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PHYLOGENETIC ANALYSIS OF HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA ISOLATES FROM EGYPT BASED ON CLEAVAGE SITE SEQUENCE ANALYSIS

By

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ABSTRACT

Avian influenza virus A (AIV) is found in most instances in birds, and rarely in humans. Since 1997, there have been isolated occurrences of the H5N1 subtype in humans. In the current investigation, the emerged highly pathogenic avian influenza subtype H5N1 was isolated from infected birds from different governorates (Kaliobya, Sharkyia, Mansora and Gharbia) over a five year period from 2010 to 2015.birds was suffering acute infection and showed high mortality rate and molecularly assessed by PCR amplification of the cleavage site and full Haemagglutinin gene then sequencing. Sequence analysis discovered some variation across various isolates from various years. Analysis of the multi-basic amino acid sequence of the isolated virus revealed the presence of the characteristic cleavage motif of the highly pathogenic H5N1 (PQ↓ (G/R) EKRRKKR↓GLF) while some strains having differences in the (G>R) a single amino acid within the cleavage site motif. The deduced amino acid sequence analysis of the isolated nine strains of HPAI (H5N1 subtype) revealed a high degree of homology.

Keywords:

Avian influenza; Hemagglutinin gene; cleavage site; sequencing.

INTRODUCTION

Avian influenza is caused by a virus member of the family Orthomyxoviridae, genus influenza virus A. Diagnosis based on the isolation of the virus followed by molecular characterization of the cleavage site to Decemberlare its pathogenicity nature. Infections in birds are characterized by a wide variety of clinical manifestations that may vary according to the species, the strain of the virus, the host's immune status, the presence of any secondary exacerbating organisms, and environmental conditions **(OIE 2012, Harfoot and Webby 2017).**

Infection in chickens with highly pathogenic avian influenza virus (HPAIV) H5N1 is similar to infection with other HPAIV viruses **(Palese *et al.,* 1974; Els *et al.,* 1989 and Perkins and Swayne 2001).** Clinical signs include anorexia, ruffled feathers, swollen hemorrhagic necrotic wattle and comb, congested legs, cyanosis, dermal hemorrhage, and coma, and may lead to peracute death without clinical signs **(Kobayashi *et al.,* 1996 and Swayne 1997 and Samah Mosad *et al.,* 2020).** Gross lesions include subcutaneous edema, mottled pancreas, petechial hemorrhage on the surface of serosa, splenomegaly, swelling in the kidney, systemic congestion and hemorrhage, pulmonary congestion and /or hemorrhage and edema with lung consolidation, conjunctival hyperemia, edema and hemorrhage of the intestinal tract**. (Abou-Rawash *et al.,* 2012 and Bakeer *et al.,* 2019).**

Avian influenza virus's pathogenicity depends on the sequence of the cleavage site (CS); HPAIV possess multiple basic amino acid residues, which is a prerequisite factor for pathogenicity in chicken **(Abolnik, 2017),** while the low pathogenic avian influenza virus (LPAIV) strains do not. Haemagglutinin (HA) sequences with monobasic cleavage site (e.g." HA1-PSIQVR-GL-HA2") are cleaved by striptease produced by respiratory and digestive tract epithelium **(Air and Laver 1989; Whittaker 2001; Chen *et al.,* 2004 and Luczo *et al.,* 2015).** Haemagglutinin gene sequences with polybasic amino acid cleavage sites (e.g." HA1-KKREKR-GL-HA2"), allow proteolysis by proteases such as furine and pro-protein-convertase6 (PC6) found in Golgi apparatus of allcells **(Horimoto *et al.,* 1994 and Thorlund *et al.,* 2011).** AIV with polybasic cleavage sites has an unlimited distribution network and may cause fatal systemic infection. A cleavage sequence containing several basic amino acids is more readily activated by cellular proteases present in a variety of cells distributed throughout the body compared with a cleavage sequence containing only a single basic amino acid, which can be cleaved by a limited range of cellular proteases. It is well-accepted that influenza viruses containing multiple basic amino acids have multiple sites of virus replication and produce more severe infections in birds and mammals **(Zambon, 2001; Kandeil *et al.,* 2017 and Luczo *et al.,* 2018).**

Since the Haemagglutinin (HA) gene is the most important gene that shape and characterize the pathogenicity of AIV, the current research focuses on the molecular characterization and genotypic analysis of HA gene in nine different AIV strains that were isolated from Egypt over a period of five years from 2010 to 2015.

