Surveillance and Identification of Avian Influenza Subtype H9 in West Delta Governorates in Egypt

Awad, A.M.[1], Sedeik, M.E.[2], Abdalla A.Seliem[3] and Suzan A. Dabour[4]

[1,2] Department of Avian and Aquatic animal, Faculty of Veterinary Medicine, Alexandria University
[3,4] Reference laboratory for veterinary control on Poultry Production (Central lab and Behera branch), Animal Health Research Institute, P.O. Box 246 - Dokki, Giza, Egypt 12618)
seddeeklab@yahoo.com

Abstract: The present Study was investigated for Surveillance and Identification of avian influenza subtype H9 in commercial chicken farms in West Delta region in Egypt (Alexandria, El-Behera, Gharbia, Marsamatrouh and Kafr El-SheikhGovernorates) during late 2012 to 2014. There were 150 flocks (142 broilers, 6 layers, 2 breeders) of different ages with different mortalities showing respiratory manifestation. Tracheal swab samples were collected and tested for avian influenza viruses (AIV) by real-time RT-PCR. 70.4%,33.3% and 0% of samples tested by rRT-PCR were positive for AI matrix gene in tested broilers, layers and breeders flocks, respectively. Highest incidence was found in broiler flocks followed by layers flocks with a total incidence of 102/150 (68%) in all chicken sectors. Subtyping using real-time RT- PCR for H9 subtypes has been done. The results were positive for H9 by percentage of 56.3%, 16.7%, 0% respectively in broiler, layer and breeder flocks were recorded with a total incidence of 54 % (81/150) for H9 in all species of chickens farms. Sequencing of HA gene from selected seven AIV (subtype H9) viruses isolated during 2012 to 2014 and the phylogenetic trees were constructed. Circulation of avian influenza continues to threaten public and animal health in Egypt, and continuous surveillance for avian influenza virus is needed.

[Awad, A.M., Sedeik, M.E., Abdalla A. Seliem and Suzan A. Dabour. **Surveillance and IdentificationAvian Influenza Subtype H9 in West Delta Governorates in Egypt.** *Nat Sci* 2015;13(2):30-36]. (ISSN: 1545-0740). http://www.sciencepub.net/nature. 6

Key words: RT-PCR; Avian influenza virus; subtype- H9; isolation; Hemagglutinin; Sequencing

1. Introduction

Avian influenza is caused by type A influenza virus of the family Orthomyxoviridae. Type A influenza viruses are serologically categorized according to antigenic difference of 2 surfaces of glycoprotein into 18 HA (H1-H18) and 11 NA (N1-N11) subtypes **(Tong *et al.*, 2012, 2013)**.

Subtypes (H1-H16) have been identified in wild and domestic birds, pigs, horse and human **(Stubbs, 1948; Swayne and Halverson, 2003; Fouchier *et al.*, 2005**). Whereas H17 &H18 were identified in new world bats **(Tong *et al.*, 2012, 2013).** An outbreak of HPAI can occur in any country, due to the transmission of LPAI strain from reservoir into poultry followed mutation presumably in gallinaceous poultry, to high pathogenicity **(OIE Manual, 2009**).

In the field, influenza A viruses infecting poultry are divided into two groups based on their apparent pathogenicity: highly pathogenic avian influenza (HPAI) which cause generalized rather than respiratory disease result in flock mortality as high as 100% and low pathogenic avian influenza (LPAI) which usually present as a much milder respiratory disease with low mortality if there are no secondary viral and /or bacterial infection or poor environment condition **(OIE Manual, 2008).**

The H9N2 virus was the first reported in 1966 in the United States **(Homme and Easterday, 1970)**. Since that, the virus has been isolated and reported many times from various countries **(Alexander, 2003; Senne, 2003)**including Hong Kong (in 1975, 1985, 1992, 1994, 1997), China (in 1994), and the Middle East (in 2001). The H9N2 virus was first isolated from chickens and domestic ducks in Jordan in 2003 **(Monne *et al.*, 2007)**.In November 2011, H9N2 infection was 1St reported in Egypt in bobwhite Quail **(El-Zoghby *et al.*, 2012)**.

