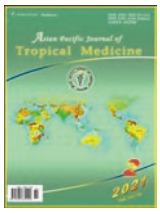

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Genomic characterization of velogenic avian orthoavulavirus 1 isolates from poultry workers: Implications to emergence and its zoonotic potential towards public health

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ABSTRACT

Objective: To carry out the genetic characterization and evolutionary analysis of three avian orthoavulavirus 1 (AOAV-1) isolates from poultry workers with respiratory symptoms.

Methods: Using Illumina MiSeq, whole-genome sequencing was carried out to assess the evolutionary dynamics of three AOAV-1 isolates. A phylogenetic and comparative analysis of all coding genes was done using bioinformatics tools.

Results: Phylogenetic analysis and genetic distance estimation suggested a close relationship among human- and avian-originated velogenic strains of genotype Ⅹ Ⅲ, sub-genotype Ⅹ Ⅲ.2.1. Several substitutions in the significant structural and biological motifs were exclusively identified in the human-originated strains.

Conclusions: To our knowledge, this is the first report of a velogenic AOAV-1 isolate from natural infection of the human upper respiratory tract. Our findings highlight the evolution and zoonotic potential of velogenic AOAV-1 in a disease endemic setting.

KEYWORDS: Avian orthoavulavirus 1; Human originated strain; Zoonotic potential; Evolution; Genotype Ⅹ Ⅲ; Poultry workers

1. Introduction

The International Committee on Taxonomy of Viruses has created a new subfamily Avulavirinae and classified previously designated avian avulaviruses into three genera: *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus*[1]. Avian orthoavulavirus 1 (AOAV-1) belongs to the genus *Orthoavulavirus* and causes Newcastle disease in multiple susceptible hosts including several avian species[2]. The disease is characterized by the rapid onset of respiratory and nervous symptoms in susceptible species of birds including chickens, pigeons, and turkeys[2].

AOAV-1 is an enveloped, non-segmented negative-sense single-stranded RNA virus with (14.9-17.4) kb in genome length[1]. Based on the clinical disease produced in chicken and typical residue patterns at the fusion protein cleavage site (^{112}RRKQR ↓ F^{117}), the strains of AOAV-1 are classified into three distinct pathotypes (lentogenic, mesogenic, and velogenic strains). The lentogenic strains produce mild infection with low or no mortality; mesogenic strains yield an intermediate virulence, cause respiratory symptoms and may affect egg quality and production; and the velogenic strains are highly virulent and cause severe nervous and respiratory infection with up to 90% mortality in infected flocks[2].

Viruses of the family Paramyxoviridae have the potential to infect a wide range of susceptible hosts including birds, reptiles, mammals, and aquatic species[3]. Besides avian species, AOAV-1 infection has been observed in unusual hosts including humans, mink, swine, and cattle, highlighting the zoonotic potential of this virus[2,4-7]. Indeed, the zoonotic potential of AOAV-1 has been recognized for more than

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70 years, which was isolated from human subjects, including poultry workers exhibiting conjunctivitis. Accidental exposure of laboratory workers to AOAV-1 by infected liquid splashed into the eyes has been associated with conjunctivitis, and in rare cases, systemic infection with symptoms including headache and general malaise. Occasionally, upper respiratory tract infection and/or general flu-like symptoms have been observed in infected humans[8,9]. Recently, two cases of AOAV-1 infection have been reported from immuno-compromised human individuals who died from deep respiratory tract infection (pneumonia) where the isolated AOAV-1 was presumed to be the cause of infection[4].

In the current report, three closely related AOAV-1 strains that were isolated from nasal swabs of poultry workers, exhibiting mild upper respiratory symptoms, were sequenced for their complete genome and genetically characterized. Unlike previous cases from immuno-compromised patients, the velogenic AOAV-1 isolates described in this study were associated with upper respiratory tract infection. Phylogenomic characterization revealed a close relationship of these human-originated isolates with strains known to exist in chickens from the same geographical region. The human isolates demonstrated several unique amino acid substitutions in the important structural and biological motifs of fusion and hemagglutinin proteins. The finding of AOAV-1 infection in association with upper respiratory tract disease in humans raises the possibility for this virus to adapt to the human host and may gain the ability to transmit and cause infection in other healthy humans. Therefore, there is a need for continuous monitoring and surveillance of AOAV-1 in humans particularly in those linked with poultry settings.

2. Materials and methods

2.1. Ethical approval

All essential procedures were approved by the Ethical Review Committee for the Use of Laboratory Animals of the University of Veterinary and Animal Sciences, Lahore, Pakistan (approval number: ERCULA/DR-1349). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

2.2. Sample collection and processing

We previously found extensive seropositivity to AOAV-1 among poultry workers in Pakistan through modified horse red blood cells haemagglutination inhibition assay[10]. Alongside blood collection from poultry workers in these studies, nasal swab samples were collected aseptically from everyone. Swabs were introduced into both nostrils and rotated 3-4 times, then placed in tubes of 2.0 mL brain heart infusion media containing 200 μg/mL gentamicin 5 μg/mL amphotericin B, stored at -80 ℃ until processing. The tubes were centrifuged at 1 000×*g* for 5 minutes. The resulting supernatant was treated with penicillin (10 000 IU/mL), streptomycin (10 000 μg/mL), amphotericin B (20 μg/mL), gentamicin (1 000 μg/mL), and kanamycin sulfate (600 μg/mL) for 20 minutes at 25 ℃ and then filtered through a 0.2 μm pore syringe filter. Nine-day old embryonated chicken eggs were inoculated with 0.2 mL of the treated sample *via* chorioallantoic sac route. Embryos were monitored daily, and all were dead within 60 hours, which is a typical characteristic of velogenic strains of AOAV-1 and pathogenic avian influenza A viruses[11]. The chorioallantoic fluid (CAF) was acquired and processed by spot agglutination test using 10% washed chicken red blood cells in saline as previously described[11]. Extracted RNA from CAF samples showing haemagglutination was tested by real-time reverse-transcript polymerase chain reaction (RT-PCR) for AOAV-1 matrix gene using M+4100 (5′-AGTGATGTGCTCGGACCTTC-3′) and M-4220 (5′-CCTGAGGAGAGGCATTTGCTA-3′) primers and M+4169 probe[12], and using M+25 and M-124 primers and M+64 probe for influenza A virus matrix gene[13].