MATERIAL AND METHODS

Virus isolation:

Sampling and study area:

Over the period 2010 to 2015, we collected tracheas from different infected flocks from different Egyptian governorates (Kaliobya, Sharkyia, Mansora and Gharbia). We collected about 30 tracheas per year from severely infected flocks and were transferred on ice to the laboratory. Tracheae of morbid or freshly died birds from poultry farms having severe clinical signs of influenza infection were swabbed. The solution was then centrifuged at 7000 rpm/ 10min/4°C, 9 days old SPF chicken eggs were candled and inoculated with 0.2 ml of the supernatant **(Xu *et al.*, 1999 and Wu *et al.*, 2008)** via the allantoic route. The eggs were incubated at 37°C in a humid chamber until embryo death (Usually within 18 hours) and the chorioallantoic fluid (CAF) was collected and clarified by centrifugation.

Virus identification:

The clarified virus from the allantoic fluid was identified by haemagglutination (HA) **(Killian 2008)** and haemagglutination inhibition (HI) assays **(Potter and Oxford 1979 and Katz *et al.*, 2009)** using monospecific antisera against H5N1. The validity of the results was assessed against negative control serum. Monospecific antisera against Newcastle disease virus (NDV) and Adenovirus were used to test the purity of the isolated H5N1 stains from extraneous haemagglutinating agents.

Amplification of the cleavage site and the full length H5 gene from all the nine isolates of HPAIV by RT-PCR amplification:

Viral RNA was extracted from the clarified allantoic fluid **(Sambrook *et al.*, 1989),** by QIAamp Viral RNA Mini Kit (Qiagen Germany, cat #52904), according to the manufacturer's instructions. The cleavage site and full-length H5 gene of the H5N1 isolates were amplified using Affinity Script One-Step RT-PCR Kit (cat # 600188). Fifty nanograms of the purified RNA were included in each reaction using specific primers for the cleavage site or the full H5 gene .The amplicons separated by electrophoresis on a 1% agarose and the size of the amplicons was determined using SynGenetool software V4.01(SynGen Corporation, Cambridge, England) using *gene* ruler 100 bp plus DNA ladder (Thermo scientific cat#SM0322).

A previously well-identified HPAIV **(Soliman *et al.*, 2016)** was included as a positive control.

Sequencing:

The complete nucleotide sequences of the cleavage sites and full-length Haemagglutinin gene of the 9 isolated strains of HPAI-H5N1 were studied in the current paper. For the preparation of the gene for sequencing, the PCR product was separated on 1% low melting agarose. The corresponding bands were purified with the Biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacturer. Sequencing was performed using BigDyeTM Terminator Cycle Sequencing Kits, following the protocols supplied by the instructions (ABI PRISM 3730XL Analyzer, Applied Biosystems). Samples were subjected to electrophoresis in an ABI 3730 XL sequencer (Applied biosystem) in a single pass sequencing process.

Analysis:

The nucleotide and deduced amino acid sequence analysis and the phylogenetic trees construction was done using DNASTAR V15. For comparison different Egyptian isolates were retrieved from the gene bank (https://www.ncbi.nlm.nih.gov/nuccore) and used for nucleotide and deduced amino acid sequence alignment using Clastal*W* algorithm as well as phylogenetic tree alignment.

Table (1): The geographic distribution and the chicken breeds of the isolated strains of (HPAI- H5N1 subtype) during 2010-2015.