The H9N2 has been recorded in the Middle East region for several years, indicating additional risk factors to the poultry industry. Although H9N2 viruses are characterized as LPAI viruses, they may cause high morbidity and mortality. However, it is a major concern that the spread of H9N2 in Egypt can negatively affect poultry health overall and increase the risk of infections of H5N1 HPAI, which is already endemic there **(Park *et al.*, 2011).**

The diagnosis of avian influenza (AI) virus infections, represents a considerable challenge due to lake of pathognomonic or specific clinical signs and their variation in different avian hosts plus the marked antigenic variations among influenza A viruses. Conventional laboratory techniques involve the isolation, identification and characterization of the

virus has proven successful in the past and remains the method of choice for at least the beginning of the outbreak. Molecular techniques are being used and in particular reverse transcriptasepolymerase chain reaction (RT-PCR) and real-time RT-PCR technologies (for direct detection of AI viral protein or genes in specimens such as swab, tissue) for rabid diagnosis **(Alexander, 2008)**.

2. Materials and Methods

Field samples

A total of 1500 tracheal and cloacal swabs were collected from 150 chicken farms) broiler, layer and broiler breeder(from 5 governorates in Egypt (Alexanderia, El-Behera, Gharbia, Marsamatrough and Kafr El-Sheikh) showing relatively moderate respiratory signs. Swab samples were collected in medium containing 50% glycerol, 50% phosphate-buffered saline (PBS), penicillin (2 × 106 U/L), streptomycin (200 mg/L), and amphotericin B (250 mg/L) (antimicrobial drugs from Lonza, Walkersville, MD, USA). Samples were chilled on ice until delivered to the laboratory (within 24 hours). All samples were stored at –80°C until used. The samples were collected from 2012 to 2014.

Virus isolation through chicken embryo Inoculation

Samples that showed a positive reaction in the partial M segment RT-PCR were grown in the allantoic cavities of 10-day-old specific pathogen–free embryonated chicken eggs. Virus titers were determined by chicken red blood cell hemagglutination assays.0.2 ml inoculums inoculated aseptically into the allantoic cavity.SPF eggs obtained from (SPF production project, KomOshim, El Fayoum, Agriculture Research Center- Ministry of Agriculture, Egypt).

Microtiter Plate Haemagglutination (HA) and HI test

This test was done according to **(OIE, 2012)** using U-shape bottomed microtiter plate and 1% chicken RBCs against 4 HA unit of H9 Egyptian virus antigens. This test was useful for the rapid detection of HA activity in harvested allantoic fluids from inoculated SPF embryonated chicken egg (ECE).

Real-time Reverse Transcription polymerase Chain Reaction (rRT-PCR)

The real–time RT-PCR for AIV was done using Quantitect probe RT_PCR kit with VLA modified protocols **(VLA, 2008).** The RNA from swab samples were extracted using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) and the rRT-PCR was done using Qiagen one step RT-PCR Kit (Qiagen, Germany).

Sequencing and phylogenetic analysis

The extracted RNAs of the seven positive AI samples were submitted to the gene analysis unit of the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, for sequencing and genetic analysis

3. Results

Results of the examined broiler chicken flocks:

A total number of 142 broiler flocks in different governorates (Alexandria, El-Behera, Gharbia, Marsamatrouh, and Kafr El-Sleikh) were subjected to virus identification by real –time RT-PCR test. Results presented in **(Table 1)** revealed that AI (matrix gene) virus was detected among broiler flocks by RT-PCR test in Alexandria, El-Behera, Gharbia, Marsa matrough, and Kafr El-Sleikh. The total incidence of AIV(70.4%) 100 out of 142 for matrix gene and (56.3%) 80 out of 142 for H9 gene within all tested broiler flocks.

