Biomedical Letters ISSN 2410-955X– An International Biannually Journal

Research Article
2019 | Volume 5 | issue 1 | Pages 33-40

ARTICLE INFO

Received
September 01, 2019
Revised
October 12, 2019
Accepted
October 21, 2019

***Corresponding Author**
Naila Siddique
E-mail
naila.siddique@gmail.com

Keywords
Avian influenza
Backyard poultry
Non-vaccinated
Pakistan
Seroconversion

How to Cite
Sharif A, Siddique N, Abbas MA, Rafique S, Farooq S, Nawaz R. Surveillance of low pathogenic Avian Influenza Virus among non-vaccinated birds in Pakistan. Biomedical Letters 2019; 5(1):33-40.

Open Access

Surveillance of low pathogenic Avian Influenza Virus among non-vaccinated birds in Pakistan

Adeela Sharif[1,2], Naila Siddique[1,2*], Muhammad Athar Abbas[1,2], Saba Rafique[1], Saba Farooq[1], Rabia Nawaz[1]

[1]National Reference Lab for Poultry Diseases, Animal Health Program, National Agricultural Research Centre, Islamabad-44000, Pakistan
[2]PARC Institute of Advanced Studies in Agriculture, Islamabad-44000, Pakistan

Abstract

Influenza viruses from wild birds can be a reason of outbreak in commercial poultry and upon exposure it may also infect humans. Extensive surveillance is required to understand the routes of transmission of virus and circulating virus subtype. In the present study the prevalence, seroconversion and biological characterization of low pathogenic Avian Influenza viruses (LPAIV) were investigated from Non-vaccinated poultry birds from different areas of Pakistan during 2014. Out of 499 serum samples seroconversion was recorded in 206 samples. During investigation out of 797 swab samples, 55 isolations through *in ovo* inoculation and 68 detections of AIV H9 via PCR were recorded. Biological characterization of randomly selected isolates revealed that all the inoculated isolates were low pathogenic. Conclusively, wild migrant birds have a significant role in the dissemination of AIVs and the backyard poultry act as an intermediate host in spreading of infection to commercial poultry. Apart from detecting LPAIV from Non-vaccinated poultry birds, the PCR was able to detect higher number of AIV which would otherwise have been neglected by routine lab methods or falsely diagnosed and treated undesirably.

Scan QR code to see this publication on your mobile device.

Introduction

Influenza viruses contain three genera, A, B, and C within the Orthomyxoviridae family. Influenza virus A is economically significant and major cause of infection in fowls and rarely distress horses, swine and humans [1]. Avian influenza viruses (AIVs) can produce a series of signs from mild illness to severe problems i.e., disturbance in egg laying mechanism or low egg production and respirational problems that finally leads to systemic disease having almost 100% mortality rates. Genetic features and virulence of infection in fowls decides either the virus will be categorized as highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI). Low pathogenic avian influenza is an infection of fowls produced by any subtype of avian influenza virus with an intravenous pathogenicity index (IVPI) less than 1.2 [2]. Avian influenza has been described a major disease of birds with zoonotic importance and may lead to mortality in humans [3].

Localized virus replication is responsible for causing various symptoms like coughing, sneezing, lethargy and diarrhea. Some LPAI epidemics especially H1, H3, H5, H6, H7 and H9 may be responsible for more severe signs like pulmonary congestion, dyspnea, and inflamed sinuses, nasal discharge and lacrimation particularly if opportunistic pathogens are involved [4]. Avian influenza (AI) and Newcastle disease (ND) are two most significant zoonotic viral disease of fowls all over the world. Both viruses mostly exhibit similar signs that must be differentially diagnosed. Newcastle disease viruses (NDVs) and avian influenza viruses (AIVs) are commonly isolated from wild duck population. Differential diagnosis of influenza illness from other pathological conditions can be very difficult [5, 6]. Avian influenza virus can be diagnosed by different techniques used in laboratories either by virus isolation/identification or serological methods [7, 8].