2.3. Whole-genome sequencing

Viral RNA from the hemagglutination-positive CAF samples was extracted using a MagMAX AI/ND viral RNA extraction kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, and the extracted RNA was stored at -80 ℃ until processing for next-generation sequencing. A barcoded library was prepared from each RNA sample using the Illumina TruSeq Stranded mRNA kit (San Diego, CA). The manufacturer's protocol was followed using the "purified mRNA input" recommendation, which skips polyA RNA enrichment and begins with RNA fragmentation and priming. An equimolar pool of libraries was made and sequenced on an Illumina MiSeq instrument using 150 bp×150 bp paired-end sequencing according to the manufacturer's protocol. Between 29947 and 82808 reads were assembled for each genome. Following FastQC, the paired-end reads were merged using FLASH version 1.2.11 and then run against the NCBI nr-protein database using DIAMOND version 0.8.33 and Kraken version 1.1. Results were visualized with MEGAN 6[14], revealing close relation to AOAV-1.

2.4. Viral genome assembly and analysis

Reads belonging to all viruses were extracted, and the sequence was assembled using SPAdes version 3.9.1[15]. From the assembly, the largest contigs (>500 bases) were extracted, and these were used as a query for BLAST against the nr-genome database. The top hit was goose paramyxovirus SF02 (NC_005036) and the second hit was Newcastle disease virus strain B1 (NC_002617). Contigs were aligned against each of these reference strains separately. Nodes were reversely complemented as required and concatenated based on alignment with the goose paramyxovirus reference strain (NC_005036). Where needed, gaps were filled based on alignment with the reference strain.

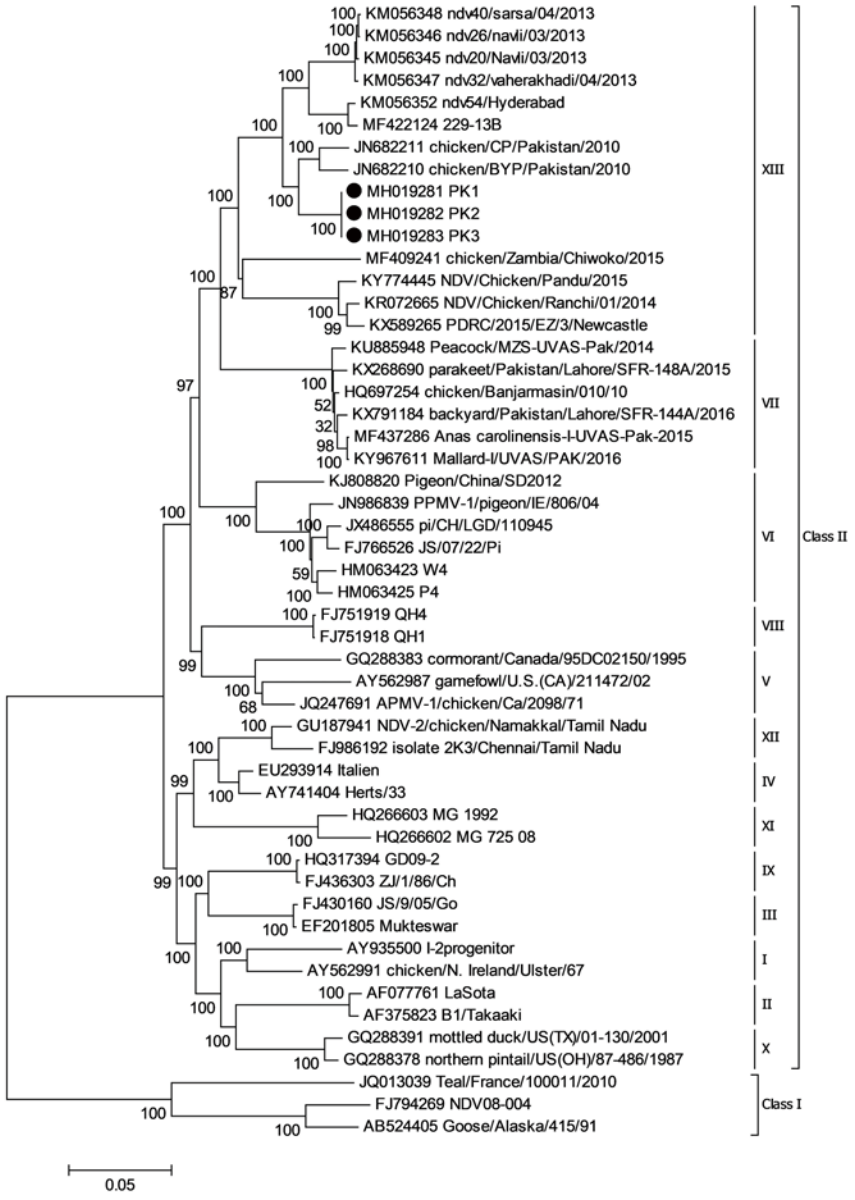

Figure 1. Phylogenetic relationship of novel human avian orthoavulavirus 1 (AOAV-1) isolates with previously described AOAV-1 strains. Aligned whole-genome sequences of isolates representative of all AOAV-1 genotypes were used to construct phylogenetic trees *via* the neighbor-joining method with 1 000 bootstrap replications.

