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Molecular Detection of Chicken Infectious Anaemia Virus from Backyard Chickens in Libya

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ABSTRACT

Background and Objective: *Chicken infectious anaemia (CIA) is primarily a disease of young chickens caused by a small DNA virus - chicken infectious anaemia virus (CIAV) belonging to the family Circoviridae. The virus is present in all major poultry producing countries of the world. The vertical transmission, SPF/vaccine contamination, highly contagious, hardy and omnipresent nature and also the potential for inducing marked immunosuppression has placed the CIAV at a global scenario reflecting considerable economic significance. In recent years, the virus has been detected and isolated from commercial chicken flocks of Libya. Moreover there is scarcity of information on epidemiological status of the infectious diseases in backyard chickens (Gallus gallus domesticus) in the Libya. Therefore the molecular detection of circulating CIAV among backyard chickens was evaluated in the current study for the first time in Libya.* ***Methods****: During March 2014, two pooled homogenised samples (consists of; thymus and spleen) from village chickens were collected then stored by using Flinders Technology Associates (FTA) filter paper for DNA extraction. Polymerase chain reaction (PCR) technique was undertaken on the samples using a pair of primers designed to amplify a 1390 base pair fragment in the VP1 (capsid protein) gene of CIAV.* ***Results****: The presence of the expected amplification products obtained by PCR was confirmed by agarose gel electrophoresis. PCR analysis detected CIAV-DNA in the two (100%) tested tissue samples.* ***Conclusion****: Apart from the previous report of serologic evidence of CIAV infection in Libyan backyard chickens, the demonstration of CIAV from naturally infection in these type of chickens has not been reported before in different breeding regions countrywide. The present report revealed presence of CIAV in study population and it could be regarded as a potential threat to chickens raising at the studied area. Under no circumstance should commercial industrial chicken varieties be allowed to come into direct or indirect contact with backyard chickens, and vice-versa. The findings of this study suggest that continuous CIAV surveillance and genetic analysis at commercial chicken and free range flocks are imperative.*

Keywords: ***Chicken Infectious Anaemia Virus, Backyard Chickens, FTA, PCR, Libya.***

INTRODUCTION

Chicken Infectious Anaemia (CIA), an emerging disease mainly of young chicken, characterised by poor weight gain, severe anaemia, aplasia of the bone marrow, lymphoid atrophy, subcutaneous and muscular haemorrhages and increased mortality, has been responsible for considerable health problems and economic losses to the poultry industry [1-4].

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The causative agent, Chicken Infectious Anaemia Virus (CIAV), first reported by Yuasa et al., in 1979 [5], and classified within the newly established genus *Gyrovirus* under the virus family *Circoviridae* [6], is the smallest DNA virus and is now being recognized as an important avian pathogen worldwide [2,7-10]. CIAV has been proven to be a potent immunosuppressive agent for very young unprotected chicks, thereby increasing their

susceptibility to secondary infections i.e., viral, bacterial and fungal agents and depressing vaccinal immunity and production performance in the field situations [11,12]. The virus seems to play a key role in the aetiology of several multifactorial diseases *viz*. haemorrhagic syndrome, haemorrhagic anaemia syndrome, infectious/aplastic anaemia, anaemia-dermatitis syndrome, gangrenous dermatitis and blue wing disease [13-17]. Certain notable characteristics such as vertical transmission, detection in SPF eggs, and its highly contagious, hardy and ubiquitous nature along with the potential for inducing marked immunosuppression have demanded the attention of global poultry production systems towards the CIAV infection [18]. CIAV diagnosis: ELISA-based assays, indirect immunofluorescence assays, and virus neutralization tests [4], but they are recommended for epidemiological study of the virus [19]. The molecular technique of PCR for the direct detection of CAV-DNA in the infected specimens has been employed for diagnosis of CIA. CIAV-DNA has been detected in various samples i.e., infected MDCC-MSB-1cells, chicken tissues such as unfixed liver and lymphoid organ homogenates, formalin fixed liver homogenate or formalin fixed paraffin embedded (FFPE) tissues (thymus, spleen and bursa of Fabricius, etc), serum and blood smears from experimentally or field infected chicks, contaminated vaccines, and in serum samples from disease free chickens [20-29].