Results of the examined layer chicken flocks

A total of six layer flocks in different governorates in Alexandria, Gharbia and El-Behera were subjected to virus identification by real –time RT-PCR. Results presented in **(Table 2).** The total incidence of AIV (33.3%) 2/6 for matrix gene and (16.7%) 1/6 for H9 infection within all tested layer flocks.

Results of the examined breeder chicken flocks:

Two vaccinated breeder flocks in El-Behera were tested for virus identification by real –time RT-PCR test **(Table 3).** Results revealed that AI (H9) virus was negative for the two tested farm in El-Behera at a percentage of (0%) 0 out of 2. Total incidence of AIV (0%) 0 out of 2 within all tested breeder flocks.

Incidence of AI virus in different examined species

Concerning the results of real –time RT-PCR test used for detection of AI virus for matrix gene (common) a percentage of 70.4%, 33.3%, 0% in broiler, layer and breeder flocks respectively and 56.3%, 16.7%, 0% in broiler, layer and breeder flocks respectively for H9 infection were recorded with a total incidence of 68%(102/150) for matrix gene (common) in all species and 54 % (81/150) for H9. **(Table 4).**

Results of virus isolation

A total of 50 flocks were inoculated into fertile SPF eggs from the 81 positive flocks for H9 (as we detect them early in RT-PCR in cycle before 30, this mean that they were high amount of virus particle that will help in results of virus isolation on SPF eggs).Results of virus isolation trails from the selected 50 flocks revealed 20H9 isolates as judged by slide HA test and HI test against reference H9 antiserum

andwere negative for H5 and NDV. Their HA titer log2 were ranging from 0 to 11.

Results of H9 sequenced isolates

From those H9 viruses, we sequenced the HA gene from the selected seven isolates from 2012-2014 and compared with original strain of Egypt and other stains from Israel and Hong kong **(Figure1).** The seven isolates found to be have an identity with each other ranging from 92.9_ 98.3% and in comparison to Israel 2008 strain it was 86.2_89% and with the original Egyptian strain of 2011-2013 by 93.9_97.4% **(Table 5).**: H9 primers used for one step conventional RT-PCR and sequence (**Table 6).**

Table (1): Incidence of AI virus in examined broilers chicken flocks

Location	rRT-PCR	
	Common matrix Ag Pos/Total (%)	H9 Pos/Total (%)
Alexandria	11/18 (61%)	7/18 (38.9%)
Behera	70/90(77.7%)	60/90(66.7%)
Gharbia	12/19(63.2%)	9/19(47.4%)
Kafr El-Sheikh	2/5(40%)	1/5(20%)
Marsamatrough	5/10(50%)	3/10(30%)
Total	**100/142(70.4%)**	**80/142(56.3%)**

Table (2): Incidence of AI virus in examined layers chicken flocks

Location	rRT-PCR	
	Common matrix Ag Pos/Total (%)	H9 Pos/Total (%)
Alexandria	0/1(0%)	0/1(0%)
Behera	2/4(50%)	1/4(25%)
Gharbia	0/1(0%)	0/1(0%)
Total	**2/6(33.3%)**	**1/6(16.7%)**

Table (3): Incidence of AI virus in examined breeders chicken Flocks

Location	rRT-PCR	
	Commonmatrix Ag Pos/Total (%)	H9 Pos/Total (%)
Behera	0/2 (0%)	0/2 (0%)
Total	**0/2(0%)**	**0/2(0%)**

Table (4): Collective results for incidence of AI in different examined species

Species	rRT-PCR	
	Commonmatrix Ag Pos/Total (%)	H9 Pos/Total (%)
Broiler	100/142 (70.4%)	80/142 (56.3%)
Layer	2/6(33.3%)	1/6(16.7%)
Breeder	0/2(0%)	0/2(0%)
Total	**102/150(68%)**	**81/150(54%)**

Table (5): H9 Nucleotide Identity between the selected seven isolates in this study 2012 – 2014 and original Egyptian strain and other different stains).