In serological tests a diagnostic tool Enzyme-linked immunosorbent assays (ELISA) have been used to detect antibodies against influenza A. Virus isolation (VI) in embryonated eggs of chicken and proceeding HA and NA subtyping by serological methods represent the paragon for AIV detection and subtype recognition. Over the last decade, PCR has enabled the rapid and accurate detection of many pathogens surprisingly detecting the very low pathogen counts [9, 10]. The fastness, speed and sensitivity of this molecular indicative tool has been considered as clinically advantageous for devising a positive diagnosis and preventing disease spread [11]. The molecular diagnostic technique provides fast and more reliable results than usual time consuming and laborious laboratory methods. The RT-PCR assay is much quicker and less labor-intensive than conventional methods used for laboratory diagnosis, such as virus isolation followed by subtype determination using hemagglutination inhibition (HI), immunofluorescence staining, and enzyme-linked immunosorbent assays [12]. Avian influenza is an important zoonotic infection and a massive threat to the poultry industry so there is a need of systematic sampling and rapid detection of infected birds to control and eradicate contagious avian viral diseases. The aim of the study was to explore the key sources that play a major role in the dissemination of AIV in commercial poultry across the different regions of Pakistan. A study conducted by Khawaja et al., 2005 to monitor the prevalence of AIV in wild birds in certain areas of the Pakistan, which were free from infection during the outbreak of November 2003. The results of that study indicated that antibodies to AIV serotype H9N2 were present in 10% of wild birds, whereas the virus was itself isolated only from 6.72% of the samples. The data provided the evidence regarding the wild birds as one of the major carrier of the AIV infection [13].

Materials and methods

Source and collection of specimens

Samples for avian influenza surveillance included sera, swabs and tissues, collected from different bird types including commercial poultry, backyard poultry and wild birds (captive and migratory). From commercial poultry samples were only collected from non- vaccinated broilers. However, backyard poultry included the samples of household birds like desi, golden and fayoumi bird types. While the migratory birds included jungle fowl, red jungle fowl and water fowl and the wild domestic fancy birds included duck, pigeon, turkey, pheasant, peacock, peafowl, partridges and eagle. Out of total 1011 samples 783 (77.4%) were received from commercial poultry, 163 (16.1%) from backyard, 45 (4.45%) domestic fancy birds and 20 (2%) from wild migratory birds were included in the study. Tissues, Swabs and Serum samples were collected for evaluation from various poultry populated areas of four provinces (Punjab, Sindh, Khyber Pakhtunkhwa and Baluchistan) along with Islamabad Capital Territory. Out of total 1011 samples 520 (52%) were collected from KPK, 236 (23%) from Punjab province, 119 (12%) from

Baluchistan, from Sindh the number of collected samples was 115 (11%) while from ICT minimum number of samples was received; 21 (2%).

Virus isolation

Tissues were processed and blended to prepare a 20% suspension in PBS (pH 7.2) solution containing Penicillin (2×10 IU/L) Gentamycin (2 ×10 IU/L) and Streptomycin (200 mg/L. The whole material was centrifuged at 2000 rpm for 10 min at 10°C. The supernatant was filtered through 0.2µm syringe filter (Biotech) and inoculated into 9-days-old embryonated chicken eggs. The eggs were incubated at 37°C for 2 days. The centrifuged material was also used for detection through PCR. The Cloacal swabs were placed in the Glycerol viral transport medium. The tubes containing swabs were vortexed, centrifuged at 2000 rpm and the supernatant was also processed for egg inoculation. After incubation of 2 to 3 days, the eggs were chilled for 3 to 4 hours and then the allantoic fluid was harvested for test through Hemagglutination (HA) activity. Hemagglutination positive allantoic fluids were additionally tested using reference antisera of NDV and AIV subtypes (H1N1, H3N1 H7N3 and H9N2) by using standard protocols of Hemagglutination Inhibition test as described in literature [14].

RNA Extraction

RNA extraction was performed using QIAamp Viral RNA mini Kit according to manufacturer's instructions (QIAamp Viral RNA mini kit, QIAGEN, USA, CAT# 52906).

RT-PCR

A one step RT-PCR protocol was used to test the samples through Invitrogen SuperScript™ One step RT-PCR kit following the manufacturer's instructions.