Traditionally in Libya, the farmers raising backyard poultry as alternative production system, most of them are kept in free range practice s and scavenge for food, with their adoptability to tolerate the harsh environmental condition. These type of birds requires less startup capital, land, and equipment than other comparable enterprises. Many residents of the city sides keep small flocks of free range chickens, mainly extensively, for domestic use and idles can acquire an revenue through poultry breeding. An important point is that there is a consistently strong market for fresh, farm-raised eggs, which called in colloquial Libyan Arabic: "*Dahee Arbee*". Nevertheless of this, a minor and inadequately information is available on the presence of contagious diseases among rural raised chickens. This may be owing to not receiving sick or dead bird to veterinary investigation. Most the cadavers are eaten by stray predators, or are abandoned, which means the most cases go unreported. There are no routine vaccination programmes against CIA in chicken production systems in Libya.

Furthermore the chickens industry in Libya seem to be affected by CIAV, based on the results of previous published studies indicated serologic evidence of the virus in commercial broilers, broiler breeders (and their progeny) and backyard chicken (*Gallus gallus domesticus*) flocks [30,31], but there is no study conducted in backyard chickens, regarding the extent of viral genome. Subsequently this will help to better understand the epidemiology of the virus and to plan effective intervention strategies for its prevention and control.

Because of resistance, omnipresent and high transmission rate of CIAV and possibility of transmitting the infection to susceptible commercial chickens, and there are still many gaps in our knowledge of the pathology, pathogenesis and epidemiology of CIAV in our country, especially in free range chickens, identify the genome in backyard chickens and estimation of its infection seem to be necessary. Therefore this study planned to describe the molecular detection of CIAV in Libyan backyard chickens for the first time.

METHODOLOGY

Data and Samples Collection

Between 9 to 23 March 2014, the data and specimens were gathered by personal visits to twelve backyard chicken farms in different villages from across Libya (6 farms from Tripoli breeding area were located in: *Wadi Al-Rabe*, *Qaser Ben Ghasheer*, *Al-Asfah*, *Al-Rashah*, *Tajoura* and *Al-Sawani*. While 6 farms from Benghazi breeding area were located in: *Al-Kwefia*, *Al-Aqouria*, *Qemines*, *Al-Abiar*, *Benina* and *Al-Rajma*). All the 12 flocks apparently healthy during the visit. Number of birds per flock is between 25 to 40. The range of birds age was 4-10 months old. The breeds were mixed (indigenous/native, imported breeds, progeny of commercial fowl strains kept as free range and hybrid-cross varieties of them). All participating flocks were raised for egg production. The birds had not been vaccinated against CIAV, and no clinical signs indicating of CIAV infection were observed previously in the various flocks. From each barn, one sacrificed bird was euthanized by cervical dislocation. Of every carcass, one organ was collected (i.e., two pooled tissue samples, composed from 6 organs in each breeding area); in Tripoli barns the pooled tissue sample was the thymus, whereas in Benghazi barns the pooled tissue sample was the spleen.

All pooled tissue samples were inoculated on the Flinders Technology Associates (FTA) filter cards [FTA is a cotton-based cellulose membrane containing lyophilized chemicals that lyses many types of bacteria and viruses], under strict aseptic conditions then stored frozen at -20 °C for subsequent DNA extraction.

DNA Extraction

CIAV-DNA was extracted from FTA punches containing samples using a commercial kit (QIAamp DNA Blood mini kit 250) Qiagen Sciences, LLC, Germantown, MD 20874. USA. After hydrating the FTA-punches-samples with 200µl H_2O, the DNA was extracted according to manufacturer's instructions.

Primers

PCR was performed using primers specific for VP1 (coded for capsid protein) that produced a band of 1390 bp. They were synthesized at Life Technologies, Grandisland, NY 14072. USA. CIAV specific primers as previously described by Hiremath, and coworkers [32]. The sequences are: VP1F 5' AGC CGA CCC CGA ACC GCA AGA A 3' VP1R 5' ATC AGG GCT GCG TCC CCC AGT ACA 3'.

