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RESEARCH ARTICLE

Clinical Investigation and Molecular Prevalence of Fowl Adenoviruses of Commercial Poultry from Division Faisalabad, Pakistan

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A B S T R A C T

Fowl adenovirus (FAdV) associated diseases have emerged as major viral diseases in all types of poultry around the globe. These diseases have been re-emerging as outbreaks throughout Pakistan in recent years, therefore, the purpose of the current study was to conduct molecular epidemiology of Fowl Adenovirus in commercial poultry depending upon different variables. The study is based on n=675 farm samples (each sample represents organ collection of 5 birds per farm) from commercial poultry around division Faisalabad, Pakistan during years 2018- 2020. Type of chicken affected, age groups, shed types, seasons and regions were assumed as risk factors associated with the prevalence of FAdVs which were analyzed using non-parametric tests. For molecular studies, liver tissues were subjected to polymerase chain reaction (PCR) by targeting hexon gene. The current study showed higher prevalence in layer type chicken among commercial chicken types, younger birds showed higher FAdV prevalence due to possible vertical transmission and higher prevalence of FAdV infection was observed in commercial poultry kept in semi-environment control sheds due to inconsistent control over the biosecurity and internal environment of the shed. This study also reported shed type and season to be significantly associated ($P<0.05$) with risk of FAdV infection.

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INTRODUCTION

Fowl adenoviruses (FAdVs) are icosahedral, non-enveloped DNA viruses, known to cause a variety of diseases in both commercial and backyard poultry. There are chiefly five genera under family Adenoviridae: Ichtadenovirus, Siadenovirus, Mastadenovirus, Aviadenovirus and Atadenovirus (egg drop syndrome (EDS) (Harrach *et al.,* 2012). Since all fowl adenoviruses (FAdVs), known as traditional avian adenoviruses share a common group antigen, hence they are categorized as genus Aviadenovirus (Meulemans *et al.,* 2004). There are 12 serotypes of aviadenoviruses which infect the chicken categorized under the species as; fowl adenovirus A (FAdV-1), fowl adenovirus B (FAdV-5), fowl adenovirus C (FAdV-4 and 10), fowl adenovirus D (FAdV-2, 3, 9 and 11), and fowl adenovirus E (FAdV-6, 7, 8a and 8b) (Hess, 2000; Mittal *et al.,* 2014).

The most important diseases related to aviadenovirus (FAdVs) infection in chicken are the inclusion body hepatitis (IBH), gizzard erosions (GE) and the hydro-pericardium syndrome (HPS) (Domanska-Blicharz *et al.,* 2011). Many among the twelve serotypes of FAdV viruses have been correlated with the outbreaks of IBH. The typical IBH affects poultry birds of usually 3-5 weeks of age and demonstrates a minimum mortality rate of 10% in uncomplicated cases. However, the stress and immunosupression induced by diseases like Chicken infectious anemia (CIA), Infectious bursal disease (IBD), and aflatoxicosis increase the severity of hepatitis and consequently mortality rate in the flocks (Von Bülow *et al.,* 1986; Singh *et al.,* 1996; Naseem *et al*., 2018). Usually, HPS clinical disease is caused by FAdV-4, grossly described by increased volume of transparent to straw color pericardial fluid, hepatitis and nephritis with higher mortality ranging 30-70% (Kim *et al.,* 2008; Schachner *et al.,* 2014). Gizzard erosions (GE) is also reported to be induced by multiple serotypes of FAdVs and often observed at necropsy examination of broiler chickens (Marek *et al.,* 2010; Gjevre *et al.,* 2013).

Despite of suitable vaccination and other biosecurity measures adopted at poultry farms, FAdV outbreaks still have been reported in various regions of Pakistan and has led to serious losses of poultry farmers throughout Pakistan recently. In recent years, various serotypes of FAdVs have been reported from different regions of Pakistan at various times; FAdV-4 and FadV-8 in 2012 Khyber Pakhtunkhawa (KPK), Azad Jammu Kashmir and Punjab (Yasmeen *et al.*, 2017), FAdV4 and FAdV-11 in 2015 from Punjab (Wajid *et al.,* 2018), novel FAdV-11 isolate's whole genome sequence (WGS) from Faisalabad division (Wang *et al.,* 2020). Since there are scattered outbreak reports of various FAdV serotypes from various regions of Pakistan, therefore, the purpose of current study was to determine the factors influencing the prevalence of Fowl adenoviruses isolated from Faisalabad division of Punjab, Pakistan.

