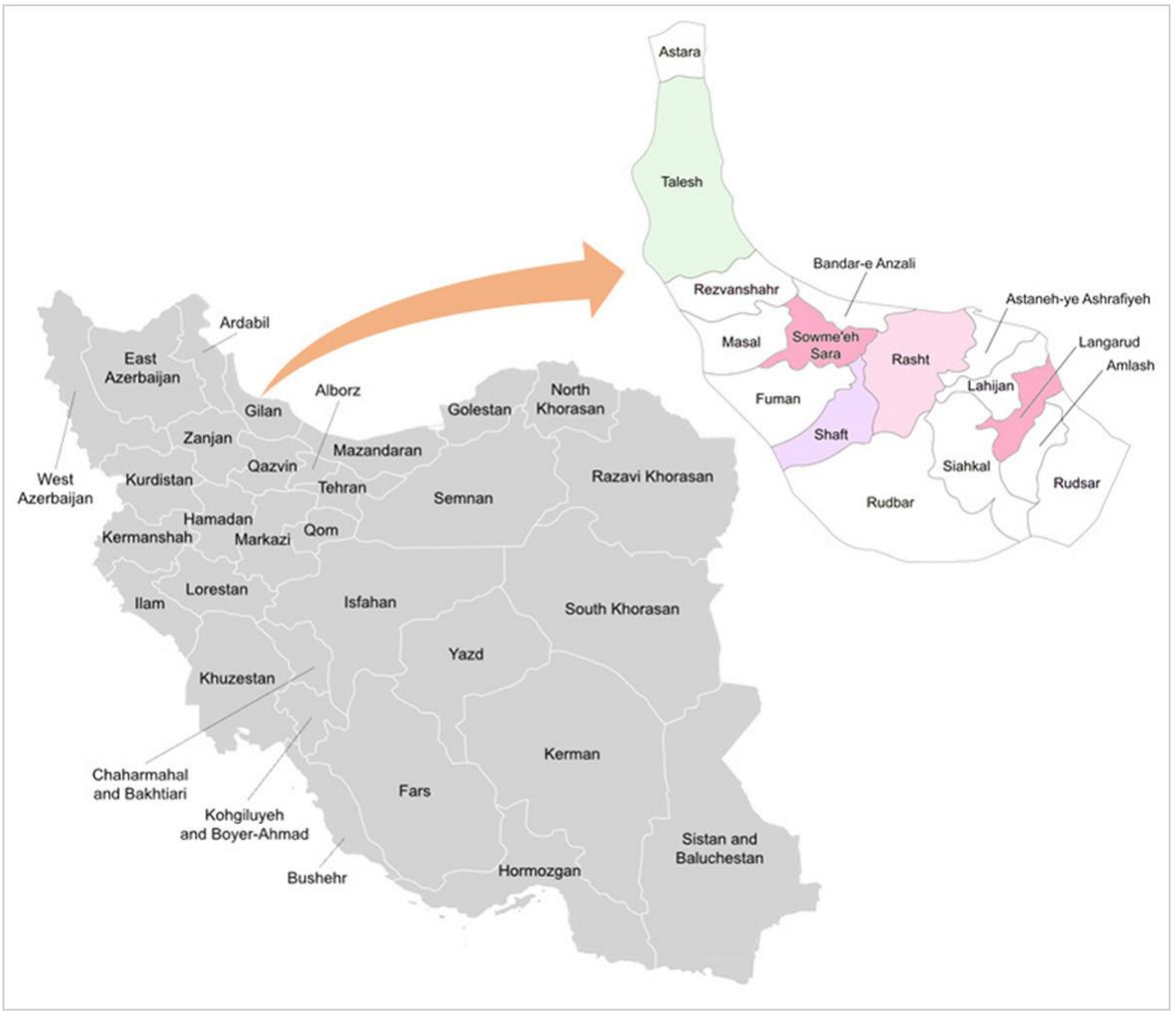

Fig. 1. Gilan province, cities where cases with complex respiratory clinical signs collected

bp read length. Trimmed reads length obtained 50-133 bp. The low-quality reads (1%) that did not pass quality control processing were removed. Non-viral reads, including the host genome and other non-viral microorganisms, were excluded. Then, 12776 reads de novo assembled using metaSPAdes. About 46% of reads were assembled into viral contigs. The 5880 reads mapped back to the viral contigs. After excluding bacterial genomes from this metagenomics analysis, nine viral families were identified, comprising 72.82% of RNA viruses, 24.32% of retroviruses, and 2.86% of DNA viruses. RNA viruses had the highest contribution to the viral etiologic agents, including paramyxoviridae, orthomyxoviridae, coronaviridae, and picornaviridae viruses (Fig. 2). Among the candidate viral reads, 4058 reads belonged to Avian Avulavirus 1, which were obtained with 95.5% and 96.5% percent similarity in nucleic acids and amino acid identity to reference virus. In the virus genome, most identified mutations were related to the genes encoding Fusion (F), hemagglutinin, and neuraminidase (HN) proteins. In this study, various subtypes of the influenza A virus and different genomic segments of H9N2, H7N9, H5N1, and H2N2 were identified, of which 119 reads were assigned to the virus. Most mutations were in segments 7 (M1, M2) and 8 (NS1, NEP) of the virus genome. Infectious bronchitis viruses with 99 reads and an average of 89.85% nucleotide identity and Sicinivirus A with six reads with high similarity were detected (Table 1). Most RT viruses are of the genus Alpharetrovirus. Although these viruses were detected by HTS data analysis, no evidence of PCR confirmation was found. More than 57% of retroviral viruses comprise avian endogenous retrovirus EAV-HP, suggesting the fossilized virus integrated into the host genome. Retroviral assign-

