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Correlation of avian influenza-H9N2 with high mortality in broiler flocks in the southwest of Tripoli, Libya

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Abstract

Background: Low pathogenic H9N2 avian influenza (LPAI H9N2) caused by the influenza A virus which belongs to the family Orthomyxoviridae. It caused mild respiratory symptoms and a drop in egg production in poultry. Outbreaks of AI-H9N2 have occurred in poultry since the 1990s in many countries in USA, Europe, and Asia. Recently, outbreaks of H9N2 in commercial chicken were recorded in Morocco, Tunisia, Libya, and Egypt. Furthermore, numerous studies demonstrated that co-infection with AI H9N2 and other pathogens results in severe respiratory illness with high mortality in broiler chickens. Outbreaks of respiratory disease with variations in mortality rate were recorded in broiler flocks growing in the southwest of Tripoli in Libya.

Aim: The present study was conducted to explain the variation of mortality rate on broiler flocks growing in the southwest area of Tripoli by detection of AI H9N2 antibodies and antigens.

Methods: A total of 453 sera samples, 60 tracheal swabs, and 60 cloacal swabs were collected from unvaccinated broiler flocks against avian influenza. Specific avian influenza type A antibodies were detected by using the Elisa test, and specific AI-H9N2 antibodies were detected by using the HI test, whereas specific AI-H9N2 antigens were detected in tracheal and cloacal swabs by using One-Step RT-PCR (M gene) technique.

Results: Respiratory diseases with high variations in mortality rate were recorded in broiler flocks growing in the southwest of Tripoli in Libya; the broiler mortality rate in Twisha farms was higher than other farms (62.2% and 11%, respectively). Whereas avian influenza type A antibodies were detected at a high level in Twisha and other farms (95.2%, and 76.7%, respectively). The positive samples for AI type A were tested for AI H9N2 using the HI test. Interestingly the percentage of AI-H9N2 antibodies was quite similar in high and low mortality regions (53.4% and 46.8%, respectively). Additionally, AI-H9N2 antigens were detected only in tracheal swabs in Twisha farm 3, Al-Maamoura, and Ber Al-Tota districts.

Conclusion: This study confirmed the endemic of AI- H9N2 in broiler flocks in the southwest of Tripoli-Libya. Also, it clarified that AI-H9N2 was not responsible for the high mortality rate by itself in broiler flocks. Moreover, this study supported the presence of other subtypes of avian influenza in the studied area.

Keywords: H9N2, Broiler flocks, Variation, Mortality rate, Libya.

Introduction

Avian influenza (AI) is one of the important respiratory diseases in poultry caused by the influenza A virus which belongs to the family Orthomyxoviridae. Influenza A viruses have several subtypes based on hemagglutinin (HA) or neuraminidase antigens. So far, there are 18 (H1-H18) subtypes and 10 neuraminidase subtypes (N1-N11) that have been recognized (Fouchier *et al*., 2005; Tong *et al*., 2012). According to pathogenicity, Avian influenza viruses (AIVs) can be grouped as highly pathogenic avian influenza (HPAI) H5 and H7 subtypes, which result in heavy economic losses to the poultry industry and low pathogenic avian influenza (LPAI) like H9N2 which usually causes minimal clinical signs and drop in egg production. Nevertheless, co-infection of H9N2 with other pathogens may result in severe respiratory disease and cause mortality of up to 65% in broiler chickens and up to 70% drop in egg production in layers and breeders (Alexander *et al*., 2009; Capua *et al*., 2009; Abdel-Moneim *et al*., 2012; Seifi *et al*., 2012; Azizpour *et al*., 2014; Kammon *et al*., 2015). LPAI H9N2 became endemic globally in many countries like Germany, Italy, Ireland, South Africa, the USA, and Korea since the 1990s. (Arafa *et al*., 2012; Al-Garib *et al*., 2016). Also, H9N2 infections have been reported in Asia (Hong Kong,