2.5. Comparative genomic characterization

Assembled sequences were aligned with AOAV-1 strains representing different genotypes accessed from NCBI (http://www.ncbi.nlm.nih.gov/) using ClustalW methods in BioEdit® version 5.0.6[16] for comparative genomic and phylogenetic analysis and prediction of deduced amino acid substitution sites for all coding genes and genetic distance. Consensus sequences from isolates belonging to the same geography isolated within a specific timeframe were used for the identification of residue substitutions. The conserved domains, functionally and structurally important motifs and unique substitutions in open reading frames were predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), Conserved Domain Prediction (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and HMMTOP (http://www.enzim.hu/hmmtop/index.php). The potential N-glycosylation sites were predicted using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc) if the encoded residue was N-X-T/S, where X denoted any residue except proline and accepted if the G-score was 0.5.

2.6. Evolutionary analysis

Phylogenetic analysis and evolutionary distance estimation were performed using MEGA® version 6.0 software[17]. A phylogenetic tree was constructed using aligned complete genome sequences from AOAVs *via* the neighbor-joining method with 1 000 bootstrap replications. To determine the nucleotide identity and divergence of isolates compared with the AOAVs belonging to genotype ⅩⅢ, sequence comparison analysis was performed using whole-genome sequences. To determine sub-genotype categories, complete *F* gene sequences from group ⅩⅢ isolates were analyzed using the maximum likelihood method with 1 000 bootstrap replications. To

estimate the mean inter-population evolutionary diversity (mean evolutionary distance/genetic distance) between sub-genotypes of ⅩⅢ, the analysis was performed using complete *F* gene sequences. This analysis was performed *via* the maximum composite likelihood method (d: Transitions+Transversions model). The rate and pattern of substitutions among sites were modeled with a gamma distribution (parameter=1 with homogenous lineage pattern)[18].

2.7. Recombination analysis

For detection of an occurrence of putative recombination events within the same genotype and/or with vaccine strains, the complete genome sequences of human-originated study strains, avian-originated AOAV-1 isolates from genotype ⅩⅢ, and vaccine strains [(genotypes Ⅱ (LaSota, GenBank accession number AF077761) and Ⅲ (Mukteshwar, GenBank accession number EF201805)] were used. Recombination analysis was performed for identification of putative breakpoints using SimPlot, GARD (http://www.datamonkey.org/GARD), DAMBE[19], and RDP4 version 4.95[20]. Initially, all enriched sequences were used to check the occurrence of recombination events. Then the outcomes shown by RDP4 were further investigated to enhance its accuracy, clarity, and reliability. This analysis employed seven different recombination algorithm methods (RDP4, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq) to reveal putative recombinant and parent isolates at $P<0.001$. Putative recombination events were assumed to have occurred only when they were consistently identified by at least four of the recombination algorithms at a probability threshold of 0.05.

3. Results

3.1. Viral agent isolation

CAF from eggs inoculated with material derived from nasal swabs, which were isolated from poultry workers, demonstrated the haemagglutination reaction to horse red blood cells and death of embryos within 60 hours, suggesting infection of embryos with a viral agent. However, the CAF tested negative for both avian influenza A virus and AOAV-1 by real-time RT-PCR. Therefore, viral RNA was extracted from CAF and analyzed by next-generation sequencing to identify the viral agent.

The individuals from whom these nasal swabs originated demonstrated serum antibody titers against the lentogenic AOAV-1 LaSota strain (GenBank accession number: AF077761). Endpoint titers of AOAV-1-specific antibody of these individuals ranged from 160 to 320 (254.0±53.3). These individuals had worked with poultry from 9 months to 8 years and age ranged from 21 to 41 years. One individual reported mild respiratory symptoms, while none of the individuals presented symptoms associated with human AOAV-1 infection, including conjunctivitis.

3.2. AOAV-1 genome assembly

Total reads of 50 to 151 nt length for the three samples were 534941 (PK1, 92.4% of all reads), 696870 (PK2, 91.70%), and 629717 (PK3, 95.70%). Visualization of DIAMOND blast analysis of merged paired-end reads (262277, 228934, and 192498 for PK1, PK2, and PK3, respectively) surprisingly suggested a close match of the sequences with *Avulaviruses*. Phylogenetic analysis of complete genome sequences showed that these three sequences clustered with AOAV-1 strains of genotype ⅩⅢ isolated from avian species from Pakistan (Figure 1). The *M* gene sequence of the isolates was aligned with the primers and probe (Table 1), which target the *M* gene that had been used for screening the CAF samples for AOAV-1. The nucleotide identity of the probe with the three human isolates was 84% (21/25) (Table 2). The complete genome sequences of these AOAV-1 isolates have been deposited in GenBank under the accession numbers MH019281 (PK1), MH019282 (PK2), and MH019283 (PK3).

3.3. Phylogenetic and evolutionary analysis

In the initial phylogenetic analysis of complete genome sequences, distinct clades were observed within genotype ⅩⅢ with AOAV-1 strains clustering based on location and host species (Figure 1). The Indian, European, Iranian, and Pakistani avian AOAV-1 strains were clustered into ⅩⅢ.1.1, ⅩⅢ.1.2, ⅩⅢ.2.1 and ⅩⅢ.2.1 sub-genotypes, as described previously[21-24]. The human-originated isolates clustered in a distinct clade closely related to avian originated ⅩⅢ.2.1 isolates from Pakistan (Figure 2).

Among human-originated AOAV-1 isolates, 0%-0.01% nucleotide divergence (99.9%-100.0% nucleotide identity) was observed. In contrast, the human-originated strains were 4.39%-4.52% divergent from Pakistani avian AOAV-1 strains during 2010, 5.80%-5.97% divergent from Indian avian AOAV-1 strains isolated during 2013, and 10.09%-10.46% divergent from Indian avian AOAV-1 strains isolated during 2014-2015 (Table 2).