The reaction mixture contained the AIV H9 specific primers along with other reagents from the Kit and it was run in a thermal cycler (Veriti, Applied Biosystems) using the temperature profile shown in (**Table 2**).

The amplified product was run on an agarose gel along with standard DNA markers at 100 V for 1 to 1.5 h as described by Sambrook et al. (1989). To verify the specificity of RT-PCR for AIV subtypes detection and NDV, specific primers were used. For this purpose, the type specific primers for each virus serotype were used in the study (**Table 1**).

Table 1: Specific primers used for PCR against AIV H9, H7, H3, H1 and NDV

Name/ID of virus	Sequence of forward primer	Sequence of reverse primer
Matrix Gene	GATGGTAGAGTATGAAAGATG	GAAACATGGTAGTTTGCCTATC
AIV [a] (H9 serotype)	ATGACACAATCAGGAATGTACC	TCAGGCGGCTTGCACAAT
AIV (H7 Serotype)	ATYAAYMSYAGRRCWGTRGG	GATCWATTGCHGAYTGRGTG
AIV (H3 Serotype)	GYATYACTCCWAATGGAAGC	ATTCTYCCTTCYACTTCDGA
AIV (H1 Serotype)	YDTCGATGCTCCRGTYCAY	TGYTCYTTRCCYACYGCWGTG
NDV [b] (Lasota)	CGAGGTTGTTGGCAGCAAA	GACTTCATATACACCTCATG

Note: a AIV, Avian Influenza Virus, **b** NDV, Newcastle Disease Virus.

Real Time PCR (q RT-PCR)

q RT-PCR was performed using Invitrogen Superscript TM III PlatinumR One Step Quantitative RT-PCR system Cat. No. 11732-020. The previously extracted RNA was used to carry out the procedure. The following procedure was carried out by using selected primers (**Table 3**) standardized protocol (**Table 4**).

Biological Characterization through Intravenous Pathogenicity Index (IVPI)

The biological characterization of AIV was carried out to evaluate the pathogenicity of virus, for this purpose 30 isolates were selected exhibiting HA activity. To observe the virulence and pathogenic potential of selected avian influenza (AI) viruses IVPI was

conducted using the standard protocols described in previous studies [15].

Table 2: Temperature Profile for one step RT-PCR

No. of cycles	Step	Temperature	Time
1	1	45°C	25 min
1	2	94°C	2 min
40	3	94°C	15 sec
	4	58°C	30 sec
	5	78°C	1 min
1	6	72°C	20 min
1	7	4°C	

Table 3: Specific primers used for Real Time PCR against AIV H9

Name/ID of virus	Matrix Gene
Sequence of forward primer	AAATGAAGACTTCTGACGAGGTCG
Sequence of reverse primer	TGGAAAAAACATTCCCTGAA
Probe	FAM-TGAAAGCCCTATAGGCGCA-TAMARA

Table 4: Temperature Profile for one step q RT-PCR

No. of cycles	Step	Temperature	Time
1	1	50°C	15 min
1	2	95°C	2 min
40	3	95°C	15 sec
	4	60°C	30 sec

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed using IDEXX AIV ELISA Test Kits. The serum samples were stored at 4ºC and in case of whole clotted blood to separate sera from blood, the samples were centrifuged at 2000 rpm for 10 minutes at 10ºC in refrigerated centrifuge machine according to the procedure in literature [15].

Results

Avian Influenza Virus (AIV) subtype H9N2 was isolated from the samples through *in ovo* inoculation. Total 55 isolates were recovered out of 797 swabs and tissue samples through *in ovo* inoculation. Positive samples were subjected to HI test for AIV subtype confirmation (i.e. AIV H9, H7, H3 and H1). The isolates were also cross checked for New castle disease virus (NDV), results revealed that all were negative for AIV H7, H3 and H1 and also there was no co infection of NDV. 68 samples were found positive for Matrix-gene through PCR and all were confirmed as subtype AIV H9. All the samples were found negative for AIV H7, AIV H3, AIV H1 and NDV through RT-PCR. The specific primer's base pair pattern is shown in **Fig.1**.