DNA Amplification Profiles

The PCR assay was carried out with HotStarTaq Plus Master mix kit (Qiagen). The PCR buffer containing 1.5mM $MgCl_2$, 200µM of each dNTPs, 0.625U/µl *Taq*DNA polymerase and 10 pmoles of each primer was used for 25µl PCR reaction. The amplification was performed under the following conditions in a thermal cycler (AB 2720 Life Technologies): The reactions were started with denaturation (Initial heat activation) at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec), primer annealing 60 °C for 30 sec, extension 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis in 2% agarose gel (100V, 30 min), and visualized under ultraviolet light after staining with ethidium bromide. The PCR products were visualized by electrophoresis on an ethidium bromide-stained 2% agarose gel (100V, 30 min), and image was visualized using an UV transilluminator.

RESULTS

Analysis of PCR amplification of the extracted DNA from homogenised tissue samples by agarose gel electrophoresis indicated DNA bands of corrected size as expected with a length of 1390 bp. The PCR analysis detected CIAV-DNA in 2 of 2 (100%) tested tissue samples, and is shown in Figure 1.

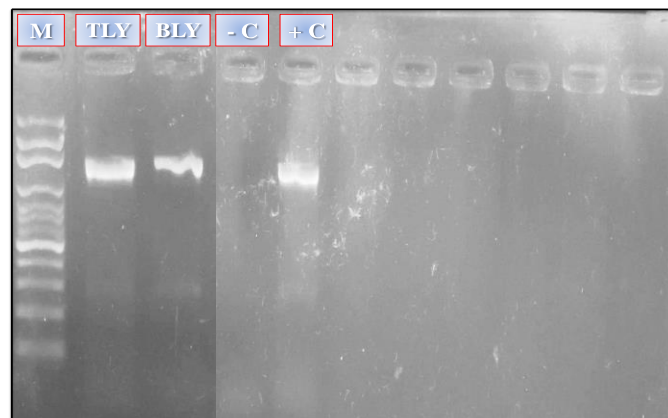

Figure 1: PCR products (1390 bp in size) of amplified CIAV-DNA extracted from tissues of examined birds: Lane: TLY is backyard tissue samples from Tripoli. Lane: TLY is backyard tissue samples from Benghazi. Lane: - C is control negative, Lane + C is control positive, and M: Size marker (500 bp ladder)

DISCUSSION

Although the number of samples was limited, our investigation revealed clearly that CIAV detected in sampling regions. Amplification of DNA extracted from the backyard chickens tissues yielded 1390 bp CIAV-specific bands. Both samples were positive for CIAV by PCR, i.e., this established that 100 percent of samples collected from backyard flocks in the two investigated breeding regions were positive for CIAV. That is mean at least one bird from one flock was infected with subclinical form.

In spite of their importance in related to production, the backyard chickens are still negligible in the scientific researches [30], as well as the available data on the prevalence of important pathogens and their effects on industrially-produced chickens are quite finite in Libya. To our knowledge, this is the first study that describes molecular detection of circulating pathogen among backyard chickens, and also is the second published research conducted in general on this subspecies in Libya.

Although the potential importance of backyard chickens in epidemiology of CIAV, few studies have been conducted globally on these chickens. For instance, in Nigeria, 75% of the studied backyard chickens were

positive for serum CIAV-DNA [33], 90% of native chickens evaluated in Ecuador presented CIAV-specific antibodies in the serum [34], in Tripoli, Libya the prevalence-within-flock ranged from 0% to 75% in backyard, and the flock prevalence was 83% [30], while the results of study undertaken in Bulgaria found that CIAV reagents were detected in all villages surveyed with seroprevalence rates ranging from 84.4% to 100% [35], and in Brazil with 30% [36]. Despite results of serological tests suggest that CIAV alone has no zoonotic significance [4], but certain notable characteristics such as vertical transmission, detection in SPF eggs, and its highly contagious, hardy and ubiquitous nature along with the potential for inducing marked immunosuppression have demanded the attention towards the significant aetiology.

The wide application of PCR particularly for CIAV diagnosis includes the direct detection of CIAV in the infected tissues, as an aid for screening and selecting specimens for virus isolation, detecting contamination with CIAV of cell line, virus preparation and vaccines, screening of breeder SPF/commercial flocks and to study molecular epidemiology of CIAV [31]. It can detect even when the concentration of the virus is very low in the field samples or to detect subclinical infections. Further, the test can be very useful where the facilities for MSB-1 cells are not available for virus isolation [24,37]. High sensitivity is achieved with a PCR, which is also most sensitive to cross-contamination [37,38]. Therefore, PCR has been the technique of choice for diagnostic purposes and is replacing the cumbersome and time consuming conventional techniques. Several investigators have used thymus, spleen, bone marrow, and liver [23,39]. Interestingly, CIAV-DNA was detected by PCR in the thymus and spleen from which CIAV could not be isolated. Todd et al., have extracted DNA from different tissues and found that the thymus always contained more viral DNA than did spleen or liver [39]. These studies indicate the importance of the thymus as target tissue for CIAV-DNA extraction.

