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Original Research Article

Prevalence of proteases and other virulence genes in APEC associated with respiratory viral infections in broilers

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ABSTRACT

Acute upper respiratory disease in chickens is a major cause of economic losses due to high mortality rates especially in poorly managed cases. Respiratory disease in poultry is initiated by variety of viruses, bacteria and fungi. The current study aims to investigate the prevalence of avian pathogenic *E. coli* (APEC), their proteases and other virulence genes in respiratory viral disease outbreaks in broiler chickens. Quantitative RT-PCR (qRT-PCR) was performed on samples from 25 farms with respiratory affections, APEC was isolated and virulence determinants in *E. coli* were investigated phenotypically and genotypically.

E. coli was isolated from different flocks (100%, n=25). They were positive to Congo red binding (100%, n=25), *iss* gene (100%, n=25), *iut*A gene (92%, n= 23), *tsh* gene (24%, n=6), *vat* gene (20%, n=5). Presence of *iss* gene and CR binding proves that all isolates are APEC. Although the entire 25 APEC isolates carried more than one virulence gene; either 2 genes (n=17), 3 genes (n=7) and 4 genes (n=1), no effect of the number of genes harbored on the mortality rates in different flocks was observed. The presence of two serine proteases genes (*tsh and vat*) was confirmed in a total of 10 isolates (40% of the isolates) with positivity to *tsh* gene (24%) and *vat* gene (20%).

qRT-PCR for detection of IBV-S1, AIV-H9, AIV-H5 and velogenic NDV-F genes revealed that 96% (n=24), 44% (n=11), 12% (n=3) and 4% (n=1) of 25 farms were positive to IBV, AIV-H9, velogenic NDV and AIV-H5, respectively. The results showed that among the 25 flocks, single viral infection was observed in 12 flocks (11 IBV and 1 AIV-H9), while mixed viral infections were detected in 13 flocks; IBV/AIV-H9 (n=9), IBV/velogenic NDV (n=3) and IBV/AIV-H9/AIV-H5 (n=1).The average mortality rate was the lowest in flocks infected with IBV, higher rates of mortality were observed in flocks infected with AIV-H9, velogenic NDV and AIV-H5. Flock age seems to affect the mortality rate in flocks infected with AIV-H9 where flocks aging 16:20, 21:25 and 26:30 days suffered from 2.38%, 8.13%, 11.48% average mortality rates, respectively.

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1. Introduction

Poultry farming is one of the primary means of supplying human beings with high quality and relatively cheap animal protein. As a consequence, there has been extensive increment in the number and capacity of poultry farming units. Unfortunately, the increase in poultry farming was associated with the emergence of many poultry diseases associated with stressful conditions and/or infectious agents.

The avian respiratory system is complicated and is characterized by very specific criteria including relatively long trachea, non-expandable lungs and the presence of poorly blood-supplied air sacs (Brown et al., 1997). These criteria represent a risk factor as they reduce the defense mechanism, increase the susceptibility to different pathogens and reduce the efficiency of treatment using antimicrobial agents.

Acute upper respiratory disease in chickens is a major cause of economic losses due to high mortality rates especially in poorly managed cases (Yashpal et al., 2004). Respiratory diseases are characterized by their multifactorial nature (Roussan et al., 2008). Various pathogens may initiate respiratory disease in poultry including a variety of viruses, bacteria, and fungi. Environmental factors may augment these pathogens to produce clinical illness. Different viral agents such as avian influenza virus (AIV), Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) are associated with variable but usually high mortality rates in broiler chickens (Haghighat-Jahromi et al., 2008; Hassan et al., 2016). These pathogens can induce disease independently or in association with each other (Yashpal et al., 2004; Roussan et al., 2008). On the other hand, infection with some other pathogens including *E. coli* and Mycoplasma species is common but disease induction requires certain circumstances and usually, these pathogens are considered secondary pathogens.