Out of 68 detections through RT-PCR the maximum detections 29 (43%) were during the summer season (June – August) while in case of total 55 isolations the maximum number 26 (47%) was also in summer season. The highest number of detections were from KPK and minimum detections were recorded from ICT and Baluchistan. In case of bird types, the maximum detections were recovered from Commercial poultry. In case of 20 randomly selected samples 15 were positive for matrix gene through qRT-PCR while 10 were positive by RT- PCR. Intravenous Pathogenic Index (IVPI) values ranging from 0.40 – 0.78 shown that all the selected isolates were Low pathogenic causing mild clinical signs in experimental birds.

Different techniques were also compared for their sensitive and specific detection, results revealed that PCR technique (8.53%) was more sensitive and specific than in-ovo inoculation technique (7%) in terms of high number of detections **Fig. 2** from 206 ELISA positive sera the maximum 108 (52%) Sero conversion was recorded in winter season and the highest Sero prevalence was observed in KPK 64 (31%) (**Table 5**). The ELISA positive samples were further subjected to HI for AIV subtypes detection and their antibody titres (MT log 2). Area wise Seroconversion given in **Table 6**.

Table 5: Area wise AIV ELISA positive samples

Province		**Punjab**			**KPK**				**Sindh**	**Baluchistan**	**ICT**	**Total**
Cities	**Rajanpur**	**Lahore**	**Rawalpindi**	**Taunsa Sharif**	**Peshawar**	**Abbottabad**	**Malakand**	**Mansehra**	**Karachi**	**Quetta**	**Islamabad**	
Positive samples	21	7	3	16	15	24	13	12	58	25	12	206
Total		47				64			58	25	12	

Table 6: Area wise sero-conversion recorded against avian influenza in MTlog2 against AIV H1, H3, H7 and AIV H9.

Ecological zones	Sero conversion recorded against AIVs MT log			
	H1	H3	H7	H9
Punjab	0.00 – 1.33	0.00 – 3.45	0.00 – 1.87	3.66 – 10.75
Sindh	0.00 – 1.66	0.00 – 2.96	0.00 – 1.45	5.33 – 8.50
KPK	0.00 – 1.00	0.00 – 4.00	0.00 – 1.46	4.86 – 11.00
Baluchistan	0.00 – 1.56	0.00 – 2.33	0.00 – 1.00	5.66 – 11.30
ICT	0.00 – 1.46	0.00 – 2.50	0.00 – 1.31	4.00– 7.50

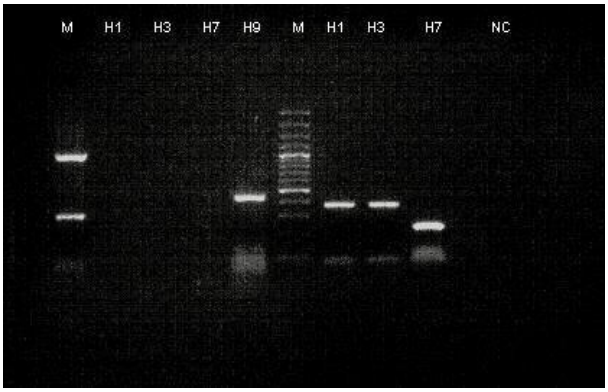

Fig. 1: Agarose Gel Electrophoresis of RT-PCR amplified AIV H9N2, Lane 1: Matrix gene positive sample 1023bp, Lane 2: H9 positive sample 471bp, Lane 3: N2 positive sample 480bp, Lane 4: NDV negative sample, Lane 5: Marker 100bp DNA step ladder, Lane 6: Matrix gene positive control 1023bp, Lane 7: H9 positive control 471bp.