MATERIALS AND METHODS

Collection of samples: Simple random sampling technique was used to collect the samples from four districts of division Faisalabad of Punjab, Pakistan. Multistage sampling design with assumption of 50% prevalence and $z = 1.96$ and $d = 0.05$ (the desired level of precision or accuracy).

$$n = \frac{1.96^{2} \times P\,exp\,(1 - P\,exp)}{d^{2}}$$

The minimum required sample size was $n = 384$. Each sample represents 5 birds from each shed/ farm. However, during our study, total of 3375 samples were collected from 675 farms. The probable risk factors contributing to the prevalence of FAdVs; such as type of chicken affected, age group, shed type, seasons, and region which may attribute to the prevalence of FAdVs were studied.

Clinical and postmortem evaluation: The liver samples in zip bags were stored at -20ºC until further processing for PCR. The initial diagnosis for Inclusion Body Hepatitis or Hydro-pericardial syndrome was based on clinical manifestations and post-mortem findings (Mittal *et al.,* 2014).

Molecular detection for FAdVs: DNA extraction was done from the liver samples. By weight, 25 mg sample will be homogenized in 500 µl Phosphate Buffered Saline (PBS). The total purified DNA was harvested with a commercial kit by Thermo-scientific called GeneJET® Genomic DNA Purification kit (Lot #00786838). The extracted DNA of each sample was stored at -20ºC until further use for PCR and sequencing.

For the current study, PCR was performed in Applied biosystems® thermocycler (Model # 2720) to amplify the FAdV hexon gene including its L1 loop region. The reaction mixture consisted of; 12.5 µl of master mix (Dream Taq Green PCR Master mix (2X), Lot # 00869160 by Thermo Scientific®), 8.5 µl of nuclease free water (Lot # 00829481 by Thermo Scientific®), 2 µl of forward and reverse primers and 2 µl of the extracted DNA sample. The two primer sets used in this study are given as:

Primer sequences and their estimated product sizes were as follows:

Primers	Sequence	PCR Product	Reference
Hexon-A	5′-CAARTTCAGRCAGACGGT-3′	897 bp	(Meulemans *et al.,* 2001)
Hexon-B	5′-TAGTGATGMCGSGACATCAT-3′		
FAdF	5′-AACTTCGACCCCATGTCGCGTCAGG-3′	480 bp	(Pan *et al.,* 2017)
FAdR	5′-TGGCGAAAGGCGTACGGAAGTAAGC-3′		

For primer set Hexon A/B, the amplification conditions in thermocycler for 35 cycles were; denaturation of DNA at temperature; 94°C for 2 minutes; annealing at 60°C for 1 minutes and the extension at 72°C for 1.30 minutes. A final step of extension was also given at temperature; 72°C for 2 minutes. For primer set FAd F/R PCR was executed under the given thermal cycling conditions; initial denaturation at temperature of 95ºC for 5 minutes, progressed by 30 cycles of denaturation; 95ºC for 45 seconds, annealing; 56ºC for one minutes and extension; 72ºC for one minutes, completed by a final elongation of 10 minutes at 72ºC. The PCR amplification processes were stopped by dropping the temperature of thermocycler to 4°C.

Statistical analysis: The prevalence percentage was calculated by formula (Christley and Thursfield, 2018):

$$\text{Prevalence percentage} = \frac{\text{No.of PCR +ve samples for FAdV in a catagory}}{\text{Total no.of samples collected in a category}} \times 100$$

The epidemiological data collected has also been analyzed statistically by using a logistic regression model later calculating Odds Ratio using C.I as 95%. Pearson Chi-square correlation was calculated to determine the correlation of factors with FAdV infection with ($p \leq 0.05$).