For genetic distance among AOAV strains from genotype ⅩⅢ, the mean inter-population evolutionary distance was found to be greatest (0.097 1) between sub-genotypes ⅩⅢ.1.1 and ⅩⅢ.2.2, with smaller distances between sub-genotypes ⅩⅢ.1.1 and ⅩⅢ.2.1 (0.084 3), sub-genotypes ⅩⅢ.1.2 and ⅩⅢ.2.2 (0.079 4), sub-genotypes ⅩⅢ.2.1 and ⅩⅢ.2.2 (0.068 4), sub-genotypes ⅩⅢ.1.2 and ⅩⅢ.2.1 (0.060 5), and sub-genotypes ⅩⅢ.1.1 and ⅩⅢ.1.2 (0.054 9) (Figure 2).

Table 1. Sequence of human Pakistani isolates of AOAV-1 compared with sequence of real-time RT-PCR probe[12].

Source	5'-sequence-3'
Probe	5'-TTCTCTAGCAGTGGGACAGCCTGC-3'
PK1	5'-TT**T**TC**C**AGCAG**C**GGGACAGC**T**TGC-3'
PK2	5'-TT**T**TC**C**AGCAG**C**GGGACAGC**T**TGC-3'
PK3	5'-TT**T**TC**C**AGCAG**C**GGGACAGC**T**TGC-3'

Mismatches are highlighted in bold.

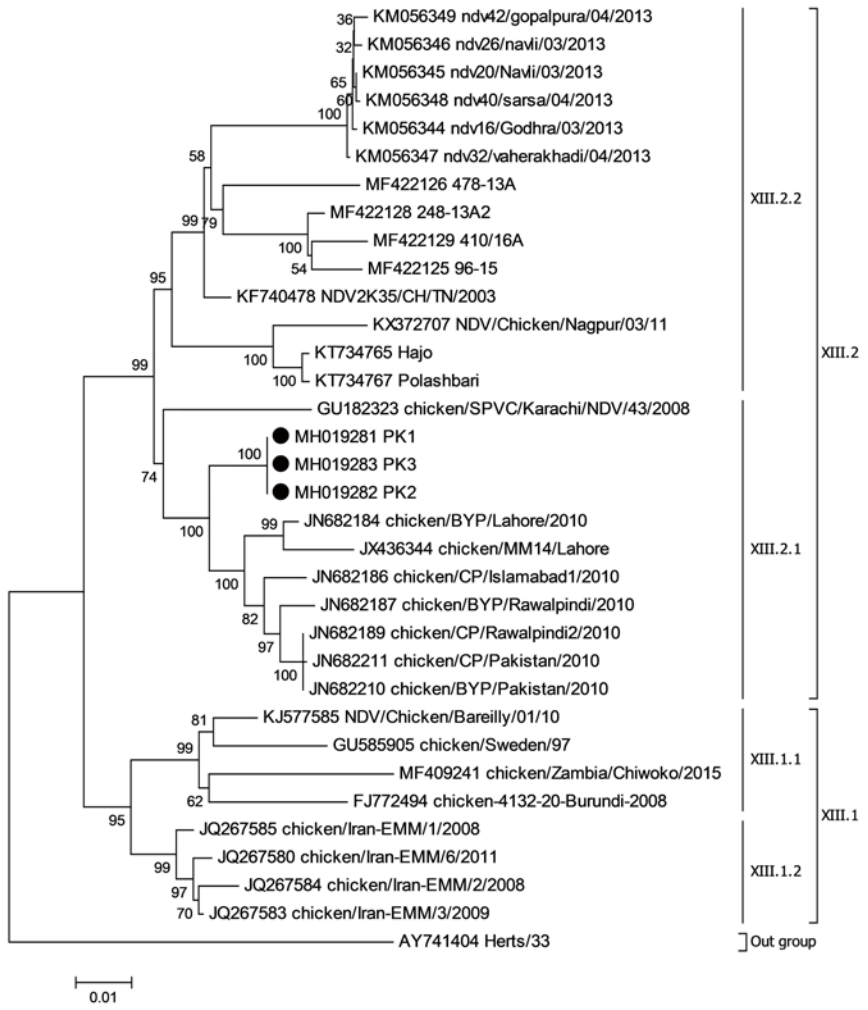

Figure 2. Phylogenetic tree was constructed using complete *F* gene sequence of under-study human originated avian orthoavulavirus 1 (AOAV-1) isolates with previously described AOAV-1 strains. Tree was constructed using the maximum likelihood method with 1 000 bootstrap replications.

Table 2. Percent divergence of complete genome of human and avian genotype ⅩⅢ AOAV-1 isolates.

Isolates	MH019281	MH019282	MH019283	JN682211	JN682210	KM056348	KM056345	KM056346	KM056347	KM056352	KR072665	KY774445	KX589265
MH019281_PK1													
MH019282_PK2	0.01												
MH019283_PK3	0.00	0.01											
JN682211_chicken/CP/Pakistan/2010	4.39	4.39	4.34										
JN682210_chicken/BYP/Pakistan/2010	4.52	4.52	4.47	2.78									
KM056348_ndv40/sarsa/04/2013	5.97	5.97	5.92	7.19	7.03								
KM056345_ndv20/Navli/03/2013	5.85	5.86	5.82	7.07	6.91	0.29							
KM056346_ndv26/navli/03/2013	5.93	5.93	5.88	7.14	6.98	0.13	0.25						
KM056347_ndv32/vaherakhadi/04/2013	5.80	5.81	5.78	7.07	6.90	0.38	0.30	0.43					
KM056352_ndv54/Hyderbad	5.90	5.91	5.89	7.11	6.94	4.60	4.46	4.57	4.40				
KR072665_NDV/Chicken/Ranchi/01/2014	10.15	10.16	10.12	11.07	11.05	11.17	11.07	11.14	11.06	10.95			
KY774445_NDV/Chicken/Pandu/2015	10.12	10.13	10.09	10.96	10.90	11.04	10.94	11.01	10.93	10.89	1.46		
KX589265_PDRC/2015/EZ/3/Newcastle	10.45	10.46	10.42	11.35	11.36	11.38	11.28	11.35	11.28	11.18	1.46	2.67	

Table 3. Comparative genomic characterization of human-originated AOAV-1 with avian-originated classical AOAV-1 isolates.