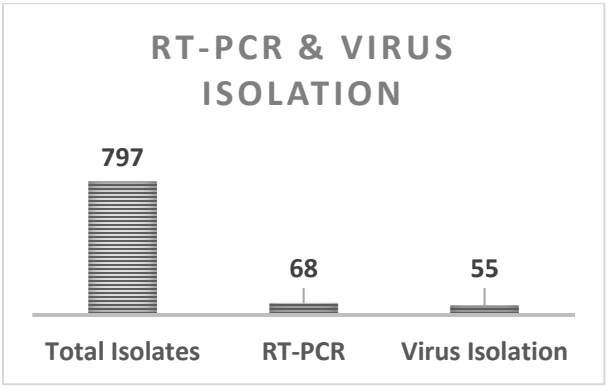

Fig. 2: Comparison of RT-PCR and Virus isolation techniques

Discussion

Avian influenza (AI) signifies one of the highest concerns for public health. It is a serious threat to the economy of poultry industry, proper vaccination and continuous surveillance are two main factors to control the disease [16]. The present study was conducted to monitor the Serosurveillance and biological characterization of LPAIV subtypes (AIV H1, H3, H7 & H9) in various non- vaccinated avian species in Pakistan. Total 1011 samples were received from different areas of Pakistan. Out of total 520 (52%) were collected from KPK, 236 (23%) from Punjab, 119 (12%) from Baluchistan, from Sindh 115 (11%) while from ICT only 21 (2%).

In spring season, the maximum number of sample 355 out of 1011 were collected, 277 in winter, 192 in autumn and 187 in the summer season. 783 (77.4%) were collected from commercial poultry, 163 (16.1%) from backyard, 45 (4.45%) captive wild birds and 20 (2%) from wild migratory birds. AIV subtype H9. During the year 2014, total 1011 samples (499 sera and 797 tissues & swabs) were collected from different regions of Pakistan. During this study 55 (7%) AIV H9 isolates were recovered from the tested samples. In the present study to the total of 55 AIVs isolations during the study year commercial poultry contributed 50 (91%), backyard poultry contributed 4 (7%) while the wild birds contributed 1 (2%).

According to the recent study the highest number (75%) of LP H9N2 isolations was recorded from the backyard poultry which shows that the LP H9N2 AIVs has not only been endemic in commercial poultry since 1999 but it has also been prevalent in domesticated poultry of Pakistan [17]. Current findings are in accordance with previous studies [18] [19]. In the current study out of 797 tissues and swabs the positive samples through *in ovo* inoculation technique 55 (7%) gave positive HA. In Hemagglutination Inhibition test AIV H9 was confirmed. All the isolates were negative for Newcastle disease virus. 7% virus isolations (VI) of AIV H9 were recorded during the study year while RT-PCR detections throughout the year was 68 (8.53%). The difference between the results of both techniques shown that PCR is more sensitive than in ovo inoculation technique. Similarly the study conducted by Siddique et al., 63% more samples were found H9N2 positive through RT- PCR that left negative after *in ovo* inoculation technique [20].

In present study out of 68 detections 53% were from the non-vaccinated commercial poultry (broiler), 7% from non- vaccinated wild birds and 40% were reported from backyard poultry during the whole year. In contrast to this study Sarwar, Muhammad [21] found the prevalence of AIV in the live bird markets of Lahore which showed only 0.012% AIV isolations were detected through PCR and VI. Another study narrated that only 1.4% samples were positive for LPAI and no isolation or detections of any HPAI was recorded in earlier study [22]. Conversely, Fereidouni, Werner [23] described the maximum number 88% of detections were from dabbling duck (wild bird). In the present study the maximum detections 29 (43%) were during the summer season (June – August) than 15 (22%) in spring (March - May), same in winter (December - February) and 9 (13.2%) in autumn (September - November).

In the study of Fereidouni, Werner [23] the highest incidence of AIV was in the month of February and November. Another study at Hazara region of Pakistan showed that the maximum number (47 %) of AIV H9 isolates were during the month of July followed by May and June (20 %). The incidence rate of disease prevalence is almost similar to the present study because it is the time period in Pakistan when there is increased humidity in atmosphere and little warmer climatic conditions that are more suitable conditions for disease occurrence. Seasonal prevalence reported in the current study also indicate the movement of wild migratory birds during those months [24-27].