Avian influenza (AI) is a highly contagious disease caused by type A influenza viruses which are members of the family *Orthomyxoviridae* (Swayne and Suarez, 2000). Based on surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA), Influenza A viruses are classified into 18 HA (H1-H18) and 11 NA (N1-N11) subtypes to date (Tong et al., 2013). According to their virulence, AIV are classified into two pathotypes; highly pathogenic AIVs (HPAIVs) and low pathogenic ones (LPAIVs). LPAIVs cause asymptomatic infections in wild aquatic birds but in domesticated poultry, infections may remain asymptomatic or produce clinical signs and lesions related to the damage of the respiratory, digestive and reproductive systems (Pantin-Jackwood and Swayne, 2009). The detection of the H9N2 AIV subtype during 2010 and 2011 in chickens was a challenge facing the Egyptian poultry industry which was already facing highly pathogenic AI outbreaks (El-Zoghby et al., 2012). Severity of AI infection does not only depend upon the virulence of virus pathotype but also upon factors such as birds age, species susceptibility, environmental and management conditions and concurrent or secondary infection with other pathogens (Brugh and Beard, 1986; Easterday et al., 1997). Although H9N2 is a virus of low pathogenicity, the co-infection with *Staphylococcus aureus, Haemophilus paragallinarum, E. coli,* or IBV can aggravate H9N2 outbreak resulting in high mortality rates (Nili and Asasi, 2002; Bano et al., 2003; Kishida et al., 2004; Haghighat-Jahromi et al., 2008).

Infectious bronchitis (IB) is one of the major components of mixed infections that produce airsaculitis in chickens which may result in condemnation of broilers during processing (Hofstad, 1984). IBV affects chickens of all ages causing respiratory, renal and reproductive disorders (Pohuang et al., 2009; Cook et al., 2012). Respiratory manifestations include coughing, sneezing, rales and nasal discharge (El-Mahdy et al., 2010). IBV can provoke ciliostasis in the host’s airways (Cook et al., 1976; Hassan et al., 2016) thus facilitates the opportunity for other related pathogens and aggravates their pathogenicity (Haghighat-Jahromi et al., 2008). Bacterial pathogens also play an important role in respiratory disease complex in domestic poultry. In many cases, the bacterial component of a respiratory disease colonizes the respiratory system only after a primary viral or environmental insult. Colonization of air sacs of chickens by *E. coli* following IBV infection is a good example of secondary bacterial invasion (Dwars et al., 2009).

Colibacillosis refers to any local or systemic infection caused entirely or partially by *E. coli*. The pathogenicity of *E. coli* is generally enhanced or initiated by predisposing factors such as mycoplasma infections, viral infections, environmental factors or immunosuppressive diseases (Dho-Moulin and Fairbrother, 1999; Ewers et al., 2003; Bopp et al., 2005). APEC infections are responsible for significant economic losses in the poultry industry (Dho-Moulin and Fairbrother, 1999; Barnes et al., 2003). Virulence gene studies are important as they aid in the characterization of pathogenic strains of *E. coli* and may eventually lead to the development of an effective vaccine or other control measures (Janben et al., 2001). *E. coli* is a normal inhabitant of the gastrointestinal tract, so scholars use different methods to differentiate pathogenic strains from normal flora. Congo red binding assay was recognized as simple phenotypic method to differentiate pathogenic from non-pathogenic *E. coli* (Qadri et al., 1988) and recommended as pathogenicity marker by (Sharma et al., 2006) to discriminate invasive from non-invasive *E. coli* strains as invasive ones develop brick red colonies onto Congo red containing media (Aniruddha et al., 2009) due to the production of curli and cellulose (Uhlich et al., 2014). *E .coli* has been known to cause respiratory syndromes alone or in conjugation with other microorganisms. APEC most likely enter and colonize the avian respiratory tract by inhalation of fecal dust leading to localized infections such as airsacculitis and pneumonia (Barnes and Gross, 1997). In certain cases, it spreads into various internal organs and typically causes pericarditis, perihepatitis, peritonitis, salpingitis, and other extraintestinal diseases.

Rapid and early detection of respiratory disease etiology as well as understanding the pathogenesis of the infectious process especially the interaction of different viruses and bacteria being involved in the respiratory disease complex in broiler chickens is crucial for effective control (Scholz et al., 1994; Yashpal et al., 2004).