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China, Korea, Pakistan, Japan, India, and Vietnam), the Middle East (United Arab Emirates, Saudi Arabia, Kuwait, Lebanon, Iraq, and Sultanate of Oman), and Africa (South Africa, Tunisia, Egypt, Morocco, and Algeria), causing widespread outbreaks in commercial chickens (Butt *et al*., 2010; Al-Garib *et al*., 2016; Kaboudi *et al*., 2019; Peacock *et al*., 2019). In several of these countries, the vaccine has been utilized to control the disease. Nevertheless, H9N2 infection has become endemic in commercial poultry in a significant number of countries. LPAI H9N2 has not only been detected in poultry but also in some human cases, being a real to human health and a global concern for public health (Body *et al*., 2015; Sikht, 2022). In Libya, the incidence and severity of respiratory disease in commercial chicken flocks have increased due to the intensification of the poultry industry, and in mid-2005 LPAI H9N2 subtype changed, believed to be one of the essential reasons for chicken respiratory diseases in Libya, as indicated by field reports (Al-Garib *et al*., 2007; Fares *et al*., 2010). Seroprevalence results showed a positive correlation between a high mortality rate and the detection of specific antibodies against AIV H9N2 (Al-Garib *et al*., 2007). Furthermore, the seroprevalence of AI (H5, H7, and H9) in broiler and layer flocks in the northwest of Libya revealed the presence of only antibodies against the LPAI H9 subtype (Fares *et al*., 2010). The last outbreaks of respiratory signs with a high mortality rate in Libyan broiler and layer hens during 2013 were described by the Co-infection of H9N2 with NDV (Kammon *et al*., 2015). The present study was conducted to explain the variation of mortality rate on broiler flocks in the southwest area of Tripoli by detection of AI H9N2 antibodies and antigens.

Materials and Methods

Field survey

Twenty-two commercial broiler flocks were studied in seven districts (Twisha, Al-Asfah, Al-Amria, Al-Maamoura, and AL-Kayekh) from Al-Jafara municipality and two districts (Wadi Al-Rabia, and Ber Al-Tota) from Tajoura municipality. All flocks were vaccinated against NDV, IBV, and (IBDV). By contrast, all the previous flocks were not vaccinated against AI. Furthermore, Poultry sheds were of low quality and suffered from insufficient ventilation in Twisha farms compared with other farms.

Serological assays

Serological assays (Elisa and HI tests) were conducted at the National Center of Animal Health in Libya.

Collection of serum samples for serological Elisa test and HI test

Four hundred fifty-three blood samples were collected randomly from different districts in the southwest of Tripoli-Libya as recommended by WHO (2002) and described by Spackman (2007) (Table 1). ELISA test to detect AI type A antibodies in serum was conducted according to the manufacturer's (X-OvO Limited, United Kingdom) instructions. An ELx800 ELISA reader with a 550 nm filter (BIO-TEK Instruments, Inc. Winooski, VT, USA) was used. The HA and HI were carried out according to the procedure described by Alexander *et al*. (1983), Alexander (2009), and OIE (2009). The HI assay has to end up completed using 96 'U'-well microtiter plates, doubling dilution in PBS, 1 % v/v crimson blood cells, and 4 HA devices of AIV antigen.

Table 1. Blood samples from Twisha district and other districts at age 38 days.