Fig. 2. Viral taxa classifications based on logarithmically normalized viral reads in tracheal virome of broiler chickens with respiratory disease complex in Iran

ments identified were probably endogenous chicken sequences or from contaminants to other samples prepared in the sequencing platform (Table 2).

DISCUSSION

The avian respiratory complex syndrome is a multifactorial disease that has had adverse health and economic effects worldwide, especially in Iran. The respiratory complex of birds is supposed to be a syndrome in which the bird displays non-specific respiratory symptoms. The disease results from several pathogens, which on the clinical feathers exhibit similar symptoms that ultimately lead to the diagnosis of the underlying cause of the disease. The most im-

Table 1. Identification of viral species in broiler chickens with complex respiratory disease

Assignment	Contigs	Reads	Coverage (%)	Depth of Coverage	Identity (%)	
					NT	AA
Avian avulavirus 1 (NC_039223.1*)	4	4058	56.5	62.5	95.5	96.5
Avian endogenous retrovirus EAV-HP (NC_005947.1*)	5	816	46.7	53.7	97.5	95.1
Avian myelocytomatosis virus (NC_001866.1*)	3	357	26.1	53.3	99.1	97.6
Y73 sarcoma virus (NC_008094.1*)	2	79	12.1	16.6	98.7	94.9
Influenza A virus-segment 7 (NC_004907.1*)	1	50	51.6	12.5	93	93.2
Fujinami sarcoma virus (NC_001403.1*)	4	49	20.6	5.6	83.4	80.8
Influenza A virus-segment 8 (NC_007380.1*)	1	19	40.6	7.4	90	87.5
Influenza A virus-segment 5 (NC_004905.2*)	1	14	18.9	6.3	94.6	92.9
Influenza A virus-segment 8 (NC_004906.1 *)	1	10	34.2	4.3	88.8	87.3
Influenza A virus (NC_007359.1*)	1	8	12.3	3.9	92	94.4
Influenza A virus-segment 2 (NC_026423.1*)	1	18	9.8	10.5	93.4	98.6
Avian coronavirus (NC_001451.1*)	1	93	1.3	33.5	92.9	0
Avian coronavirus (NC_001451.1*)	1	6	0.9	3.2	86.8	90.9

* Reference Genome

portant infectious agents affecting respiratory complex syndrome are viruses that have been identified in several studies. Also, immunosuppressive factors, environmental conditions, and inadequate control and prevention strategies can be considered virulent factors involved in the disease. Common viral agents in this disease are *avian influenza virus, avian orthoavulavirus* type 1, and avian infectious bronchitis virus. Bacterial agents such as *Mycoplasma, Escherichia coli,* and *Ornithobacterium rhinotracheale* have been identified (22, 23). Since 2009, different genotypes of viral diseases common in poultry respiratory complex syndrome have identified using serological and molecular detection methods in Iran and neighboring countries.

Newcastle disease (ND) is one of the most serious viral infections in the Iranian poultry industry. The Newcastle disease virus dominant genotype (NDV) in Asian countries is Class 2 and genotype VII. Circulation of genotype VII virus pathogens periodically has caused considerable economic losses. The highly variable subgenotypes of the virus are supposed to be the country's long-term presence of genotype VII. In this study, in addition to identifying the virulent strains of NDV with subgenotype VIIl, other viruses involved in the respiratory complex, including Avian influenza virus (AIV) and Infectious bronchitis virus (IBV), have also been identified. Since 1998, the dominant influenza virus serotype that has been endemic in our country is the H9N2 serotype identified from various birds. Most strains of this serotype are G1-W, which is widespread in Iran and the Middle East. The Avian influenza virus has a high zoonotic potential for the outbreak and spread of disease in human societies and animal welfares. Therefore, identification of livestock virome is essential for detecting potential zoonotic pathogens in regards to One Health strategies. In this research, in addition to identifying the dominant H9N2 virus serotype, the H7N9 and H5N1 serotypes were also identified that resembled influenza virus strains in Hong Kong, China, and Korea. Newcastle disease and Avian influenza viruses are notable concerns of the industry due to economic losses to the poultry industry due to the rapid spread of infections and high mortality rates. Isolated IBV in the analysis was most similar to isolates from Iran in 2012. The virus increases the severity of the disease in concomitant infections.