2. Material and methods

Field samples Samples were collected from moribund chickens (5-10 chickens per flock) from 25 broiler chicken farms of variable ages located at Beni-Suef governorate during the period from March to May 2017. Selected flocks suffered from respiratory manifestations and gross lesions suggestive for possible respiratory viral infection (gasping, sneezing, rales, nasal discharge, bronchial casts, and mortalities were the common findings in the selected flocks, swollen infraorbital sinuses either unilateral or bilateral, tracheal congestion and exudations were evident in some flocks). Flock history and clinical data were recorded. Samples from trachea, bronchi, and lungs from each flock were collected for bacteriological examination and tracheal swabs were collected for virological examination.

1. **Tracheal swabs:** They were collected on sterile phosphate buffer saline (PBS) pH 7.2. five to ten swabs were clarified by centrifugation at 2000 rpm for 10 min in screw capped sterile tubes (OIE, 2008), pooled and kept frozen at -80°C for viral detection.
2. **Organs:** Samples from trachea, bronchi, lung and air sacs were collected aseptically for bacteriological examination.

Detection of viral RNA using real time RT-PCR (qRT-PCR)

Viral RNA was extracted from tracheal swabs of each flock using Biospin virus RNA extraction kit (BioFlux) according to the manufacturer's instructions. qRT-PCR **f**or AIV-H9, IB, AIV-H5, and ND gene detection, a uniplex qRT-PCR reaction was adjusted as recommended by *SensiFAST™ Probe Lo-ROX Kit* instruction. Primers used are shown in (Table 1). qRT- PCR mixture for each reaction included 2x SensiFAST probe Lo-ROX One-Step Mix (10µl),10 µM forward primer and reverse primers (0.8µl, each),10 µM probe (0.2µl), reverse transcriptase (0.2µl), riboSafe RNase inhibitor (0.4µl),template RNA (5.0µ) and RNase free water (2.6µl). Thermal profile included one cycle for reverse transcription (45°C/10min), one cycle for initial denaturation (95°C/2min), 40 cycles of amplification each included denaturation at 95°C/5 sec and an annealing/extension step at 54°C/30sec (for AIV-H5 and vNDV) or 60°C/60sec.(for AIV-H9 and IBV).

Table 1. Primers and probes used in qRT-PCR

Virus	Target gene	Oligonucleotide	Primer Sequence (5`-3`)	Reference
IBV	S1	GU391 (F primer) GL533 (R primer) Probe	GCTTTTGAGCCTAGCGTT GCCATGTTGTCACTGTCTATTG CACCACCAGAACCTGTCACCTC	Callison et al., 2006
AIV-H9	H9	F primer R primer Probe	GGAAGAATTATTTATTGGTCGGTAC GCCACCTTTTTCAGTCTGACATT ACCAGGCCAGACATTGCGAGTAAGATCC	Ben Shabat et al., 2010
AIV-H5	H5	LH1 (F primer) RH1 (R primer) Probe	ACATATGACTACCCACARTATTCAG AGACCAGCTAYCATGATTGC CCCTAGCACTGGCAATCATG	Slomka et al., 2007
vNDV	F	F primer R primer Probe	GGAAGAATTATTTATTGGTCGGTA GCCACCTTTTTCAGTCTGACATT AAGCGTTTCTGTCTCCTTCCTCCA	Wise et al., 2004

Bacteriological examination

***E. coli* isolation, morphological and biochemical identification** The collected samples were cultivated under aseptic conditions into Tryptone soy broth (TSB). All inoculated media were incubated aerobically at 37°C for 12 hours. Loopfulls from the inoculated broth were streaked onto Tryptone soya agar (TSA) and MacConkey's agar then incubated aerobically at 37°C for 24 hours. All recovered isolates were identified morphologically and biochemically according to the following tests; oxidase, TSI, indole production, citrate utilization and motility test (Quin*n et al., 2*002).

***E. coli* pathogenicity testing**

Phenotypically Congo red binding assay was used for phenotypic evaluation of *E. coli* virulence.