Location		No. of birds	No. of samples
Twisha district			
Twisha Farm (1)	H1	10,000	15
	H2	10,000	15
	H3	10,400	15
	H4	10,000	15
	H5	10,000	16
	H6	11,200	15
	H7	10,400	15
Twisha Farm (2)	H1	9,625	21
	H2	9,625	19
	H3	9,625	24
Twisha Farm (3)	H1	8,500	15
	H2	8,500	15
	H3	8,400	15
	H4	8,500	15
Total		134,775	230
Other districts			
Wadi Al-Rabia farm 1		28,000	29
Wadi Al-Rabia farm 2		27,000	36
Al-Asfah farm 1		11,000	22
Al-Asfah farm 2		21,000	26
Al-Amria farm		6,500	25
Al-Maamoura farm		21,500	26
AL-Kayekh farm		11,000	26
Ber Al-Tota farm		18,000	33
Total		144,000	223

Molecular technique assays to detection of avian influenza type A antigen

Highly sensitive monoclonal antibodies rapid test

One hundred twenty tracheal and cloacal swabs were collected for Rapid Tests from broilers suffering from mild to severe clinical symptoms (Table 2). Antigen Rapid AIV Ag Test Kit was used according to the manufacturer's (BIO NOTE, KOREA) instructions. Positive samples of rapid tests were confirmed by

one-step RT-PCR (M gene). Virus antigen detection by molecular technique was done at the Institute of Veterinary Research of Tunisia, 20 Rue Djebel Lakhdar La Rabta 1006 Tunis. Individual plastic swabs for molecular technique were collected from the wall of the trachea and cloacal, according to Spackman (2007) and Spackman *et al*. (2002). Swab immerse in viral transport media tube containing brain–heart infusion broth with penicillin G (10,000 Units/ml), streptomycin (2 mg/ml), kanamycin (0.6 mg/ml), gentamicin (1 mg/ml), and amphotericin B (0.02 mg/ml). Swabs were eliminated using a disinfectant solution, and transport media tubes were Labeled and stored separately at -70°C for the following analysis. RNA was extracted from tracheal and cloacal swabs of individual birds according to the manufacturer's instructions (Qiagen, GmbH - Germany) using QIAamp viral RNA mini Kit. One-step RT-PCR was performed using an Access RT-PCR System kit (Qiagen, Germany) according to the manufacturer's procedure. Universal primers were used to determine the presence of influenza A viruses (Table 3). PCR was carried out in a final reaction volume of 25 µl using a 0.2 ml thin wall PCR tube. A master mix of 5 samples was prepared and liquated in 20 µl quantities in each PCR tube. A 5 µl sample of RNA was added in each tube to make the final volume of 25 µl. The amplified segments were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) using 1× Tris/borate/ethylenediaminetetraacetic acid buffer at room temperature at gradients of 5 v/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. 100 and 1,000 bp ladder (Qiagen, GmbH – Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Table 2. Describes the rapid test specimen at age 38 days.

Location	Tracheal swabs	Cloacal swabs
Twisha districts		
Twisha Farm (1)	10	10
Twisha Farm (2)	10	10
Twisha Farm (3)	10	10
Other districts		
Al-Amria	10	10
Al-Maamoura	10	10
Ber Al-Tota	10	10

Statistical analysis

Data were managed and analyzed using SPSS, version 17.0 (SPSS Inc., Chicago, IL). Data were performed using analysis of variance (ANOVA). Statistical significance was designated for differences with *p*-values less than or equal to 0.05.

Ethical approval

All samples were collected according to the local and international standards of the care and use of animals in research.

Results

Clinical signs and postmortem

The samples were collected prissily from broiler farms suffering from severe to mild respiratory signs, but the severity of respiratory signs varied from farm to farm. By contrast, postmortem examination did not provide clear evidence of a specific disease. Only the trachea and upper respiratory tract were congested with an accumulation of little mucus. The lung was also congested. The kidney and spleen were quite an enlargement. The thoracic and abdominal air sacs were slightly thickened and whitish.

Mortality rates

A high broiler mortality rate (62.2%) was recorded on Twisha farms at Al-Jafara municipality. By contrast, a low broiler mortality rate (11%) was recorded on Al-Asfah, Al-Amria, Al-Maamoura, and AL-Kayekh farms at Al-Jafara and municipality, and Wadi Al-Rabia, and Ber Al-Tota farms at Tajoura municipality (Table 4).