Virus mane	Year of isolation	Chicken breed	Governorate
A/chicken/ Kaliobya /ch1.12.3/2010 (H5N1)	**December-2010**	**Cobb**	**Kaliobya**
A/chicken/ Kaliobya /ch1.12.5/2010 (H5N1)	**December-2010**	**Baladi**	**Kaliobya**
A/chicken/ Sharkyia /ch2.1.6/2011(H5N1)	**January-2011**	**Lohmann**	**Sharkyia**
A/chicken/ Kaliobya /ch2.1.7/2011 (H5N1)	**January-2011**	**Cobb**	**Kaliobya**
A/chicken/ Mansora /ch2.2.12/2011 (H5N1)	**February-2011**	**Arbo**	**Mansora**
A/chicken/ Kaliobya /ch2.1.10/2011 (H5N1)	**January-2010**	**Lohmann**	**Kaliobya**
A/chicken/ Kaliobya /ch2.2.18/2011 (H5N1)	**February-2011**	**Lohmann**	**Kaliobya**
A/chicken/ Kaliobya /ch11.5.9/2014 (H5N1)	**May-2014**	**Cobb**	**Kaliobya**
A/chicken/ Gharbia /ch15.1.12/2015 (H5N1)	**January-2015**	**Baladi**	**Gharbia**

Table (2): Primer sequences used for amplification of the cleavage site and the full length Haemagglutinin gene of Egyptian AI isolates.

Name	Target	Sequencing primer	Size of the amplicons
CsH5-f	Cleavage site	5'–CCTCCGAATATGCGTAG -3'	315 bp
CsH5-r		5' - TACCAACCGTCTACATGCCG -3'.	
H5-f	H5 gene	5' - AGCAAAAGCAGGAAAGGTTAAAAGGA-3'.	1710 bp
H5-r		5' - TAGCAACAAGGAGAGTTTTTGAACAACC -3'.	

RESULTS

Virus isolation and characterization:

The clinical symptoms of AIV infection in chicken farms included a sudden halt in egg production, petechial hemorrhage (spots-like) on the shafts on one or both, and cyanosis of combs. In the postmortem examination (PM), there was Hemorrhage of the muscular tissue, clear coronary fat and peritoneal petechial hemorrhage. The mortality percentage exceeds 90% of the birds within 3 days. Dead birds were delivered to the laboratory on ice, with individual tracheal swabs for virus isolation.

Hemagglutination test:

The CAF obtained from all inoculated SPF eggs was initially subjected to slide HA test to ensure he presence of haemagglutinating agents. The samples gave clear positive HA results with titer ranging from 9 -12 log2 that depends mainly on the virus titer in the collected samples.

Hemagglutination inhibition test:

The CAF of each sample was diluted (4 $\log_2$ HA units) and used for HI test using anti-H5N1 monospecific antisera, Newcastle disease virus antisera, or Adeno virus antisera. All the test samples were negative to the monospecific antisera against Newcastle disease virus or Adenovirus, but all samples were strongly positive using the monospecific antisera against H5N1. The titer ranged from 5 - 10 $\log_2$.

RT-PCR:

The H5 cleavage site and the full-length H5 gene of the HPAIV were targeted by unique primers during molecular investigation by RT-PCR on SPF eggs with embryo mortalities between

18-24 hours and positive HA and HI. The cleavage site was identified as a distinct, highly intense band with a molecular size of 315 bp, while the full H5 gene was identified as a band of 1710 bp .

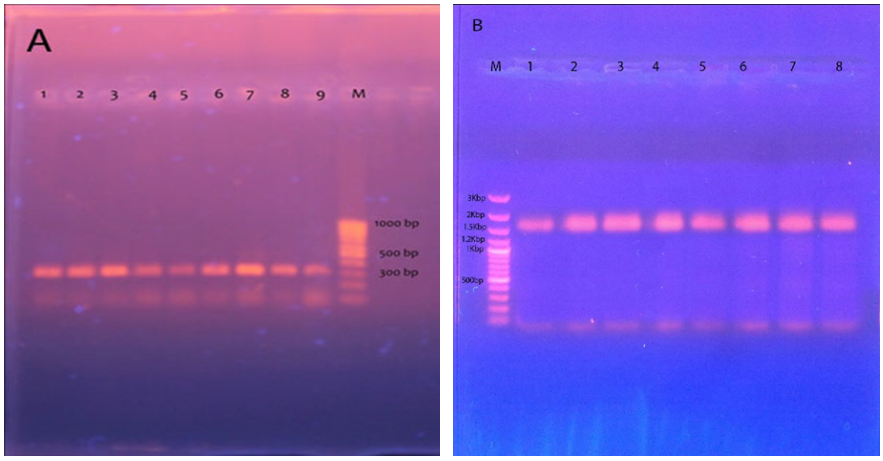

Fig. (1 A): Representative amplification of the cleavage site and amplification of the full Haemagglutinin genes from the selected isolates for sequencing **(B).** M: gene ruler 100 bp plus DNA marker.