Different methods for virus detection in clinical specimens, such as isolation and molecular detection (PCR & Real-time PCR), are available with advantages and disadvantages. In recent years, researchers could discover the relationships and interactions of pathogens by molecular studies of viral populations in organs (Virome). Since the advent of sequencing technologies and other advanced laboratory technologies, high throughput data, complexity, and multiplicity of available bioinformatics tools, the management of processes and metagenomics analyzes have

Table 2. Viral families and assignments

Virus	Families	Genome	Assignment (Reference Genome)	Reads
	Paramyxoviridae	ssRNA(−)	Newcastle disease virus isolates JSD0812, complete genome	4058
	Picornaviridae	ssRNA(+)	Sicinivirus 1 strain UCC001, complete genome	6
			Influenza A virus (A/Hong Kong/1073/99(H9N2)) segment 7, complete sequence	50
			Influenza A virus (A/Korea/426/1968(H2N2)) segment 8, complete sequence	19
RNA virus	Orthomyxoviridae	ssRNA(−)	Influenza A virus (A/Shanghai/02/2013(H7N9)) segment 2 polymerase PB1 (PB1) and PB1-F2 protein (PB1-F2) genes, complete CDs	18
			Influenza A virus (A/Hong Kong/1073/99(H9N2)) segment 5, complete sequence	14
			Influenza A virus (A/Hong Kong/1073/99(H9N2)) segment 8, complete sequence	10
			Influenza A virus (A/goose/Guangdong/1/1996(H5N1)) polymerase (PA) and PA-X protein (PA-X) genes, complete CDs	8
	Coronaviridae	ssRNA(+)	Avian infectious bronchitis virus, complete genome	93
			Avian infectious bronchitis virus, complete genome	6
			Avian endogenous retrovirus EAV-HP	816
			Avian myelocytomatosis virus	357
			Y73 sarcoma virus	79
RT virus	Retroviridae	ssRNA(+)	Fujinami sarcoma virus	49
		into a linear	Rous sarcoma virus	40
		dsDNA	Rous sarcoma virus	35
			Atlantic salmon swim bladder sarcoma virus	26
			Avian leukosis virus	24
			UR2 sarcoma virus	4
	Phycodnaviridae	dsDNA	Micromonaspusilla virus 12T genomic sequence	72
			Shigella phage pSs-1, complete genome	40
	Myoviridae	dsDNA	Enterobacteria phage P88, complete genome	35
			Enterobacteria phage RB3, complete genome	2
DNA virus	Herpesviridae	dsDNA	Gallidherpesvirus 2, complete genome	4
			Enterobacteria phage BP-4795, complete genome	2
			Enterobacteria phage cdtI, complete genome	2
	Siphoviridae	dsDNA	Enterobacteria phage mEp460, complete genome	2
			Salmonella phage 9NA, complete genome	5
			Ralstonia phage RS138 DNA, complete genome	2
			Stx2-converting phage 1717, complete prophage genome	2

grown exponentially. Each of these steps in viral data analysis may be performed with different bioinformatics tools or different algorithmic approaches, each with different assumptions, input data, and parameters. With the advent of NGS technology, metagenomics, and bioinformatics, continuous disease monitoring has gradually been resolved. Metagenomics-based detection methods are highly sensitive and do not target any specific pathogens. These technologies comprehensively scan the entire genome of the specimens, and consequently, the pathogens can be identified regardless of whether they are included in the surveillance plan.

In recent years, the prevalence of concurrent infections has received increasing attention, as in years 2014 to 2015, ND virus, AI virus, and IB virus diagnosed, 60, 34, and 55 percent, respectively (24). From 2015 to 2016, all three viruses detected 67%, ND virus and IB virus 48%, and IB virus alone 38%, and the most common respiratory complex syndrome virus was poultry IB virus (25). In this study, all three ND viruses (46.99%), AI virus (27.03%), and avian IB virus (25.99%) were identified, with ND and AI viruses having the most effect on poultry respiratory complex syndrome.

The results showed that it could be used as a diagnostic and molecular epidemiology tool. In this study, the metagenomics methods identified respi-

ratory viruses, including ND virus, AI virus, and IB virus, which could determine their genotype by the extent of coverage and essence of viruses. More herds should be evaluated to detect pathogenic agents of diseases individually in different parts of the country and identify new viruses. Interestingly, viruses from the order Caudovirales, Herpesvirales, Ortervirales, Picornavirales, and the family Phycodnaviridae have been identified that researchers should continue to study more closely the role of these viruses and evaluate the importance of these viruses in respiratory syndrome. The results of the metagenomics analysis of respiratory viruses show that identifying different viruses using second-generation sequencing technology is of great importance in the accurate detection and monitoring of poultry respiratory diseases, so the use of this technology for the continuous monitoring of livestock diseases is a necessity. Besides, effective measures should be taken in the field of herd biosecurity and vaccination strategies. Hence, to prevent disease occurrence, the dominant strains of respiratory complex syndrome viruses in the herd should be continuously identified. This is the first study of metagenomics to detect the causes of respiratory syndrome in Iran. In this study, we were able to detect different viral agents in the trachea. This study aims to contribute to the epidemiology of infectious agents in the population. It is further recommended that such studies be continued on different flocks, different provinces, and different clinical conditions.

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