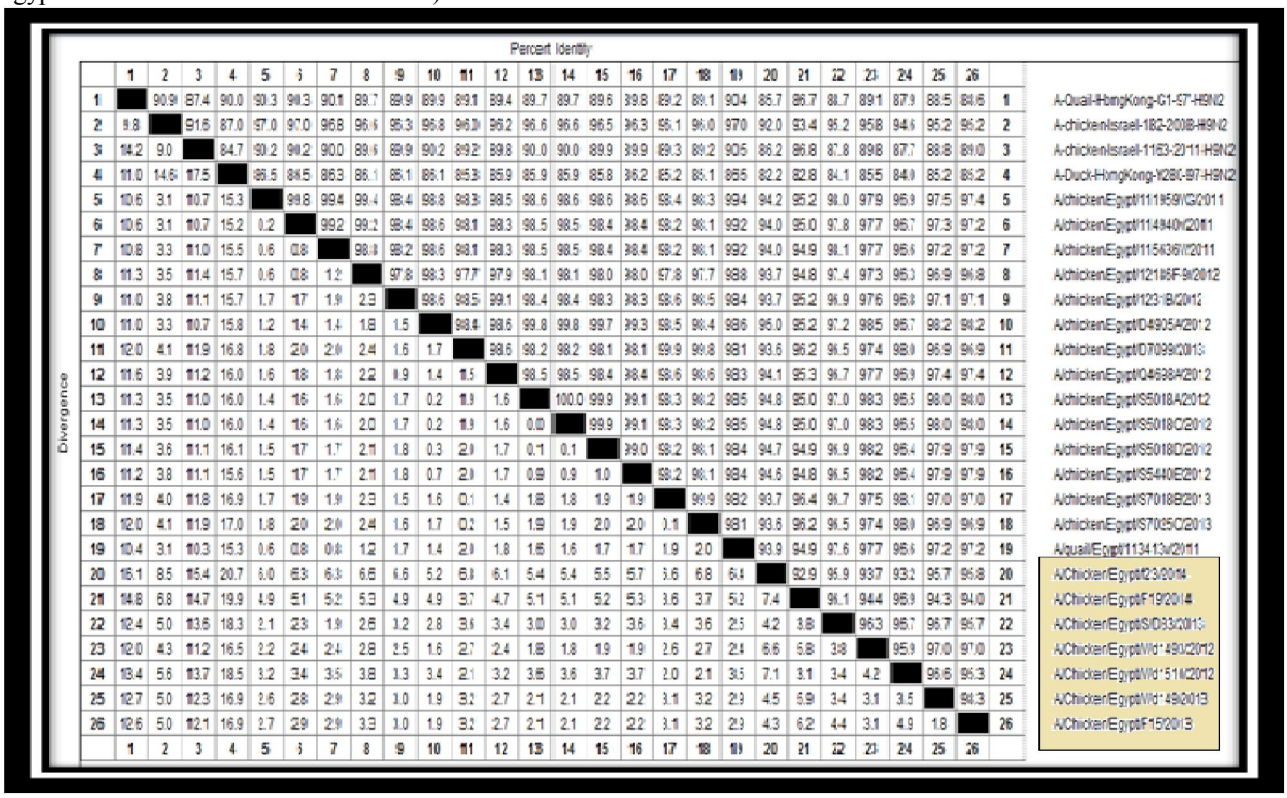

Table 6: Sequence of H9 primers used for one step conventional RT-PCR and sequence.