In the current study the highest number of AIV positive detections were from KPK 34 (50 %), Punjab 29 (43%), Sindh 3 (4.4 %) and from Baluchistan and ICT 1 (1.50%). The present study was in line with the study conducted by Muhammad, Muhammad [24] the highest number of prevalence were recorded from different cities of province KPK 9.75 % from Abbottabad, 8.4 % from Mansehra and 6.8 % from Haripur. In the current study out of total samples 499 were sera sample that were subjected to ELISA for initial screening of antibodies against AIV. The number of positive sera was 206 (41%) across the year. A study conducted by Fereidouni et al., (2010) in Iran showed the results of seroprevalence against AIV was (35.5%) during the year 2003, (21%) in 2004 and (57.4%) in 2007.

Seroprevalence rate observed in present study was corroborating with seroprevalence rate year 2003. Another study conducted in Pakistan showed 40% AIV seroprevalence rate. The seroprevalence is almost in line with the prevalence rate of AIV [28] . In Iran Mohammadi, Masoudian [29] studied the sero monitoring of AIV H9 and reported the sero prevalence rate as 80%. Similarly another study reported by Nooruddin, Hossain [30] from Bangladesh shown 10% sero prevalence. According to the study conducted by Ghaniei, Allymehr [31] the percentage of AIV H9 positive sera was 40 %. The seroprevalence is almost similar to the present study. In the current study out of 206 positive samples 56 % from commercial poultry, 36% from backyard and 8% recorded in wild bird species.

More positive samples were from non-vaccinated commercial poultry followed by backyard and least from wild birds. According to the study conducted by Hadipour et al., in 2011 the seroprevalence of AIV H9 in backyard poultry was 62.9% and in ducks (wild birds) the seroprevalence was 78.4%. Fereidouni, Werner [23] documented the seroprevalence in water birds 48.5%. Similarly, it was reported that seroprevalence in broiler was 26% and in layer it was 23%. The season wise sero prevalence in the current study revealed that the maximum sero-conversion was recorded in winter season with 108 (52%) sera positive, followed by spring season 63 (31%), than in summer 21 (10%) and least in autumn 14 (7%) [32].

Excitingly, Munster, Baas [33] in 2007 reported the highest Seroconversion in autumn season and lowest in spring season in contrast to study. Sera with positive ELISA were further subjected to HI for antibody titer detection against specific subtype of AIV. The mean HI titres against AIV subtype H9 was 11.30. In the present study the biological characterization of LPAIV were also carried out to assess the virulence and severity of avian influenza viruses through Intravenous Pathogenicity Index (IVPI). The IVPI values ranging from 0.40 – 0.78. All the isolates were categorized as Low pathogenic avian influenza viruses.

Another study demonstrated that the H9N2 influenza virus developed intravenous pathogenicity after the introduction of a pair of dibasic amino acid residues into the cleavage site of the HA and then consecutively passaged in chicks [34]. The results shown that mutated (H9N2) killed 75% of chickens when inoculated intravenously. In the current study 797 tissues and swabs were also cross checked against Newcastle disease virus to differentially diagnose the acute respiratory pathogens. No co- infection was recorded as no sample was positive for NDV. According to the study of Mehrabanpour, Rahimian [35] the high percentage of respiratory pathogens (especially AIV and NDV) mostly found as co infection hence concluded that there was a strong need

of differential diagnosis ,continuous and strict monitoring and also proper vaccination schedule. The results of current study indicate the persistent exposure of the birds to low pathogenic avian influenza viruses so there is a need of vaccination program amongst the poultry birds. As the Avian influenza is an important zoonotic infection and it is a massive threat to the poultry industry hence there is a need of systematic sampling and rapid detection of infected birds to control and eradicate contagious avian viral diseases from the country.

Conclusions

The present study concluded that there is a persistent exposure of the birds to low pathogenic avian influenza viruses so there is a need of vaccination program, systematic sampling and rapid detection of infected birds to control and eradicate contagious avian viral diseases from the country. Molecular techniques are able to detect higher number of pathogens which would otherwise have been missed by routine lab methods or incorrectly identified and treated undesirably so, advanced and sensitive diagnostic techniques should be used for diagnosis of various respiratory pathogens instead of conventional methods.