Genotypically Detection of virulence genes (*iss*, *iut*A, *tsh*, and *vat*) in different *E. coli* isolates was performed as following: DNA was extracted by boiling method according to (Delicato et al., 2003). A single colony grown on EMB agar was cultured in TSB medium for 12 hours at 37°C. 1.5 ml of the broth medium was centrifuged at 1500 rpm for 10 minutes. The pellet was resuspended in 250 µl nuclease-free water and followed by boiling in a water bath for 5 minutes followed by centrifugation at 1500 rpm for 10 minutes. The supernatant containing DNA was collected in a new sterile 1.5 ml tubes for PCR and stored at -20°C. PCR was prepared using gene-specific primers (Table 2) in 25 µl reactions prepared according to MyTaq™ Red Mix (BIOLINE) instructions. Thermal profile included one cycle of initial denaturation (94°C/5min), 35 cycles of amplification (94°C/30 sec., 52:54°C/ 45 sec., 72/45 sec.) and a final extension at 72°C /10 min. PCR products were separated by electrophoresis in 1.5%

agarose in TAE buffer then the DNA bands were visualized using the UV-trans-illuminator.

Table 2. Primers used for amplification of *iss*, *iut*A, *tsh* and *vat* genes of *E. coli*.

Gene	Primer Sequence 5´-3´		Annealing temperature*	Amplified product	Reference
Iss	F	ATGTTATTTTCTGCCGCTCTG	54°C	266 bp	(Yaguchi *et al.*, 2007)
	R	CTATTGTGAGCAATATACCC			
*iut*A	F	GGCTGGACATGGGAACTGG	55°C	300 bp	
	R	CGTCGGGAACGGGTAGAATCG			
tsh	F	GGTGGTGCACTGGAGTGG	52°C	620 bp	(Delicato *et al.*, 2003)
	R	AGTCCAGCGTGATAGTGG			
vat	F	AACGGTTGGTGGCAACAATCC	54°C	420 bp	(Restieri *et al.*, 2007)
	R	AGCCCTGTAGAATGGCGAGTA			

*: Modified to improve the quality and intensity of the PCR product.

3. Results

In this study, 25 broiler chicken flocks suffered from respiratory affections were examined for respiratory viruses AIV and IBV infection. These flocks were collected from the Beni-Suef governorate during March to May 2017. Flock history is summarized in (Table 3). Organs (trachea, lung and air sac) were processed for RNA extraction and qRT- PCR. *E. coli* was isolated, biochemically identified, virulence determinants were detected phenotypically and genotypically.

qRT-PCR detection of IB, AI and ND viruses in different flocks

Tissues from trachea, lung and air sacs were tested for IBV S1 gene, AIV HA gene (H5/H9) and velogenic NDV F gene. Summary of respiratory viral genes detection is illustrated in (Table 4). IBV was detected in 96% (n=24), H9 AI virus was detected in 44% (n=11), velogenic NDV was detected in 12% (n=3) and H5 gene was detected in one flock (4%). Mixed respiratory viral infection (at least 2 viruses) was detected in 52% (13/25) of the flocks. AIV-H9 with IBV has been found to be the most common mixed infection (9 flocks) representing 69.23% of mixed infected flocks (13 flocks) and 36% of total investigated flocks (25 flocks). Single IBV infection was detected in 44% (11/25) while single H9 virus infection was detected in only one flock (4%). Flocks infected with IBV showed the lowest mortalities (except flock #3 in which the mortality rate was 10.6%). The highest mortality rates were observed in AIV-H5 infected flock then in vNDV infected flocks then in AIV-H9 infected flocks. Flock age seems to affect the mortality rate in flocks infected with AIV-H9 where flocks aging 16:20, 21:25 and 26:30 days suffered from 2.38%, 8.13%, 11.48% average mortality rates, respectively (Table 5).

Results of biochemical identification of *E. coli* isolates recovered from chickens with respiratory disorders All of the 25 isolates of *E. coli* were all positive for indole production and methyl red (MR). They were all negative for oxidase, citrate utilization, hydrogen sulphide (H_2S) on TSI, voges prauskeur (VP) and urea hydrolysis tests. All tested strains of *E. coli* fermented glucose (with acid and gas production), lactose, mannose, arabinose, sorbitol, maltose and mannitol. Concerning the other non-biochemical tests, all isolates were positive for motility tests and grown onto MacConkey's agar media giving pink colonies. Then they were given green metallic sheen onto eosine methylene blue agar (EMB).

Detection of virulence traits of *E. coli* isolates

All *E. coli* isolates (n= 25, 100%) were Congo red positive (CR^+) All of the 25 isolates were subjected for screening by PCR targeting four virulence genes and proteases *iss*, *iut*A, *tsh*, *vat*. As shown in (Table 6), 25 isolates were carriers for *iss* gene with a prevalence of 100%, 23 isolates were carrier for *iut*A gene with a prevalence of 92%, 6 isolates were carriers for *tsh* gene with a prevalence of 24% and 5 isolates were carriers for *vat* gene with a prevalence of 20%.