Sequencing of the cleavage site and the full-length Haemagglutinin genes:

The full-length H5 gene sequence of the 9 isolated strains of HPAI-H5N1 along with the retrieved sequence was aligned based on both the nucleotide and deduced amino acid sequence and the phylogenetic tree has been constructed. Concerning the cleavage site, only 2 substitutions were found among the studied strains of HPAI-H5N1 where G>R in one isolate (Chicken-1.12.3-CLEVB-2010) this mutation is found in the first isolate of H5N1 in china (Goode-GD-97) isolate and in one isolate of 2013 (Chicken-13133S-2013). The remaining studied isolates retain the cleavage site motif signature of GERRRKKR. Based on the nucleotide sequence, all Egyptian isolated were grouped into four closely related groups all belonging to clade 2.2. With a difference not exceeding 2% of the total nucleotide compositions when deduced amino acid was aligned and construction of the phylogenetic tree was conducted Fig. (4) However, the isolated were gathered in only 3 groups with all the currently studied isolated grouped in the 2nd group. It was noticed also the GD isolate of China (The first isolated H5N1

strain in GD provincial of China) was widely apparat from all the Egyptian isolates with on the bases of nucleotide or deduced amino acid sequence.

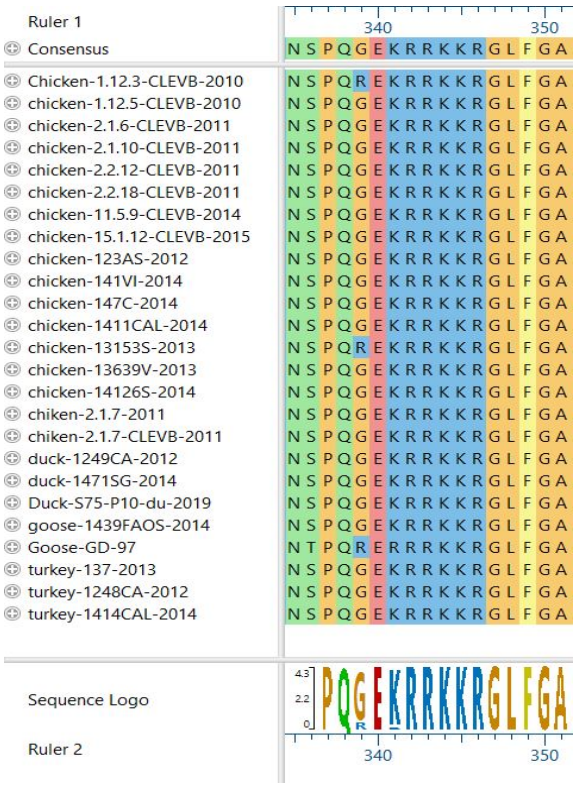

Fig. (2): Deduced amino acid alignment of the cleavage motif of the isolated HPAI-H5N1 strains in comparison with other Egyptian AI strains. Note that, the (GEKRRKKRG) sequence with entirely conserved among all isolates.