Serological assay

Two hundred thirty blood samples from Twisha farms and 223 blood samples from different farms were exanimated by using the ELISA technique to detect AI type A antibodies. The percentage of positive antibodies against AI type A in Twisha farms was 95.2%, whereas in different farms was 76.7% (Table 5). Similar rates of antibody titration against AI type A were recorded in Twisha farms 1, 2, and 3 (96%, 94%, and 95%, respectively). Also, in AL-Asfah, farm (1) was 91%, and farm (2) was 96% (table 5). By contrast, different percentages of antibody titration against AI type A with significant differences ($p < 0.05$) were recorded in the Ber Al-Tota farm 36.4%, Wadi Al-Rabia farm (1) 75.9%, Al-Amria farm 100%, Al-Maamoura farm 88.5%, and AL-Kayekh farm 34.6% (Table 5 and 6). The positive

Table 3. Polymerase chain reaction primers used for the influenza A virus and H9 subtype.

Type of primers	Primer's name	Sequences	References
Universal	Forward M52C	5′- CCT CTA ACC GCG GTC GAA ACG - 3′	Fouchier *et al*., 2000
	Reverse M253R	5′ - AGG GCA TTT TGG ACA AAK CGT CTA - 3′	
Specific	Probe FAM H9	5′-TTCTGGGCCATGTCCAATGG-3′	Spackman *et al*., 2002
	H9F	5′-ATGGGGTTTGCTGCC-3′5′-	

samples for AI type A were tested for subtypes H9N2 using the HI test. H9N2 antibodies were detected in 53.4% of samples collected from Twisha farms and 46.8% of samples collected from other farms. This result refers to a significant decrease ($p < 0.05$) by two folds in the number of positive samples to AI H9N2 antibodies compared with positive samples to AIV type A antibodies in the studied area (Tables 5 and 6).

Virological tests

Rapid test to detect AIV type (A): Tracheal and cloacal swabs were collected from farms with positive serology to AIV type A at age 38 days. All swabs collected from Twisha farms were negative to AIV type A, except tracheal swabs collected from farm 3 were positive to AIV type A. By contrast, all tracheal and cloacal swabs collected from Al-Amria, Al-Maamoura, and Ber Al-Tota district were positive to AIV type A.

Table 4. The mortality rates of broiler birds at 38 days old.

No. of birds	No. of death birds	Accumulative mortality rate (%)
Twisha district		
134,775	83,879	62.2%
Other districts		
144,000	15,765	11%

Molecular diagnosis of avian influenza

The positive rapid test samples were confirmed by using a one-step PCR technique to detect AI H9N2. As illustrated in (Table 7). Only tracheal swabs collected from Twisha-farm (3), Al-Maamoura, and Ber Al-Tota districts were positive for AI type A and the H9N2 subtype by rRT-PCR. At the same time, tracheal swabs collected from Al-Amria districts were negative for AI type A and AI H9N2. Moreover, all cloacal swabs were negative for AI type A and H9N2 using the same technique (Table 7; Figs. 1 and 2).

Discussion

This study appears a high variation in broiler mortality rate between Twisha farms compared with other farms (62.2% and 11%, respectively). At the same time, all broiler flocks suffer from mild to severe respiratory signs. Furthermore, the postmortem examination does not refer to specific diseases that can explain the difference in broiler mortality rate between flocks. The seroprevalence of AI types A antibodies are detected at a high level in the Twisha district, suffering from a high mortality rate, and different districts suffering from a low mortality rate (95.2% and 76.7%, respectively) using the Elisa test. This result is in accordance with Al-Natour *et al*. (2005) in Jordan who indicated that the seroprevalence of AI antibodies is 71%; Barbour *et al*. (2006) in Lebanon reports that seroprevalence

Table 5. Comparative between AIV type A detected by Elisa test and specific antibodies AI H9N2 detected by HI test in Twisha district.