Regions	Human-originated AOAV-1			Avian-originated AOAV-1
	PK1 (MH019281)	PK2 (MH019282)	PK3 (MH019283)	
NP	108-1577 (1470)	113-1582 (1470)	108-1577 (1470)	122-1591 (1470)
Non-coding (NP-P)	1578-1878 (301)	1583-1890 (308)	1578-1858 (281)	1592-1892 (301)
P	1879-3066 (1188)	1891-3078 (1188)	1859-3046 (1188)	1893-3080 (1188)
Non-coding (P-M)	3067-3281 (215)	3079-3293 (215)	3047-3261 (215)	3081-3295(215)
M	3282-4376 (1095)	3294-4388 (1095)	3262-4356 (1095)	3296-4390 (1095)
Non-coding (M-F)	4377-4535 (159)	4389-4547(159)	4357-4515 (159)	4391-4549 (159)
F	4536-6197 (1662)	4548-6209 (1662)	4516-6177 (1662)	4550-6211 (1662)
Non-coding (F-HN)	6198-6403 (206)	6210-6415 (206)	6178-6383 (206)	6212-6417 (206)
HN	6404-8119 (1716)	6416-8131 (1716)	6384-8099 (1716)	6418-8136 (1716)
Non-coding (HN-L)	8120-8372 (253)	8132-8384 (253)	8100-8352 (253)	8137-8386 (250)
L	8373-14987 (6615)	8385-14999 (6615)	8353-14967 (6615)	8387-15001 (6615)

Table 4. Amino acid characterization of avian and human AOAV-1 isolates from Pakistan and India.