Conflict of interest

Authors have no conflict of interest. No financial interest relating to the topic of this article have been declared.

Acknowledgement

This work was supported by National Reference Laboratory for poultry disease (NRLPD), NARC Islamabad. Pakistan

References

[1] Sangsiriwut K, Uiprasertkul M, Payungporn S, Auewarakul P, Ungchusak K, Chantratita W, et al. Complete Genomic Sequences of Highly Pathogenic H5N1 Avian Influenza Viruses Obtained Directly from Human Autopsy Specimens. Microbiol Resour Announc. 2018;7:e01498-18.

[2] Lee C-W, Saif YM. Avian influenza virus. Comparative immunology, microbiology and infectious diseases. 2009;32:301-10.

[3] van den Brand JM, Verhagen JH, Veldhuis Kroeze EJ, Van de Bildt MW, Bodewes R, Herfst S, et al. Wild ducks excrete highly pathogenic avian influenza virus H5N8 (2014–2015) without clinical or pathological evidence of disease. Emerging microbes & infections. 2018;7:1-10.

[4] Siddique AB, Rahman S, Hussain I, Muhammad G. Frequency distribution of opportunistic avian pathogens in respiratory distress cases of poultry. Pak Vet J. 2012;32:386-9.

[5] Ge J, Deng G, Wen Z, Tian G, Wang Y, Shi J, et al. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. Journal of virology. 2007;81:150-8.

[6] Waseem H, Jameel S, Ali J, Saleem Ur Rehman H, Tauseef I, Farooq U, et al. Contributions and challenges of high throughput qPCR for determining antimicrobial resistance in the environment: a critical review. Molecules. 2019;24:163.

[7] Alexander D. Avian influenza–diagnosis. Zoonoses and public health. 2008;55:16-23.

[8] Waseem H, Ali J, Sarwar F, Khan A, Rehman HSU, Choudri M, et al. Assessment of knowledge and attitude trends towards antimicrobial resistance (AMR) among the community members, pharmacists/pharmacy owners and physicians in district Sialkot, Pakistan. Antimicrobial Resistance & Infection Control. 2019;8:67.

[9] Lee M-S, Chang P-C, Shien J-H, Cheng M-C, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. Journal of virological methods. 2001;97:13-22.

[10] Ali J, Ali N, Wang L, Waseem H, Pan G. Revisiting the mechanistic pathways for bacterial mediated synthesis of noble metal nanoparticles. J Microbiol Methods. 2019.

[11] Phipps L, Essen S, Brown I. Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. Journal of virological methods. 2004;122:119-22.

[12] Chae W, Kim P, Hwang BJ, Seong BL. Universal monoclonal antibody-based influenza hemagglutinin quantitative enzyme-linked immunosorbent assay. Vaccine. 2019;37:1457-66.

[13] Khawaja J, Naeem K, Ahmed Z, Ahmad S. Surveillance of avian influenza viruses in wild birds in areas adjacent to epicenter of an outbreak in federal capital territory of Pakistan. Int J Poult Sci. 2005;4:39-43.

[14] Miller PD, de Silva TI, Leonard H, Anthias C, Hoschler K, Goddard K, et al. A comparison of viral microneutralization and haemagglutination inhibition assays as measures of seasonal inactivated influenza vaccine immunogenicity in the first year after reduced intensity conditioning, lymphocyte depleted allogeneic haematopoietic stem cell transplant. Vaccine. 2019;37:452-7.

[15] Liu K, Gu M, Hu S, Gao R, Li J, Shi L, et al. Genetic and biological characterization of three poultry-origin H5N6 avian influenza viruses with all internal genes from genotype S H9N2 viruses. Archives of virology. 2018;163:947-60.

[16] Awadin W, Said H, Abdin S, El-Sawak AA. Pathological and Molecular Studies on Avian Influenza Virus (H9N2) in Broilers. Asian Journal of Animal and Veterinary Advances. 2018;13:232-44.