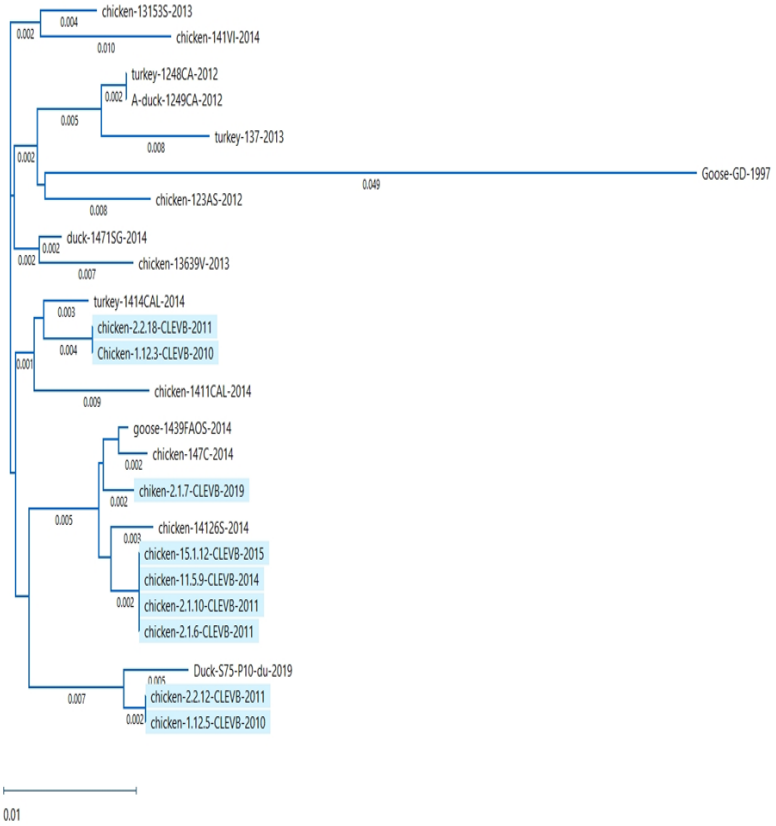

Fig. (3): The phylogenetic tree constructed based on nucleotide sequence of the full H5 of the strains isolated in the current study (blue labelled) as well as other Egyptian isolates retrieved from the gene bank covering the period from 2010-2019.

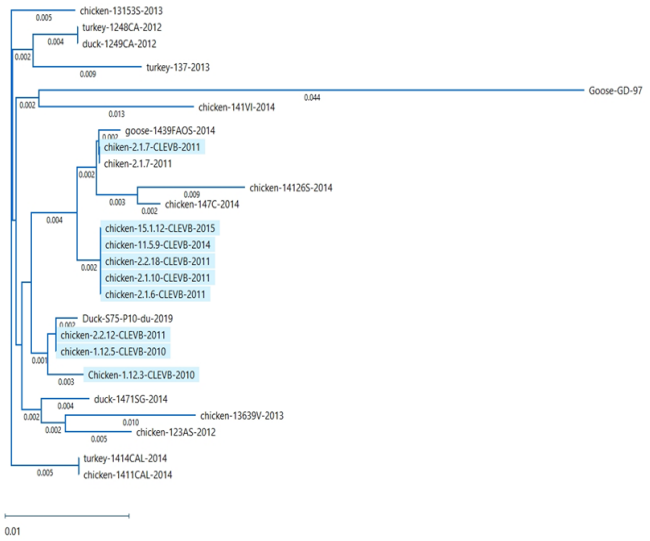

Fig. (4): The phylogenetic tree constructed based on the deduced amino acid sequence of the full H5 of the strains isolated in the current study (Blue labeled) as well as other Egyptian isolates retrieved from the gene bank covering the period from 2010-2019.

DISCUSSION

Avian influenza viruses of the H5N1 subtype have migrated from Southeast Asia to central Asia and the Middle East to Europe and Africa since they first surfaced in 2003. They do this by infecting wild birds and animals. Each year, new influenza viruses appear, and this causes significant genetic variation among H5N1 viruses. **(Wu, 2008 and Erfan 2020).**

Highly pathogenic AIV is defined as having an intravenous pathogenicity index of at least 1.2. **(Hoffmann *et al.*, 2007).** Only the strains of the H5 and H7 subtypes of HPAIV that caused a horrific outbreak of the disease in poultry were still present. **(Capua and Mutinelli, 2001).** The Haemagglutinin (HA) precursor protein (HA_0) has been hypothesized to contain a polybasic cleavage motif with subtilisin-sensitive endoproteolytic characteristics as a potential indication for HPAIV. **(Perdue and Suarez 2000).** The monobasic composition at this motif is

revealed by AIV strains that possess little pathogenicity, nevertheless, and becomes targeted by tissue-specific, trypsin-like proteases. **(Bogs *et al.*, 2010).**