Proteins	Functional motifs	AOAV-1 isolates	Glycosylation sites	Comparative residue substitutions
NP	N-N self-assembly motif (^{322}FAPAEYAQLYSFAMG^{336})	Human originated	^{19}NGT^{21}, ^{116}NRS^{118}, ^{141}NGT^{143}	^{21}T, ^{93}K, ^{110}N, ^{111}V, ^{122}E, ^{168}I, ^{174}V, ^{223}H, ^{404}S, ^{425}R, ^{431}P, ^{464}P, ^{485}I
		Pakistan_2010	^{19}…^{21}, ^{116}…^{118}, ^{141}…^{143}	^{21}T, ^{93}K, ^{110}N, ^{111}V, ^{122}E, ^{168}I, ^{174}V, ^{223}H, ^{404}S, ^{425}R, ^{431}P, ^{464}P, ^{485}I
		IND_2014-15	^{19}…^{21}, ^{116}…^{11}8, ^{141}…^{143}	^{21}V, ^{93}K, ^{110}S, ^{111}V, ^{122}D, ^{168}V, ^{174}I, ^{223}Q, ^{404}G, ^{425}Q, ^{431}S, ^{464}L, ^{485}T
		IND_2013	^{19}…^{21}, ^{116}…^{118}, ^{141}…^{143}	^{21}T, ^{93}K, ^{110}N, ^{111}M, ^{122}E, ^{168}I, ^{174}V, ^{223}H, ^{404}G, ^{425}L, ^{431}L, ^{464}S, ^{485}T
P	RNA editing motif (GGGAAAAA)	Human originated	^{125}NKS^{127}, ^{288}NVS^{290}	^{11}D, ^{12}I, ^{22}S, ^{56}G, ^{76}S, ^{93}T, ^{97}T, ^{102}G, ^{115}D, ^{135}P, ^{285}I, ^{293}S, ^{301}S, ^{306}I, ^{342}T, ^{348}M, ^{371}K
		Pakistan_2010	^{125}…^{127}, ^{288}…^{290}	^{11}E, ^{12}L, ^{22}N, ^{56}S, ^{76}P, ^{93}A, ^{97}P, ^{102D}, ^{115}N, ^{135}P, ^{285}M, ^{293}S, ^{30}1F, ^{306}I, ^{342}T, ^{348}M, ^{371}R
		IND_2014-15	^{125}…^{127}, ^{288}…^{290}	^{11}D, ^{12}I, ^{22}S, ^{56}S, ^{76}P, ^{93}A, ^{97}P, ^{102}G, ^{115}N, ^{135}S, ^{285}M, ^{293}G, ^{301}S, ^{306}I, ^{342}S, ^{348}V, ^{371}K
		IND_2013	^{288}…^{290}	^{11}D, ^{12}I, ^{22}S, ^{56}S, ^{76}P, ^{93}A, ^{97}T, ^{102}G, ^{115}N, ^{135}P, ^{285}M, ^{293}S, ^{301}S, ^{306}V, ^{342}T, ^{348}I, ^{371}K
M	M late domain (^{23}FPIV^{26}), Bipartite nuclear localization motif (^{247}KKGKKVTFDKIEEKIRR^{263})	Human originated	^{207}NVT^{209}, ^{265}NLS^{267}, ^{306}NAS^{308}	^{7}V, ^{68}V, ^{73}V, ^{75}V, ^{77}V, ^{147}K, ^{166}S, ^{192}A, ^{216}S, ^{221}V, ^{261}I, ^{363}R
		Pakistan_2010	^{207}…^{209}, ^{265}…^{267}, ^{306}…^{308}	^{7}V, ^{68}V, ^{73}V, ^{75}V, ^{77}V, ^{147}K, ^{166}S, ^{192}A, ^{216}S, ^{221}V, ^{261}I, ^{363}R
		IND_2014-15	^{207}…^{209}, ^{265}…^{267}, ^{306}…^{308}	^{7}I, ^{68}I, ^{73}V, ^{75}A, ^{77}V, ^{147}R, ^{166}N, ^{192}A, ^{216}P, ^{221}I, ^{261}I, ^{363}R
		IND_2013	^{207}…^{209}, ^{265}…^{267}, ^{306}…^{308}	^{7}V, ^{68}V, ^{73}F, ^{75}V, ^{77}M, ^{147}R, ^{166}S, ^{192}T, ^{216}P, ^{221}V, ^{261}L, ^{363}K
F	Signal peptide (^{1}MGSKPSTRIPVPLRLITRV^{19}), Cleavage motif (^{112}RRQKRF^{117}), Fusion peptide (^{117}FIGAVIGSVAL GVATAAQITAAAALI^{142})	Human originated	^{84}NRT^{87}, ^{191}NNT^{193}, ^{366}NTS^{368}, ^{447}NIS^{449}, ^{471}NNS^{473}, ^{541}NNT^{543}	^{1}……^{19}, ^{20}M, ^{28}P, ^{32}L, ^{52}I, ^{69}M, ^{74}E, ^{108}A, ^{201}R, ^{226}T, ^{265}G, ^{282}L, ^{342}D, ^{476}N, ^{479}D, ^{494}R, ^{510}V, ^{522}S
		Pakistan_2010	^{84}…^{87}, ^{191}…^{193}, ^{366}…^{368}, ^{447}…^{449}, ^{471}…^{473}, ^{541}…^{543}	^{1}……S…..^{19}, ^{20}I, ^{28}L, ^{32}L, ^{52}I, ^{69}M, ^{74}Q, ^{108}T, ^{201}K, ^{226}I, ^{265}G, ^{282}L, ^{342}N, ^{476}N, ^{479}D, ^{494}R, ^{510}V, ^{522}S
		IND_2014-15	^{84}…^{87}, ^{191}…^{193}, ^{366}…^{368}, ^{447}…^{449}, ^{471}…^{473}, ^{541}…^{543}	^{1}…N……I..L..I..^{19}, ^{20}M, ^{28}Q, ^{32}I, ^{52}V, ^{69}M, ^{74}E, ^{108}T, ^{201}K, ^{226}T, ^{265}G, ^{282}L, ^{342}D, ^{476}S, ^{479}N, ^{494}K, ^{510}I, ^{522}A
		IND_2013	^{84}…^{87}, ^{191}…^{193}, ^{366}…^{368}, ^{447}…^{449}, ^{471}…^{473}, ^{541}…^{543}	^{1}……G……^{19}, ^{20}M, ^{28}L, ^{32}L, ^{52}I, ^{69}V, ^{74}E, ^{108}T, ^{201}K, ^{226}T, ^{265}S, ^{282}I, ^{342}N, ^{476}S, ^{479}D, ^{494}R, ^{510}I, ^{522}A
HN	Hydrophobic signal anchor (^{25}FRIAVLLLMIMILAISAAAL^{44}), Hexapeptide motif (^{234}NRKSCS^{239}), Haemagglutinin active motif-I (^{314}FPVYGGL^{320}), Haemagglutinin active motif-II (^{399}GAEGRI/V^{404})	Human originated	^{119}NNS^{121}, ^{341}NNT^{343}, ^{433}NRT^{435}, ^{508}NIS^{510}	^{25}……..^{44}, ^{49}R, ^{54}H, ^{57}T, ^{69}R, ^{77}G, ^{84}I, ^{165}T, ^{259}D, ^{290}I, ^{310}D, ^{347}D, ^{353}Q, ^{432}H, ^{434}R, ^{479}H, ^{508}N, ^{522}I, ^{540}A, ^{569}V
		Pakistan_2010	^{119}...^{121}, ^{341}…^{343}, ^{433}.K.^{435}, ^{508}S..^{510}	^{25}……..^{44}, ^{49}G, ^{54}H, ^{57}T, ^{69}K, ^{77}S, ^{84}I, ^{165}A, ^{259}D, ^{290}I, ^{310}D, ^{347}D, ^{353}Q, ^{432}H, ^{434}K, ^{479}H, ^{508}S, ^{522}T, ^{540}V, ^{569}V
		IND_2014-15	^{119}...^{121}, ^{341}…^{343}, ^{433}.K.^{435}, ^{508}S..^{510}	^{25}…A..L.I.....I.V...^{44}, ^{49}R, ^{54}R, ^{57}A, ^{69}K, ^{77}S, ^{84}I, ^{165}A, ^{259}E, ^{290}V, ^{310}D, ^{347}E, ^{353}R, ^{432}Y, ^{434}K, ^{479}H, ^{508}S, ^{540}V, ^{569}V
		IND_2013	^{119}...^{121}, ^{341}…^{343}, ^{433}.K.^{435}, ^{508}…^{510}	^{25}..I……..I…..T..^{44}, ^{49}R, ^{54}R, ^{57}A, ^{69}K, ^{77}G, ^{84}V, ^{165}A, ^{259}E, ^{290}V, ^{310}G, ^{347}E, ^{353}R, ^{432}Y, ^{434}K, ^{479}Y, ^{508}N, ^{522}T, ^{540}A, ^{569}D
L	Domain interact with P protein (^{13}IILPESHLSSPLV^{25}), Domain-Ⅰ (^{637}FITTDLQYCLNWRYQT^{653}), Domain-Ⅱ (^{709}YIVSARGGIEGL CQKCWTMISIAAI^{733}), Domain-Ⅲ (^{746}CMVQGDNQVIAVTR^{759}), Domain-Ⅳ (^{816}KDGAILSQVLKNSSKL^{831}), ATP binding motif (K21AXGXG), Polymerase associated motif in domain-Ⅲ (^{749}QGDNQ^{753})	Human originated	^{259}NLS^{261}, ^{463}NLS^{465}, ^{827}NSS^{829}, ^{1083}NYS^{1085}, ^{1122}NRS^{1124}, ^{1248}NIS^{1250}, ^{1899}NLS^{1901}, ^{2091}NLS^{2093}	^{59}L, ^{103}V, ^{222}G, ^{259}N, ^{281}Q, ^{329}N, ^{341}I, ^{346}G, ^{457}E, ^{514}D, ^{613}N, ^{621}S, ^{882}A, ^{947}A, ^{1003}V, ^{1159}R, ^{1387}G, ^{1461}I, ^{1523}S, ^{1550}V, ^{1712}K, ^{1938}V, ^{2004}Q, ^{2056}L, ^{2068}V, ^{2107}K, ^{2120}Q, ^{2124}R, ^{2172}K, ^{2202}S
		Pakistan_2010	^{259}…^{261}, ^{463}…^{465}, ^{827}…^{829}, ^{1083}…^{1085}, ^{1122}…^{1124}, ^{1248}…^{1250}, ^{1899}…^{1901}, ^{2091}…^{2093}	^{59}L, ^{103}V, ^{222}N, ^{259}S, ^{281}R, ^{329}D, ^{341}V, ^{346}E, ^{457}E, ^{514}D, ^{613}D, ^{621}N, ^{882}S, ^{947}A, ^{1003}V, ^{1159}K, ^{1387}R, ^{1461}V, ^{1523}S, ^{1550}V, ^{1712}K, ^{1938}I, ^{2004}R, ^{2056}M, ^{2068}V, ^{2107}R, ^{2120}R, ^{2124}K, ^{2172}T, ^{2202}N
		IND_2014-15	^{259}…^{261}, ^{463}…^{465}, ^{827}…^{829}, ^{1083}…^{1085}, ^{1122}…^{1124}, ^{1248}…^{1250}, ^{1899}.I.^{1901}, ^{2091}…^{2093}	^{59}I, ^{103}V, ^{222}D, ^{259}D, ^{281}R, ^{329}D, ^{341}V, ^{346}E, ^{457}E, ^{514}N, ^{613}D, ^{621}N, ^{882}S, ^{947}T, ^{1003}I, ^{1159}K, ^{1387}R, ^{1461}V, ^{1523}N, ^{1550}I, ^{1712}S, ^{1938}T, ^{2004}R, ^{2056}M, ^{2068}I, ^{2107}R, ^{2120}R, ^{2124}K, ^{2172}T, ^{2202}N
		IND_2013	^{259}…^{261}, ^{463}…^{465}, ^{827}…^{829}, ^{1083}…^{1085}, ^{1122}…^{1124}, ^{1248}…^{1250}, ^{1899}…^{1901}, ^{2091}…^{2093}	^{59}L, ^{103}I, ^{222}D, ^{259}S, ^{281}Q, ^{329}D, ^{341}V, ^{346}G, ^{457}D, ^{514}D, ^{613}N, ^{621}N, ^{882}S, ^{947}A, ^{1003}I, ^{1159}K, ^{1387}R, ^{1461}V, ^{1523}S, ^{1550}I, ^{1712}N, ^{1938}T, ^{2004}R, ^{2056}M, ^{2068}I, ^{2107}R, ^{2120}R, ^{2124}R, ^{2172}T, ^{2202}N