RESULTS

Clinical and postmortem evaluation: Since this study involved random sampling technique, the clinical observations and postmortem findings had been quite varied throughout Faisalabad division over the period of 2018- 2020 (Fig. 1 and 2). The birds of PCR positive (Hexon L1 region) samples in some of the cases appeared clinically healthy with no gross lesions of internal organs. The clinical signs such as; depression, ruffled feathers, loss of skin turgor (dehydration), anorexia, respiratory distress and non-uniformity in the bird body weights of a flock were commonly observed to associated with FAdV infections (later confirmed with post-mortem examination and PCR testing of hexon gene). The mortality rate for FAdV-11 infected flocks ranged from 0-35%, while FAdV-4 infected flocks showed mortality range of 30-80%. The classic postmortem presentation of inclusion body hepatitis (IBH) alone was found in several PCR positive samples showed hepatomegaly and presentation of necrosis on liver surfaces of variable intensity. IBH, however, remained persistent findings in several samples that were accompanied with varying clinical signs and gross alterations. The accompanying lesions were; edematous lungs, nephritis, splenomegaly, or development of hydropericardium with misshapen heart (in FAdV-4 infections exclusively). Total 11 FAdV isolates (partial hexon cds) have been reported to NCBI gene bank from

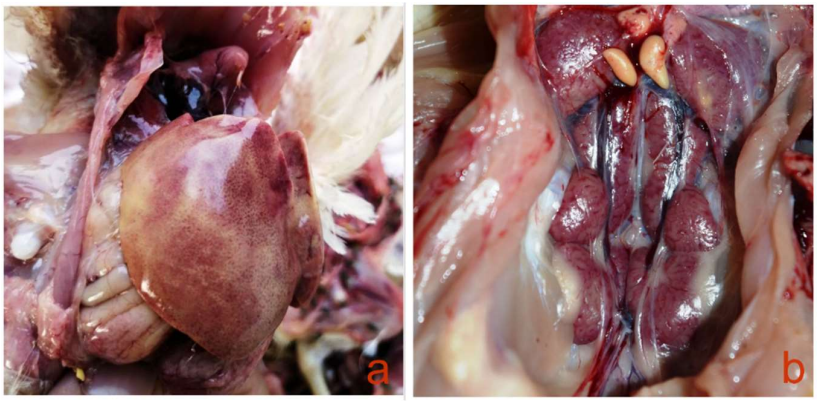

Fig. 1: Presents lesions associated with FAdV clinical infection (seen in both IBH and HPS), a) FAdV infected chicken showing necrotic foci on liver surface, b) Nephritis associated with FAdV infection.

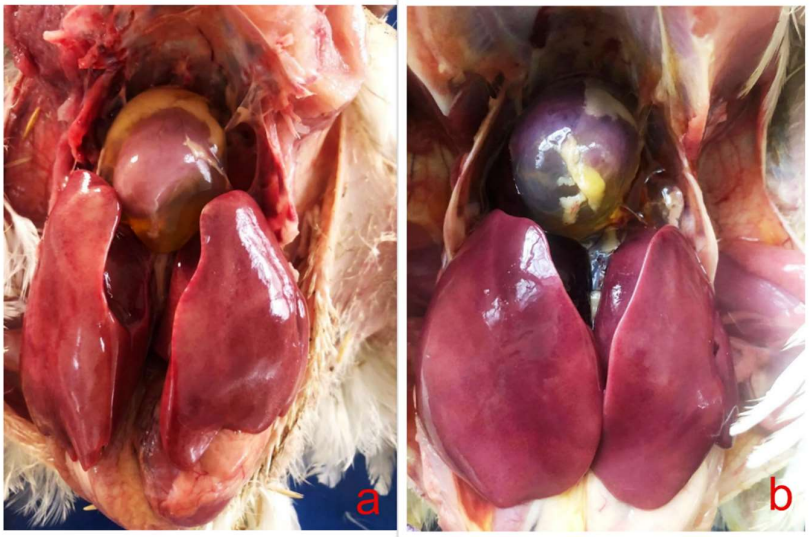

Fig. 2: Shows livers and heart infected with FAdV-4 infection, a) shows misshapen heart due to increased pericardial fluid, b) shows increased pericardial fluid around the heart.