Table 3. Clinical history and mortalities of examined chicken flocks

Sample No	Month of collection	Age (days)	Number of birds in each flock	Mortality	
				Number	%
1	March, 2017	25	2500	715	28.6
2		30	5000	610	12.2
3		28	4000	425	10.6
4	April, 2017	28	13000	170	1.3
5		21	3000	50	1.7
6		30	2500	25	1
7		23	2000	96	4.8
8		25	3000	135	4.5
9		24	5200	720	13.8
10		23	6000	534	8.9
11		34	2500	85	3.4
12		27	1000	255	25.5
13		23	3000	25	0.83
14		34	2000	210	10.5
15		28	5000	105	2.1
16		20	1000	15	1.5
17	May, 2017	11	3000	23	0.77
18		14	4000	45	1.13
19		18	4000	250	6.3
20		23	5000	310	6.2
21		18	1250	15	1.2
22		26	4000	2600	65
23		17	5500	30	0.5
24		33	7000	2260	32.3
25		30	4000	430	10.75

Table 4. qRT-PCR detection of IB, AI and ND viruses in different flocks

Sample no	IBV S1 gene	Low pathogenic AIV-H9 HA gene	Velogenic NDV F gene	Highly pathogenic AIV-H5 HA gene	Mortality (%)
1	■		■		28.6
2	■	■			12.2
3	■				10.6
4	■				1.3
5	■	■			1.7
6	■				1
7	■				4.8
8	■				4.5
9	■	■			13.8
10	■	■			8.9
11	■				3.4
12	■		■		25.5
13	■				0.83
14		■			10.5
15	■				2.1
16	■	■			1.5
17	■				0.77
18	■				1.13
19	■	■			6.3
20	■				6.2
21	■	■			1.2
22	■	■		■	65
23	■	■			0.5
24	■		■		32.3
25	■	■			10.75
Total number of positive flocks (%)	**24 (96%)**	**11(44%)**	**3 (12%)**	**1(4%)**	

■	Positive		Negative

Table 5. Age determinant of mortality among flocks infected with respiratory viruses

Age range (day)	IB				IB/H9				IB/ND			
	No	**Average**	**Min**	**Max**	**No**	**Average**	**Min**	**Max**	**No**	**Average**	**Min**	**Max**
10-15	2	0.95%	0.77%	1.13%	-	-	-	-	-	-	-	-
16-20	-	-	-	-	4	2.38%	0.5%	6.3%	-	-	-	-
21-25	4	4.08%	0.83%	6.20%	3	8.13%	1.7%	13.8%	1	28.6%	-	-
26-30	4	3.75%	1.00%	10.6%	2	11.48%	10.75%	12.2%	1	25.5%	-	-
>30	1	3.4%	-	-	-	-	-	-	1	32.3%	-	-

Table 6. Phenotypic and genotypic detection of virulence markers in *E. coli* isolated from viral respiratory disease outbreaks

Sample no	APEC isolation	Congo red binding assay	*E. coli* virulence genes				Total no of detected genes per isolate	Mortality (%)
			iss	*iut*A	*tsh*	*vat*		
1							3	28.6
2							3	12.2
3							2	10.6
4							3	1.3
5							2	1.7
6							4	1
7							2	4.8
8							2	4.5
9							3	13.8
10							2	8.9
11							2	3.4
12							2	25.5
13							3	0.83
14							3	10.5
15							2	2.1
16							2	1.5
17							2	0.77
18							3	1.13
19							2	6.3
20							2	6.2
21							2	1.2
22							2	65
23							2	0.5
24							2	32.3
25							2	10.75
Total number of positive flocks (%)	**25 (100%)**	**25 (100%)**	**25 (100%)**	**23 (92%)**	**6 (24%)**	**5 (20%)**		

	Positive	Negative

4. Discussion

Poultry is considered one of the main sources of animal protein in most countries all over the world. In Egypt, the respiratory disease complex is one of the major problems facing the poultry industry. Respiratory affections in poultry are very complex especially whenever viral ones are incriminated, as they usually involve more than one pathogen (Roussan et al., 2008). Respiratory colibacillosis is caused by secondary infection with pathogenic *E. coli* after primary infectious either viral or bacterial or environmental stresses such as elevated ammonia or CO2 levels and dust in the atmosphere (Barnes and Gross, 1997; Gomis et al., 2001).