Freshly harvested hens from representative governorates were used as samples for detailed molecular analysis of the H5 gene's cleavage site. The farms where the samples came from had fatalities that were nearing 100%, with the vast majority of cases presenting few signs. Hemorrhagic patches on the shank and coronary tissue were prevalent symptoms in several farms with rather low mortalities. These results were also reflected in the virus titration; several of the isolates produced very high HA titers, which were confirmed by back titrating the virus, as well as very high titers for the HI titer utilizing 4HA units. The high viral load in the farms or the lack of effective vaccination programs may be contributing factors to these findings. Additionally, some inadequate vaccination protocols might trigger immune stressors, which can ultimately result in vaccination-induced mutations that allow the virus to evade the immune response. **(Cameron *et al.*, 2008).**

The isolates were assigned for detailed molecular analysis employing amplification of the HA gene's cleavage site. Every tested strain had a positive amplicon at about 300 bp for the CS, confirming that each strain represented as HPAI H5N1.

All nine H5N1 isolates originating from Egypt are classified as highly pathogenic avian influenza due to the presence of a particular amino acid sequence PQGE (R/G/) RRKKR↓GLF at the cleavage site in the HA molecule, indicating their high virulence **(Horimoto *et al.*, 1995 and Luczo *et al.*, 2015)** and representing the clade 2.2. Other slightly different sequences have been seen in other clades (Clade 2.3) whereas the sequence QRERRRKKR or QRESRRKKR were isolated from 2003 -2007 in South East Asia **(Susanti *et al.*, 2008).**

This cleavage sequence was slightly different from that of A/HK/156/97(H5N1) virus PQRERRRKKR↓G as the 3rd amino acid was substituted from R in Hong Kong strains to G in all Egyptian strains except only 2 isolated **(Subbarao *et al.*, 1998).** Avian influenza viruses with high and low levels of pathogenicity differ in their cleavage sequence, the former possess multiple basic amino acid residues, while the latter does not. A cleavage sequence containing sundry basic amino acids is more readily activated by cellular proteases present in a variety of cells distributed throughout the body compared with a cleavage sequence containing only a single basic amino acid, which can be cleaved by a limited range of cellular proteases. HA sequences with monobasic cleavage site (e.g. HA1-PSIQVR-GL-HA2) are cut by tryptase yielded from respiration and digestive tract epithelia **(Whittaker 2001; Chen *et al.*, 2004).**

On the other hand however, the presence of polybasic amino acids at the cleavage site (e.g. HA1-KKREKR-GL-HA2) allow the proteolysis process to be advocated by proteases such as furin and pro-protein-convertase 6 (PC6) found in Golgi apparatus of all cells **(Horimoto *et al.*, 1994).** AI virus with polybasic cleavage sites have an unlimited distribution network and may cause fatal systemic infection.

The strains exhibiting elevated virulence markers have been detected solely in a pair of the 17 delineated Haemagglutinin subtypes present in avian populations, manifesting sporadically but inducing catastrophic morbidity in domestic avian assemblages. **(Horimoto *et al.*, 1995).** The process of ubiquitous cleavage is attributed to the addition of supplementary basic amino acids at the cleavage site. **(Senne *et al.*, 1996 and Munch *et al.*, 2001),** having a minimal amino acid motif composed of R/L-X-R/L-R. Prior to the present time, this genetic alteration had solely been detected in avian strains belonging to the H5 and H7 classifications, which were not previously believed to be pathogenic to humans. In 1997, a significant breakthrough occurred in Hong Kong when 16 individuals contracted an avian H5N1 influenza virus, thereby overcoming a major obstacle. **(CDC 1998; CDC 2006 and CDC 2011).**

Phylogenetic analysis of the 9 isolates strains revealed that all samples were grouped together with 99 % confidence except isolate chicken-1.12.3-CLEVB-2010 which showed some degree of heterogeneity. These strains shared similar amino acid sequences at the cleavage site {PQ↓ (G/R) EKRRKKR↓GLF} and also the same HA titer (11 log 2) and HI titer.

Deleted: ¶

CONCLUSION

To summarize, the Egyptian isolates obtained from various governorates exhibited negligible variations in both HA and HI titer, and demonstrated a low level of diversity in terms of nucleotide and amino acid sequence, particularly at the cleavage site of the HA gene and full H5 gene.

Compliance with Ethical Standards:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest:

Each author claims the right to declare that either had any conflict of interest.

Ethical approval:

"All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted."

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