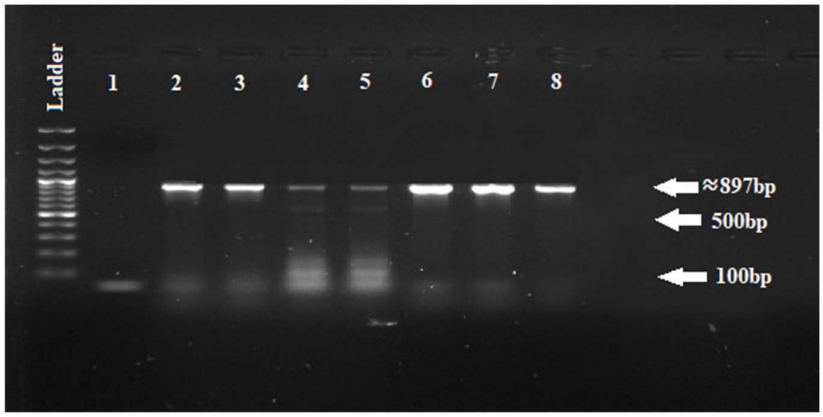

Fig. 3: Molecular identification of Adenoviruses amplified with set Hexon A/B (product size≈897bp , Ladder of 100bp, 1=negative control, 2-8 = samples

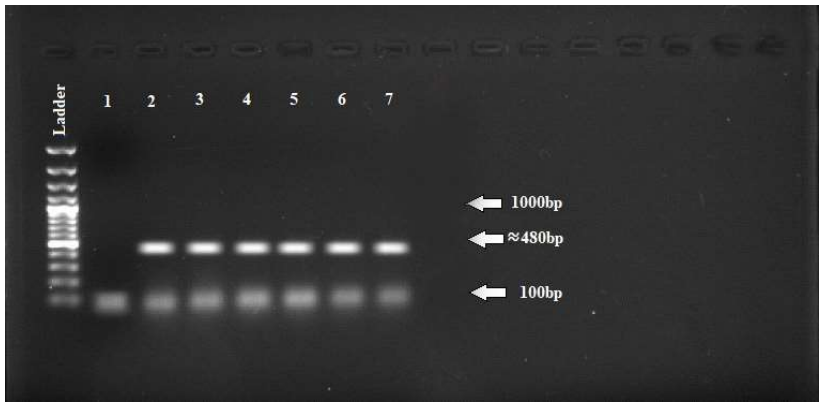

Fig. 4: Molecular identification of Adenoviruses amplified with set Fad F/R at ≈480bp Ladder of 100bp, 1=negative control, 2-7=Samples

Table 1: Chi square analysis of risk factors assumed to be associated with adenovirus of poultry

Factor	Level	PCR based Prevalence (%)	CI (95%)	*p*-value
District	Faisalabad	13.60	10.82-16.96	
	Toba Tek Sing	11.10	5.96-19.79	0.086
	Jhang	24.3	15.76-35.5	
	Chiniot	13.00	6.12-25.66	
Age groups (Broilers)	Week 1	21.57	12.49-34.63	0.409
	Week 2	11.76	6.83-19.44	
	Week 3	11.11	6.15-19.26	
	Week 4	15.79	9.27 -2.56	
	Week 5	10.96	5.66-20.16	
	Week 6	21.05	8.51-43.33	
Age groups (Layers)	Week 1-10	24.59	15.51-36.68	0.229
	Week 11-20	17.02	8.89-30.14	
	Week 21-30	6.06	1.68-19.61	
	Week 31-40	17.65	8.35-33.52	
	Week 41-50	16.67	4.7-44.81	
	Week 51-60	4.35	7.7-20.99	
	Week 61-70	16.67	3.01-56.35	
Age groups (Native chicken)	Week 1-10	14.82	5.91-32.47	0.862
	Week 11-20	0	0-56.15	
	Week 21-30	0	0-48.99	
	Week 31-40	0	0-65.76	
	Week 41-50	0	0-56.15	
	Week 51-60	16.67	3.01-56.35	
	Week 61-70	0	0-56.15	
Shed type	Open	14.43	8.11-21.14	0.031
	Semi	26.69	0-0	
	Control	12.84	10.13-16.15	
Type of floor (layers)	Floor	13.83	11-17.25	0.544
	Cage	15.61	11.28-21.21	
Bird type	Broiler	13.87	10.86-17.55	0.526
	Layer	16.20	11.88-2.17	
	Native	10.42	4.53-22.17	
Year	2018	10.11	10.56-23.79	0.095
	2019	16.67	13.37-20.59	
	2020	12.66	7.02-21.76	
Season	Summer	13.58	9.67-18.71	<0.000
	Autumn	13.11	6.79-2.38	
	Winter	4.11	2.18-7.62	
	Spring	28.74	22.53-35.87	