In the present study, 25 farms of broiler chickens were examined with respiratory tract infections. Mortality rates in the flocks under investigation ranged from 0.5 to 65% with the highest mortality rate found in a flock suffering AIV-H5 and velogenic ND. qRT-PCR for detection of IBV-S1, AIV-H9, AIV-H5 and velogenic NDV-F genes in tracheal swabs revealed that 96% (n=24), 44% (n=11), 12% (n=3) and 4% (n=1) of 25 farms are positive to IBV, AIV-H9, velogenic NDV, and AIV-H5, respectively. Single viral infection was observed in 12 flocks (11 IBV and 1 AIV-H9), while mixed viral infections were detected in 13 flocks; IBV/AIV-H9 (n=9), IBV/velogenic NDV (n=3) and IBV/ AIV-H9/ AIV-H5 (n=1). Mixed infection with IB and/or AIV-H9 viruses is the most common situation in the examined flocks with positivity in 21/25 flocks (84%) followed by 3 flocks (12 %) suffering IBV/ND and one flock (4%) suffering IBV/AIV-H5 infection. It is worth mentioning that qRT-PCR assay alone cannot differentiate between field and vaccine strains of IBV and S1 gene sequencing is thought to be the only method used to discriminate between all IBV strains. High prevalence of IBV either in a single or mixed infection was previously reported by (Abdel-Moneim et al., 2006*;* Hassan et al., 2016). The average mortality rate was the lowest in flocks infected with IBV, higher rates of mortality were observed in flocks infected with AIV-H5, velogenic NDV, and AIV-H9, respectively. Although IBV is usually a cause of mild infection and low mortality rates, some of the investigated flocks suffered from more than 10% mortality. Poor management and mixed infection with other respiratory pathogens could be the explanation of increased mortality in some flocks infected with IBV (Nili and Asasi, 2002, 2003).

The onset of clinical disease between started at 11-34 days of age. The highest number of flocks had respiratory disorders at 16:30 days of age (20 flocks) compared to younger (n=2) and older (n=3) flocks with higher incidence in flocks aging 20:30 days of age (17 flocks).

The age of birds seems to affect the mortality rate in flocks infected with AIV-H9 where flocks aging 16:20, 21:25 and 26:30 days suffered from 2.38%, 8.13%, 11.48% average mortality rates, respectively. Co-infection with IBV and AIV-H9 has resulted in 0.5-13.8% mortality rate even in flocks vaccinated against both pathogens. On the other hand single IBV infection resulted in 0.77: 10.6% mortality with average mortality around 4%. In one flock infected with AIV-H9 alone respiratory signs, tracheal caseation and 10.5% mortality rate, unlike what is known about low virulence nature of H9 subtype, were observed. This data highlights the ability of AIV-H9 to produce respiratory infection and bronchial casts in broilers as previously reported (Naeem et al., 1999; Nili and Asasi, 2002; Aamir et al., 2007*;* Hassan et al., 2016). The differences between the natural and the experimental infections with AIV-H9 suggest that secondary agents may play an important role in the pathogenesis of AIV-H9N2 in most cases (Nili and Asasi, 2002; Perk et al., 2004; Aamir et al., 2007). Co-infection with *E. coli* (Bano et al., 2003) can exacerbate AIV-H9N2 infection in chickens possibly by secretion of trypsin-like proteases by bacterial stimulation of host cells to produce or secrete more proteases, or destruction of endogenous cell protease inhibitors (Mancini et al., 2005) and suppression of the immune system due to stress by bacterial infection (Kishida et al., 2004) need to be further investigated.