Location		No. of collection samples	Percentage + ve sample Elisa	No. of + ve sample to Elisa	Percentage + ve sample HI	No. of + ve sample HI
Twisha Farm (1)	H1	15	86.7%	13	46.2%	6
	H2	15	93.3%	14	78.6%	11
	H3	15	93.3%	14	85.7%	12
	H4	15	100%	15	60%	9
	H5	16	100%	16	43.7%	7
	H6	15	100%	15	40%	6
	H7	15	100%	15	53.3%	8
Total Farm (1)		106	96%	102	57.8%	59
Twisha Farm (2)	H1	21	95.2%	20	65%	13
	H2	19	100%	19	57.9%	11
	H3	24	87.5%	21	28.6%	6
Total Farm (2)		64	94%	60	50%	30
Twisha Farm (3)	H1	15	86.7%	13	30.8%	4
	H2	15	93.3%	14	50%	7
	H3	15	100%	15	60%	9
	H4	15	100%	15	53.3%	8
Total Farm (3)		60	95%	57	49%	28
Overall		230	95.2%	219	53.4%	117

Table 6. Comparative between AIV type A detected by Elisa test and specific antibodies AI H9N2 detected by HI test in different districts.

Location	No. of collection samples	Percentage +ve sample Elisa	No. of +ve sample Elisa	Percentage + ve sample HI	No. of + ve sample HI
Wadi Al-Rabia House 1	29	75.9%	22	32%	7
Wadi Al-Rabia House 2	36	97.2%	35	43.4%	15
Al-Asfah House 1	22	91%	20	31%	6
Al-Asfah House 2	26	96%	25	40%	10
Al-Amria	25	100%	25	84%	21
Al-Maamoura	26	88.5%	23	34.8%	8
AL-Kayekh	26	34.6%	9	55.6%	5
Ber Al-Tota	33	36.4%	12	66.7%	8
Overall	223	76. 7%	171	46.8	80

Table 7. Describe the results of tracheal swabs by RT-PCR test.

Type of test	Twisha district	Other districts	
	Farm 3	Al-Maamoura	Ber Al-Tota
	TS	TS	TS
Influenza A	+ ve	+ ve	+ ve
H9	+ ve	+ ve	+ ve

of AI antibodies detected on 19 out of the 24 farms investigated. Furthermore, the wide distribution of AI type A in all studied flocks is a strong indicator of low biosecurity at the farm level in the studied area. Moreover, this result supports previous data published in Libya by Al-Garib *et al*. (2007), Fares *et al*. (2010), and Kammon *et al*. (2015). A positive correlation between a high mortality rate and the detection of specific antibodies against AI-H9N2 has been reported in Libya (Al-Garib *et al*., 2007). Moreover, several studies demonstrated that AI-H9N2 viruses have various pathogenic and mortality effects, from low to high, depending on several factors (Pazani *et al*., 2008). That is why avian AI H9N2 is implicated in inducing different mortality between broiler flocks growing southwest of Tripoli in Libya. On the other hand, the HI test in this study shows that nearly 50% of AIV type A antibodies are positive for AI-H9N2 in high and low motility rate flocks in the same and/ or different regions. The present study interferes with data demonstrating the positive correlation between the presence of H9N2 and high motility in Libya broiler flocks by Al-Garib *et al*. (2007). Furthermore, AI-H9N2 antigen is detected in some studied flocks by using a one-step PCR technique. The detection of AI-H9N2 in Libya has been reported by Kammon *et al*. (2015). This result denies the responsibility of H9N2 by itself in the difference in mortality between broiler flocks in Twisha farms compared to other farms. Those results agree with studies demonstrating that AI-H9N2 viruses have various pathogenicity effects and mortality rates from low to high, depending on environmental conditions, the strain of chickens, age of the affected birds, the health status of commercial chickens, secondary infection, route of infection, as well as sub strains of AIV viruses (Zanella *et al*., 2001; Vasfi Marandi *et al*., 2002; Kianizadeh *et al*., 2006; Pazani *et al*., 2008). High mortality rates of up to 65% are recorded in chickens suffering from a combined infection of H9N2 with *Mycoplasma gallisepticum* and/ or *Escherichia coli* (Vasfi Marandi *et al*., 2002). Also, variable mortality ranging from 5% to 90% is reported in poultry depending on the age of birds, and the presence of secondary infections such as Pasteurella multocida, *Ornithobacterium rhinotracheal*, and Mycoplasmas was reported in Italy (Zanella *et al*., 2001). The different broiler mortality between Twisha farms and other farms could be due to the poultry sheds being low quality in Twisha farms, the presence of HPAI, virulent strains of H9N2, and mixed infection of H9N2 with high virulence viruses like APMV-1 (Newcastle disease) which is capable of elevating mortality up to 70% in unvaccinated flocks in Libyan poultry farms as mentioned by Kammon *et al*. (2015). The present results descend outbreaks on poultry fields in Libya. Therefore, continuous monitoring of the poultry flocks is required to avoid high mortality rates. The seroprevalence comparative of AI type A antibodies with AI H9N2 in the present study are strong indicators of the presence of another subtype of AIV type A (HPAI and LPAI) in studied areas (Al-Jafara and Tajoura municipalities). These concerns had been raised by Al-Garib *et al*. (2007); and Kammon *et al*. (2015). In terms of public health aspects, H9N2 may pose a threat risk to human health and life after being isolated from human flu-like illness in China in 1994, in Hong Kong in 2003,