P≤0.05 indicate significant difference.

this study viz; FAdV-4 (MN754024.1), FAdV-11 (MN754018.1 to MN754023.1 and MW525217.1 to MW7525219.1). One FAdV-11 (PkFAd18/ MN428137.1) whole genome sequence has also been reported.

Prevalence of FAdV infection: During the study period (2018- 2020), 675 farms samples of division Faisalabad out of which 485 (14.37%) samples were PCR positive for Hexon L1 gene region of FAdV (Fig. 3 and 4). Within the division, district Jhang showed 24.3% PCR positive samples for FAdV infection which was the highest prevalence among all districts. District Faisalabad had 13.6%, Jhang Chiniot had 13.00%, and Toba Tek Singh had 11.10% PCR positive samples for Hexon L1 gene of FAdV (Table 1). Among the chicken types, the highest PCR based prevalence was recorded in layers (16.20%), while broilers showed 13.87% and native chickens showed only 10.42% PCR positive samples for FAdV. In year wise prevalence, the highest prevalence was recorded in 2019 as 16.67% commercial poultry were PCR positive for FAdVs infections. While the lowest prevalence of FAdV was 10.11%, which was recorded in 2018. In broilers, the highest prevalence of FAdV infection was observed within 1st week of age (21.57%) indicating vertical transmission. In layers, the highest prevalence of FAdV infection was observed within 1-10 weeks of age as 24.59% also indicating vertical transmission of infection. On the other hand, lowest prevalence was recorded in broilers in 5th week of age (10.96%) and layers of 51-60 week (4.35%). In native chicken, the highest prevalence of FAdV infection was observed within 51-60 weeks of age (16.67%). Whereas, none of sampled native chicken were PCR positive for FAdV or showed any clinical signs in age between 11- 50 weeks and 61-70 weeks. During current study, the highest prevalence of FAdV infection was observed in commercial poultry kept in semi-environment

Table 2: Binary logistic regression analysis for confirmation of risk factor of adenovirus infection in poultry

Factor	Level	Odd's ratio	*p*-value	CI (95%)	
				Lower	Upper
District	Faisalabad	0.491	0.021	0.268	0.899
	Toba Tek Sing	0.390	0.036	0.161	0.942
	Jhang (Reference category)	-	-	-	-
	Chiniot	0.468	0.143	0.169	1.293
Age groups (Broilers)	Week 1	2.234	0.112	0.828	6.027
	Week 2	1.083	0.869	0.419	2.801
	Week 3	1.016	0.975	0.379	2.721
	Week 4	1.523	0.390	0.584	3.974
	Week 5 (Reference category)	-	-	-	-
	Week 6	2.167	0.253	0.576	8.152
Age groups (Layers)	Week 7-10	3.21	0.161	0.619	17.82
	Week 11-20	3.100	0.288	0.385	24.952
	Week 21-30	3.100	0.390	0.235	40.895
	Week 31-40	0.705	0.780	0.060	8.262
	Week 41-50	5.054	0.040	1.079	23.673
	Week 51-60 (Reference category)	-	-	-	-
	Week 61-70	3.18	0.162	0.629	16.06
Shed type	Control	0.958	0.892	0.513	1.787
	Semi	1.959	0.074	0.937	4.095
	Open (Reference category)	-	-	-	-
Type of Space (layers)	Cage	1.153	0.545	0.728	1.824
	Floor (Reference category)	-	-	-	-
Bird type	Broiler	1.385	0.510	0.526	3.643
	Layer	1.663	0.316	0.615	4.495
	Native (Reference category)	-	-	-	-
Year	2018 (Reference category)	-	-	-	-
	2019	1.779	0.037	1.036	3.056
	2020	1.289	0.542	0.570	2.913
Season	Summer	3.665	0.001	1.696	7.917
	Autumn	3.522	0.013	1.297	9.563
	Spring	9.409	<0.000	4.473	19.791
	Winter (Reference category)	-	-	-	-