Virulence represents a balance among multiplicity factors, some related to the microorganisms and others related to the host defense. Virulence in microorganisms is associated with the capacity to attach and colonize the site of infection with damage to the host tissues. The presence of several factors concerning with the virulence of *E. coli* strains was studied in poultry suffering from colibacillosis such as hemolytic activity, Congo red uptake, haemagglutination activity, hydrophobicity (salt agglutination activity), serum resistance, enterotoxigenic and verotoxigenic activities, invasiveness property and production of heat-stable toxins (Radwan, 2000). There are a variety of virulence factors implicated in promoting the extra-intestinal diseases in avian species including adhesions, iron acquisition systems, hemolysis, anti-bactericidal factors (outer membrane protein A, LPS, K1-capsule, and colicin production), and toxins (heat-stable toxin and flagella toxin), aerobactin and temperature-sensitive hemagglutinin (Ewers et al., 2003; Mellata et al., 2003).

In the current study, biochemical identification of isolated bacteria according to (Farid *et al.*, 1981; Sedhom, 2000) proved that *E. coli* isolation was successful in 100 (n=25) of the samples. More interestingly, 100% of the samples also carry phenotypic and genotypic determinant of virulence. PCR was applied on 25 isolates of *E. coli* targeting four virulence genes *iss*, *iut*A, *tsh* and *vat*. All of the 25 isolates were carriers for *iss* gene with a prevalence of 100%, 23 isolates (92%) are carrier for *iut*A gene. Six isolates (24%) are a carrier for *tsh* gene and five isolates (20%) are a carrier for *vat* gene. Presence of *iss* gene, as well as CR binding, proves that all isolates are APEC (McPeake et al., 2005; Dissanayake et al., 2014). Although, the entire 25 APEC isolates carried more than one virulence gene; either 2 genes (n=17), 3 genes (n=7) and 4 genes (n=1), no effect of the number of genes harbored on the mortality rates in different flocks was observed.

The serine protease autotransporters from *Enterobacteriaceae* (SPATE) constitute a superfamily of virulence factors whose members resemble those belonging to the trypsin-like serine proteases. They are highly prevalent among enteropathogens including Shigella and all *E. coli* pathotypes (Kaper et al., 2004; Yen et al., 2008). Both *tsh* and *vat* are present in *E. coli* from avian sources and found in *E. coli* isolated from human hosts (Li et al., 2010).

In this study, we observed the presence of two serine proteases genes (*tsh and vat*) in a total of 10 isolates (40% of the isolates) with 24% of the studied isolates being positive to *tsh* gene and 20% positive for *vat* gene. High mortality rates (8.9:13.85%) were observed in five AIV-H9 infected flocks. *vat* gene was detected in *E. coli* recovered from two AIV-H9N2/IBV infected flocks with the highest mortalities (12.2 and 13.8%) among AIV-H9 infection detected in the current study. On the other hand, *tsh* gene was detected in *E. coli* collected from one AIV-H9N2/IBV infected flock with only 1.7% mortality and one AIV-H9 single-infected flock with 10.5% mortality. This may indicate that *vat* gene has a superior effect on the pathogenicity of AIV-H9 than *tsh* gene. It is worth mentioning that *E. coli* isolated from five flocks (all suffered AIV-H9N2/IBV infection), harbored no *tsh* or *vat* genes. The average mortality in these flocks was 4.85%, a lower than flocks infected with E. coli carrying vat gene. Among these five flocks, high mortality rates (8.9 and 10.7%) were noted in two flocks despite the absence of *tsh* and *vat* genes. This may be related to the presence of other proteases or other management related factors. Proteolytic cleavage of hemagglutinin is required for cell entry by receptor-mediated endocytosis and plays a key role in pathogenicity of influenza virus, at the cleavage site, which is not susceptible to cleavage by intracellular proteases but is cleaved by extracellular serine (trypsin-like) proteases by (Kishida et al., 2004; Bottcher et al., 2006; Chaipan et al., 2009; King et al., 2009).

5. Conclusions

Various avian respiratory viruses have been detected in flocks under investigation with a high prevalence of IBV. Mixed infection especially IBV/AIV-H9 was the most prevalent situation. The highest mortality was observed in AIV-H5, vNDV, AIV-H9 then IBV infected flocks. Age of the flocks plays a role in mortality, especially in AIV-H9 infection. All *E. coli* isolates recovered from flocks suffering viral respiratory diseases are avian pathogenic based on CR binding assay and virulence gene detection. The highest mortality in AIV-H9 infection was observed in flocks where *vat* gene is prevalent.

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