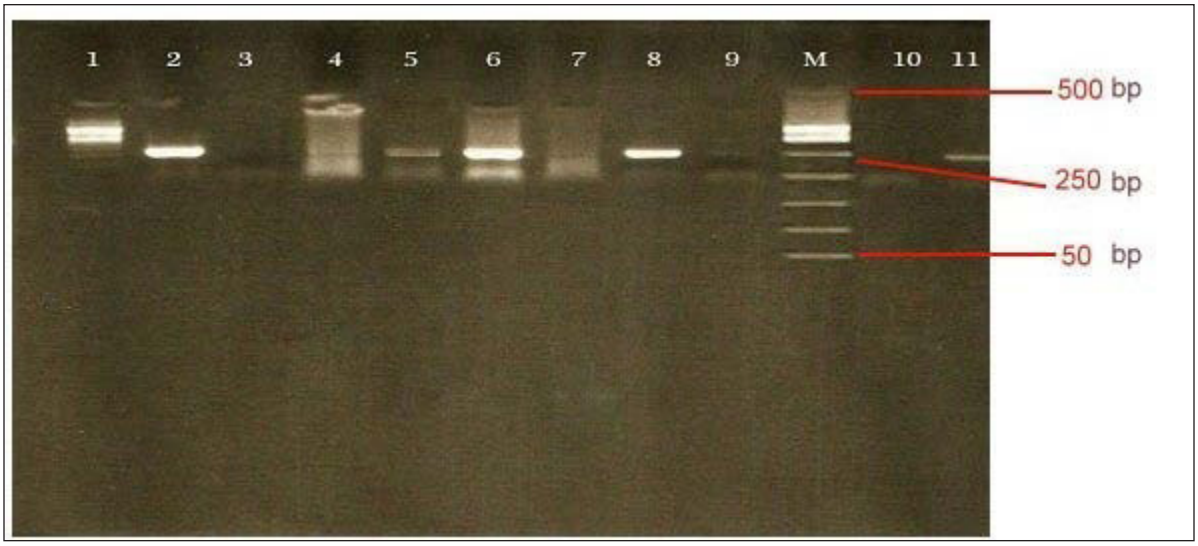

Fig. 1. Electrophoresis for avian influenza A virus. Lanes 2, 5, 6, 8 = avian influenza A virus positive flocks (positive; band at 240 bp). Lane 1, 3,4,7,9 = avian influenza A virus negative flocks. Lane *M* = Ladder marker. Lane 10 = negative control. Lane 11 = avian influenza A virus positive control (positive; band at 240 bp).