P≤0.05 indicate significant difference.

control sheds (26.69%). While lowest prevalence was observed in environment-controlled (EC) sheds (12.84%). In layer birds, the highest prevalence of FAdV infection was observed in layers kept in floor systems (13.83%) of the tested birds were positive for Hexon L1 region. While lowest prevalence was observed in cage systems (15.61%).

FAdV risk evaluation: The current study observed shed type and season to be significantly associated (P<0.05) with the risk of FAdV infection. Association of region (districts of division Faisalabad) and year as associated risk factors with FAdV were close to significant association because *p* value was not far from the set (5%) probability i.e p=0.086 and p=0.095, respectively as compared to the other assumed risk factors (Table 1). Logistic regression further explored the level /category of each assumed risk factor considered to be significantly associated risk factor with FAdV infection (Table 2). The study found none of the districts appearing as potential risk factor because odd ratios were less than reference district (Table 2). Values of these odds were significant (P<0.05) for Faisalabad and Toba Tek Singh while non-significant (P>0.05) for Chiniot. Broilers of week 1 and week 6 of age showing more than 2 odds (P>0.05) likeliness to get infected with FAdV. In case of age groups of layers, there were more than 5 odds (P>0.05) associated with age group of 41-50 weeks. And there was there were more than 3 odds (P>0.05) likeliness of getting infection a likeliness to FAdV infection associated with rest of the layer age groups. Semi-open shed type showed 1.959 odds of getting infection than to open shed type while control shed type (OR=958; *p*=0.892, CI=0.513-1.787) was found less likely to get FAdV infection. Cage type space for layer showed higher than required odds of getting infection compared to floor type. Taking into consideration of bird type for this infection, both of broilers and layer were at higher odds than to native bird while this difference stood non-significant (P>0.05). In case of year, 2019 showed significant 1.779 odds while year 2020 showed non-significant 1.289 odds of getting infection compared to the reference category (year 2018). Among seasons, there was significant (P<0.05) higher odds compared to refence category (winter season). Spring season showed 9.409 odds followed by summer (OR=3.665) and autumn (OR=3.522).

DISCUSSION

Clinical and postmortem evaluation: In current investigation, FAdV (hexon gene) has also been identified through PCR detection in clinically healthy chicken with no gross alterations in the internal organs which is similar to some of the previous studies and suggestions (Schachner *et al*., 2021). Hepatomegaly (IBH) and liver surface necrosis of variable degree were common findings in several clinically infected samples (later confirmed with PCR) also suggested by Fitzgerald (2020). Other accompanying lesions were either, edematous lungs also found by Kaján *et al*. (2019), Fitzgerald (2020), nephritis or development of hydropericardium (Meng *et al*., 2019) with flabby and misshapen heart.