3.4. Comparative genomic and residue characterization

All six coding genes of the human-originated AOAVs shared the same nucleotide length with avian-originated AOAVs from genotype ⅩⅢ. However, a 7 nt insertion (PK2) and 20 nt deletions (PK3) were observed in the non-coding region between *NP* and *P* genes whereas, all three human isolates contained a 3 nt insertion in the non-coding region between *HN* and *L* genes (Table 3). Based on predicted amino acid sequences, the cleavage activation site of the fusion protein from the human isolates was found to be ^{112}RRQKR ↓ F^{117}, consistent with velogenic strains of AOAV-1. The combination of these findings and the time until death in embryonated eggs indicate that these isolates are velogenic strains.

Amino acid residue analysis of all coding genes revealed conservation of most of the functional motifs among classical genotype ⅩⅢ AOAVs strains originating from avian hosts and the human originated AOAVs isolates (Table 4). A few amino acid substitutions were found in two motifs, the signal peptide motif in the F protein and the hydrophobic anchor region in the HN protein; however, variation was also observed in these motifs within the avian AOAV-1 strains. The number of glycosylation sites in all coding genes (3 in *NP*, 2 in *P*, 3 in *M*, 6 in *F*, 4 in *HN* and 8 in *L*) was conserved except in the *HN* coding sequence of a Pakistani strain isolated in 2010 and another Indian strain isolated in 2015, where only three glycosylation sites existed due to substitution at position 508 (N508S). Besides, a negligible substitution (R434K) was identified in one glycosylation site of the HN protein (^{433}NRT^{435}) in all avian-originated AOAVs as compared to human-originated AOAVs. A total of 28 single amino acid substitutions were exclusive to human-originated AOAV-1 strains as compared to avian-originated classical AOAV-1 strains isolated from Pakistan and India.

3.5. Detection of potential recombination events

SimPlot showed similarities in non-coding intergenic regions among selected strains. No potential recombination events for the human isolates were found by detecting putative recombination events or breakpoints integrated shown in RDP4 software (data not shown).

4. Discussion

The present report on AOAV-1 infection in humans provides evidence of the zoonotic potential as well as a continued evolution among genotype ⅩⅢ viruses. The study not only identifies a deficiency in the M-gene-based screening assay used for the initial identification of AOAV-1 but also signifies the importance of advanced sequencing tools (*e.g.* next-generation sequencing) and associated bioinformatics pipelines for the identification of other unknown or unleashed viruses of public health significance.