CIAV origin for backyard chickens is presumed, taking into consideration its widespread incidence in the chicken industry. Previously, one solely published serological study in Libya was determining the presence of specific antibodies to the virus in backyard chickens [30]. Accordingly, this study was carried out also on backyard chickens and was more comprehensive because CIAV positive chickens were detected using molecular technique.

The time required for maternal antibodies to decay is about 3 weeks [40-42]. After 3 weeks of age, the virus cannot produce clinical signs of disease in chicks but, as a result of the accompanying immunosuppression, made them susceptible to secondary bacterial, viral, and fungal infections, and also increase of morbidity and mortality of these diseases with decrease response to the vaccination [43]. In the current study the bird aged 4-10 months, this mean the infection was subclinically. Subclinical infections with CIAV reduced development of antigen specific cytotoxic T lymphocytes [44] and also adversely-affected macrophage function [45], thus resulted in increased susceptibility to diseases caused by other infectious agent and is one of the most significant mortality which is caused by CIAV infection.

Depending on the results of serological profile of the affected flocks in preceding paper [30], and the findings of the current investigation with the genome detection of the examined flocks clearly enough demonstrated the presence (both clinical and subclinical forms) of CIAV infection in a great extent, on the basis of these results, it can be assumed that CIAV is at least a part of lower growth performance, inadequate response to vaccination, and is another one of the important causes of immunosuppression in Libyan village chicken populations.

In Libya there are several villages and majority of villager family is rearing these backyard chickens to produce the egg for their own consumption. These birds are raising in a free range manner. On the other hand there are many industrial chicken houses nearby these villages. Diseased backyard chickens constitute a potential risk in transmission of the virus to commercial industrial chicken flocks resulting in economic impacts for chickens industry sector. Consequently control of CIAV in free range chickens should be an integral part of any measures to control this virus in commercial chickens, and vice-versa. The uncontrolled movement of free range chickens as well as their scavenging nature predisposes them to CIAV infection as the virus is known to persist in the environment [2]. Hence there is necessity for providing adequate immunity of chicken flocks by use of vaccine in these birds weeks before egg production as a means of averting the acute disease in their offspring's with sound management, hygiene and

strict biosecurity practices will be of immense help in preventing young chicks from early exposure to CIAV as well as co-infections with other lymphocidal agents especially Infectious Bursal Disease Virus (IBDV) following suitable vaccination programs, so as to limit immunosuppression and reduce the economic losses [31].
The emergence of new serotypes cannot be excluded and would have important consequences for vaccine efficacy and serodiagnosis, so we advise to perform experimental studies and constant monitoring of this virus in the field for emergence of any new variants and consequent change in pathogenicity [31].
FTA technology had developed as a simple method to collect samples and extracting DNA for further analysis of DNA in various fields [46]. The present study it goes without shed light upon that the use of FTA for the collection of field tissue samples and simultaneous inactivation of pathogens is feasible, as well as allows the movement of specimens for long distance without interference with the approach of molecular detection. It is cheap and easy to handle.

CONCLUSION

CIAV may have gone undetected for years in rural backyard chickens flocks in most parts of the world, being plays a vital role as a harbour of the virus to commercial chickens. This is the first report that describes the molecular detection of circulating CIAV in Libyan chicken flocks. The findings presented in the current study clearly exhibited that CIAV-DNA is detected in the study area. Implying that this virus is considered to be a threat to village chicken production system. In addition, there could be a spillage over effect in which backyard chickens can serve as source of infection for the growing small scale poultry farms in the country or vice-versa. The present study provides a basis for future epidemiological researches on CIAV in Libyan chicken populations, although more sequences data are required globally, existing results thus emphasize the importance of CIAV. Consequently, focus must be made on the molecular epidemiology CIAV and to further advocate and achieve molecular analyses so as to advise appropriate control schemes for this economical significant chickens pathogen.

DISCLOSURE STATEMENT

The authors declare that there is no conflict of interest.

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