Prevalence and risk factor evaluation of FAdV infection: Despite the molecular detection and outbreak reports of several FAdVs in Pakistan (Zia *et al.,* 2019; Wang *et al.,* 2020), not much is known about the prevalence of FAdVs in any region of Pakistan through accessible scientific literature. However, one study in China during 2015-2018 showed in 15 provinces there were 155 (55.4%) PCR positive samples out 280 suspected clinical cases (Chen *et al*., 2019). In an Indian study, 69 samples had been found PCR positive for Hexon gene out of 194 suspected samples of IBH. Moreover, the study suggested the association of IBH in 5 different Indian states with isolate MK933773 (FAdV-11) (Shinde *et al*., 2020). In contrast to our findings several other scientists have shown greater prevalence in broilers than layers and native chicken (Kim *et al*., 2008; Lim *et al*., 2011). Moreover, one Korean study showed native chicken (12.5%), broilers (2.5%) and layers (6.7%) PCR positive for FAdV (Jeong *et al*., 2018), this study further explains that higher chances of vertical transmission of FAdVs due to minimum usage of FAdV vaccination at breeder level in native chicken and low levels of maternal antibodies in the susceptible age. Similar situation of non-vaccination of breeder flocks of commercial birds has been reported in various regions of Pakistan. In current study, lowest PCR based FAdV prevalence was observed in environment-controlled (EC) sheds because of better biosecurity measures, ventilation and temperature can be achieved in EC sheds than the semi-control ones. Moreover, natural and/or mixed type of ventilation and abrupt thermal shifts (especially during growth period) are also predisposing causes of FAdV infection associated with semi-environment control houses (Eregae, 2014).

Our study showed non-significant risk association between the type of chicken and FAdV infection similarly in a Canadian study, researchers found no association between FAdV clinical disease and the type of affected chickens (Pettit and Carlson, 1972). Our study concluded that, higher prevalence of FAdV infection was observed in broilers of first week age due to possible vertical transmission also suggested by (Grgić *et al*., 2006) and broilers of 6-week age due to decline in maternal antibodies and probable reactivation of latent virus at this time also indicated by (Wang and Zhao, 2019). Association of age with infection reported in our study was in line with findings of Grgić *et al*. (2006), and Choi *et al*. (2012). Egg production stress has been reported to be associated with infection in another study (El-Tholoth *et al*., 2019) and layers of age group week 61-70 weeks were also at the significantly highest risk of FAdV infection as the early moulting activity takes place at 50-67 weeks age in Pakistan (Ahmad *et al*., 2014; Jayasinghe *et al*., 2020) or bird marketing at this age might have involved biosecurity breech (in terms of transportation cages, vehicles, and entry of outsiders) causing horizontal transmission of FAdV infection as suggested by Fitzgerald *et al*. (2020). Previous studies (Ahmad *et al*., 2014; Fitzgerald *et al*., 2020) also noted significant association of native chicken showing significant association of age with infection.

Although, there have been scientific literature published on the molecular detection of FAdVs discussing presence of different FAdV subtypes in outbreaks from Pakistan (Mansoor *et al.,* 2009; Yasmeen *et al.,* 2017; Wajid *et al.,* 2018; Wang *et al.,* 2020). None of the previous studies have reported about the risk of FAdV involved in

any of the geographical locations in Pakistan. On the other hand, significance of geographical location in the FAdV, IBDV, and CIAV associated risk has been endorsed by Eregae (2014) in their PhD dissertation.

Conclusions: Current investigation from division Faisalabad (2018-2020) suggested that FAdV can also be isolated from clinically healthy birds through PCR detection. The current study showed higher prevalence in layer type chicken among commercial chicken types, younger birds showed higher FAdV prevalence because of vertical transmission and poor maternal antibody titers against FAdVs. Higher prevalence of FAdV infection was observed in commercial poultry kept in semi-environment control sheds due to inconsistent control over the biosecurity and internal environment of the shed. From all age group studied in the commercial chicken, broilers aged 1 week and layers of 1-10 weeks were at higher FAdV infection risk. Among layers and native chicken, birds during egg production age (21- 40 weeks) and at early moulting/ culling age (51-70 weeks) had higher FAdV infection risk. It is thus dire need to consider the antigenic characters and pathogenic behavior of these FAdV isolates in the selection of FAdV vaccines and while incorporating FAdV vaccination in the vaccination schedules of the flocks for optimum protection of birds.

Authors contribution: IZ has conducted the parameters of research, applied statistical analysis and prepared original draft of the manuscript, MKS planned the study layout and helped statistical analysis, MTJ, SUR and MA have contributed to refine the writing of the manuscript.

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