Based on evolutionary distance and phylogenetic analyses, the three human AOAV-1 isolates clustered together with avian originated strain from Pakistan. The study isolates had an average nucleotide distance of 7.9% (ⅩⅢ.1.1), 6.0% (ⅩⅢ.1.2), 2.8% (ⅩⅢ.2.1), and 6.1% (ⅩⅢ.2.2) and therefore was classified within sub-genotype ⅩⅢ.2.1 as per recently proposed classification criteria[24]. Historically, the most ancestral strain of genotype ⅩⅢ AOAV-1 was firstly isolated from a cockatoo in India in 1982. Over a period of time, the virus is believed to have an ongoing evolution and this is fairly evidenced by the identification and classification of variants or sub-genotype within genotype ⅩⅢ from countries including Iran[21], Pakistan[25], Bangladesh[22], and India[23]. It is important to indicate that the previous viruses of genotype ⅩⅢ have exclusively been reported from multiple avian hosts; nevertheless, to the best of our knowledge, this is the first evidence of isolation, identification, and genomic characterization of genotype ⅩⅢ (ⅩⅢ.2.1) viruses among human in close contact with poultry.

The isolates encoded typical cleavage motif of the F protein and exhibited mean time-to-death in embryonated chicken eggs, consistent with the definition of velogenic virulent AOAV-1 strains. Theoretically, virulent viruses need only a short period of time for replication in a new environment and therefore demonstrate accelerated rates of adaptation[26,27]. This aspect could be correlated with the zoonotic potential of these velogenic strains for causing infection in humans. In addition, some substitutions were exclusively observed in human-originated strains, highlighting the capability of AOAV-1 as a mutable RNA virus for host adaptation and survival of fitness in the new environment. In fact, a continuous accumulation of several point mutations leading to amino acid substitutions combined with host immune pressure drives the evolution of AOAV-1 and may contribute to the emergence of novel virulent strains with a potentially altered host tropism[22-24]. Notably, several nucleotide substitutions in the functional motifs of the F protein signal peptide and HN protein hydrophobic signal anchor were observed in the human isolates, similar to a fatal human AOAV-1 isolate[4]. This is important because F protein mediates fusion between the viral lipid membrane and the host cell membrane, and the HN protein promotes fusion between the two membranes through interaction with the F protein. Therefore, further investigation of the effect of these substitutions on viral attachment and/or replication of these strains in human and avian cell types is warranted.

Previous studies have demonstrated the ability of AOAV-1 to replicate in hamsters, rats, guinea pigs, cattle, swine, sheep, and non-human primates[28,29]. When passaged intracerebrally in hamsters, a virulent strain of AOAV-1 was able to infect hamsters by multiple routes and cause disease in mice, sheep, cattle, and rhesus macaques[30-32]. Together, these findings in the past decades support the possibility for AOAV-1 to adapt to a mammalian host. Indeed, the use of AOAV-1 as a vaccine vector and oncolytic

therapy in humans and other mammals shows the reality of this potential[33]. Until the last decade, only one natural infection of a non-avian livestock animal with AOAV-1 had been described in a bovine calf[30]. Since 2009, increasing numbers of natural infection of mammals with AOAV-1 have been described, including healthy sheep and swine demonstrating deadly respiratory symptoms, and mink with encephalitis[5,31,34]. Notably, the more recent isolations from mammals have been associated with the disease, and viruses isolated from mink and cattle were velogenic strains.

Human infection with AOAV-1 was first described in 1943 by Burnet and others following an accidental ocular exposure of a laboratory worker to a vaccine strain of AOAV-1. Since then, additional laboratory exposures, as well as occupational exposure in commercial poultry workers have produced clinical symptoms mainly of conjunctivitis. Two cases of deep respiratory infection with AOAV-1 have been implicated as the cause of death in immuno-compromised humans[4], highlighting the zoonotic potential of AOAV-1 in susceptible individuals. Evidence of AOAV-1 causing upper respiratory symptoms in humans is little[35-37] and has not been described in detail till now. In the present study, we sequenced and genetically characterized three virulent AOAV-1 isolates from one individual with mild upper respiratory symptoms and from two individuals demonstrating no symptoms and reporting no recent respiratory symptom. To our knowledge, this is the first report of a velogenic AOAV-1 isolate from natural infection of the human upper respiratory tract.

Evidence for the ability of AOAV-1 to contribute to serious illness and death in humans is rare, but it has repeatedly been suggested that this virus should be included as a suspect in the inexplicable respiratory disease of non-avian species[4,5,31]. The new evidence presented in this study further highlights the need for further investigation of AOAV-1 evolution in mammals to investigate its zoonotic potential. Monitoring the incidence of AOAV-1 and its genetic diversity in humans may prove to be an important tool in predicting or preventing the adaptation of AOAV-1 to not only human lungs but also in other mammals as suggested in previous studies[31]. The ability to adapt to these diverse hosts raises concerns that AOAV-1 replicating in mammals, including humans, may acquire the ability to spread between mammals of the same species and/or cause more severe morbidity than historically observed. Indeed, the AOAV-1 isolated from a patient with fatal respiratory failure led to severe pneumonia when experimentally inoculated to cynomolgus macaques[32]. Continued surveillance of AOAV-1 in mammals, especially humans who are likely to contract the virus, is advisable to be prepared for possible epizootics. In addition, studies aimed at understanding the pathogenesis of AOAV-1 in mammalian hosts, including non-human primates[32], should be further pursued to better understand the potential for AOAVs to cause mild or severe infection in healthy individuals and immuno-compromised patients, respectively.

We characterized three human-originated velogenic AOAV-1 strains and found them closely related to those strains isolated from avian species within the same geography. The highest genetic homology of viruses from different origins (human and avian species) raised the concern towards the adaptation of novel host with implications of its zoonotic potential. It may suggest that the evolutionary dynamics of the virus occur naturally to adapt to the environment or a new host or perhaps confer virulent viruses with additional evolutionary advantages to allow persistence in nature.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

SVK and MZS designed the study. MZS, MR, AR, AA performed virus isolation and genome sequencing. RHN, LL, SKC, AS, IA, BMG analyzed the data. SVK, RHN and MZS wrote the initial draft manuscript all authors revised the manuscript.

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