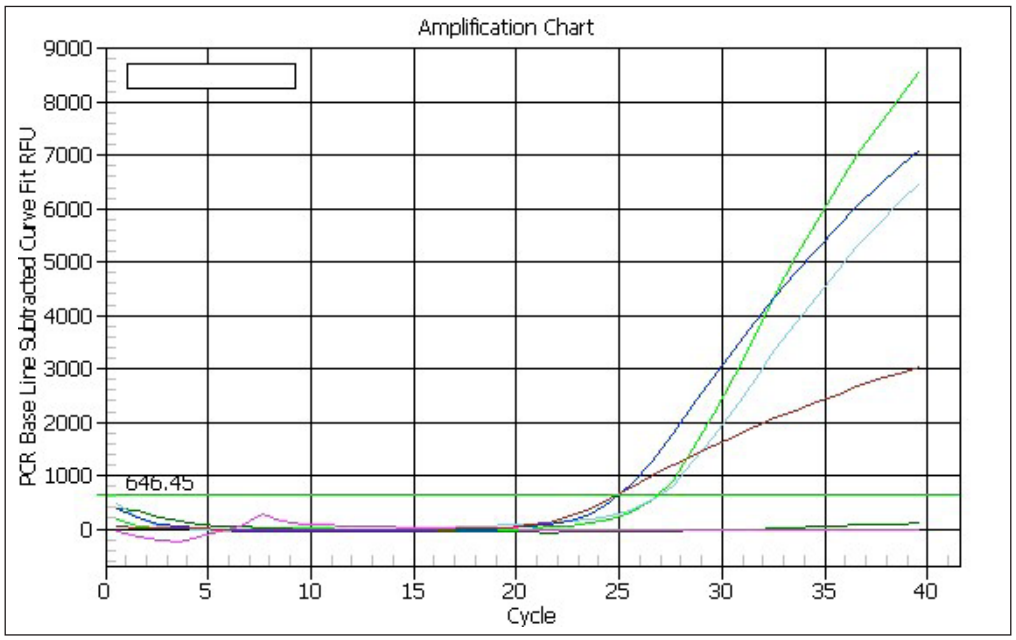

Fig. 2. Real-time PCR amplification and cycling chart.

in Bangladesh in 2006, in Egypt in 2006, in Oman in 2009, and Pakistan in 2003 (Gomaa *et al*., 2015; Pusch and Suarez, 2018; Ali *et al*., 2019; Peacock *et al*., 2019; European Food Safety Authority *et al*., 2023).

Conclusion

The prevalence of AI type A virus antibody was ubiquitous and widely distributed in all investigated broiler flocks. It was 95.2% in the Twisha district and 76.7% in different districts. Furthermore, AI H9N2 was 53.4% in the Twisha district and 46.8% in different districts. According to RT-PCR results, AI-H9N2 antigen was detected in some studied flocks. All those results clarified that H9N2 became endemic in broiler flocks growing southwest of Tripoli-Libya, and it was not responsible for the high mortality rate by itself in broiler flocks. Moreover, this study supported the presence of other subtypes of AI in the studied area.

Since direct contact with poultry is common in Libya, this suggests that transmission of the H9N2 influenza virus into humans is not impossible.

Conclusion

Libyan Veterinary Animal Health must develop intervention strategies through routine serological surveillance programs in poultry for early detection of the causative agent. Phylogenetic analysis studies must conduct to provide information on the origins of Libya isolates. Surveillance and epidemiological studies in different avian species must start to identify the incidence of AI disease. Developing a poultry vaccine program is necessary to combat AI infection. Exchange information with neighboring countries to eliminate the spread of disease. Animal Health Authority with the Ministry must collaborate with Human Health in Libya to prevent human infections.

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