[17] Naeem K, Ullah A, Manvell R, Alexander D. Avian influenza A subtype H9N2 in poultry in Pakistan. British Medical Journal Publishing Group; 1999.

[18] Aamir U, Wernery U, Ilyushina N, Webster R. Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology. 2007;361:45-55.

[19] Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine. 2007;25:5637-44.

[20] Siddique N, Naeem K, Ahmed Z, Malik SA. Evaluation of RT-PCR for the detection of influenza virus serotype H9N2 among broiler chickens in Pakistan. International Journal of Poultry Science. 2008;7:1122-7.

[21] Sarwar M, Muhammad K, Rabbani M, Younus M, Sarwar N, Ali M, et al. Prevalence of avian influenza viruses in live bird markets of Lahore. J Anim Plant Sci. 2013;23:388-92.

[22] Hansbro PM, Warner S, Tracey JP, Arzey KE, Selleck P, O'Riley K, et al. Surveillance and analysis of avian influenza viruses, Australia. Emerging Infect Dis. 2010;16:1896.
[23] Fereidouni SR, Werner O, Starick E, Beer M, Harder TC, Aghakhan M, et al. Avian influenza virus monitoring in wintering waterbirds in Iran, 2003-2007. Virology journal. 2010;7:43.
[24] Muhammad A, Muhammad S, Sarzamin K, Qureshi M, Naeem K, Muhammad R, et al. Prevalence of avian influenza and its economic impact on poultry population of Hazara region Pakistan. Sarhad Journal of Agriculture. 2010;26:629-33.
[25] Ali J, Hameed A, Ahmed S, Ali MI, Zainab S, Ali N. Role of catalytic protein and stabilising agents in the transformation of Ag ions to nanoparticles by Pseudomonas aeruginosa. IET nanobiotechnology. 2016;10:295-300.
[26] Ali J, Sohail A, Wang L, Rizwan Haider M, Mulk S, Pan G. Electro-Microbiology as a Promising Approach Towards Renewable Energy and Environmental Sustainability. Energies. 2018;11:1822.
[27] Ali J, Wang L, Waseem H, Sharif HMA, Djellabi R, Zhang C, et al. Bioelectrochemical recovery of silver from wastewater with sustainable power generation and its reuse for biofouling mitigation. Journal of Cleaner Production. 2019;235:1425e37.
[28] Subtain S, Chaudhry ZI, Anjum AA, Maqbool A, Sadique U. Study on pathogenesis of low pathogenic avian influenza virus H9 in broiler chickens. Pakistan Journal of Zoology. 2011;43.
[29] Mohammadi A, Masoudian M, Nemati Y, Seifi S. Serological and RT-PCR assays for detection of avian influenza of domestic pigeons in Kavar area (Fars province, Iran). Bulgarian Journal of Veterinary Medicine. 2010;13:117-21.
[30] Nooruddin G, Hossain M, Mohammad M, Rahman MM. Sero-epidemiology of avian influenza virus in native chicken in Bangladesh. Int J Poult Sci. 2006;5:1029-33.
[31] Ghaniei A, Allymehr M, Moradschendi A. Seroprevalence of avian influenza (H9N2) in broiler chickens in Northwest of Iran. Asian Pacific journal of tropical biomedicine. 2013;3:822-4.
[32] Verhagen JH, Lexmond P, Vuong O, Schutten M, Guldemeester J, Osterhaus AD, et al. Discordant detection of avian influenza virus subtypes in time and space between poultry and wild birds; Towards improvement of surveillance programs. PLoS One. 2017;12:e0173470.
[33] Munster VJ, Baas C, Lexmond P, Waldenström J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS pathogens. 2007;3:e61.
[34] Abolnik C. Molecular epidemiology of Newcastle disease and avian influenza in South Africa: University of Pretoria; 2007.
[35] Mehrabanpour M, Rahimian A, Shoshtari A, Fazel P, Kariminejhad E, Jula GRM. Molecular Identification of Avian Respiratory Viral Pathogens in Commercial Broiler Chicken Flocks with Respiratory Disease in Shiraz-Iran during 2009-2010. International Journal of Animal and Veterinary Advances. 2011;3:300-4.