Primer	Sequence
F1-6 (forward)	TAG CAA AAG CAG GGG AAT TTC TT
R-1320 (reverse)	ATC TTG TAT TTG GTC ATC AAT C

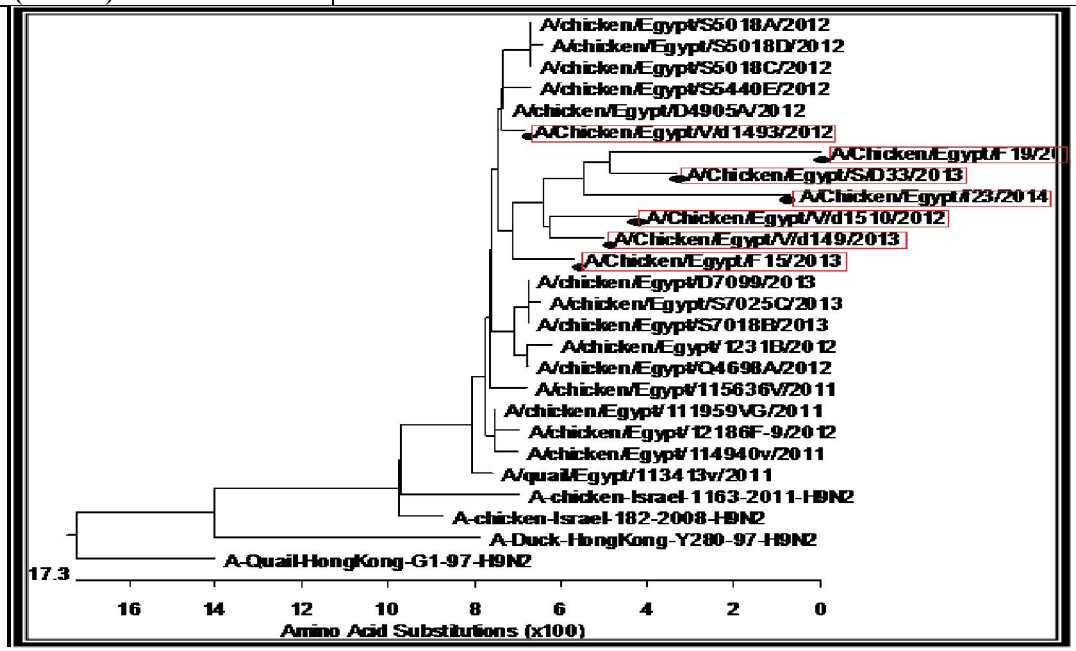

Figure (1): Phylogenetictree of selected seven H9isolates in the present study and other LPAI (H9N2) for the HA gene (indicated by black dots).

4. Discussion

H9N2 viruses of gallinaceous poultry spread from Asia to most Eurasian countries during the 1990s **(Aamir *et al.*, 2007 and Alexander, 2007).**

These viruses have circulated widely and cause only low-grade disease in gallinaceous poultry. An unanswered question is whether prior infection with H9N2 viruses reduces the lethality and therefore the detectability of HP H5N1 infection in poultry in the field, thereby facilitating virus spread. In Israel, H9N2 influenza viruses were first detected in poultry in May 2000, when these viruses caused signs of mild respiratory disease and a drop in egg production, mainly in 120 chickens and turkeys **(Guan *et al.*, 2000 and Banet-Noach *et al.*, 2007).**

Globally, after the initial outbreak of H9N2 in Israel viruses were not detected again until December 2001, and they continued to circulate in poultry until April 2003, causing disease outbreaks in chickens, geese, ostriches, and turkeys. A heterologous inactivated vaccine was widely used to control H9N2 virus spread. H9N2 viruses were introduced for the third time in February 2003 and were isolated sporadically through 2006, primarily from chickens **(Guan *et al.*, 2000 and Banet-Noach *et al.*, 2007).**

Low pathogenic AI H9N2 was first reported in Egypt in November 2011; the isolated virus was closely related to viruses of the G1-like lineage isolated from neighboring countries, indicating possible epidemiological links **(El-Zoghby *et al.*, 2012).**

Selim, (2013) concluded that if vaccinated chickens with H5N2 are infected with HPAI H5N1 virus during the protection period after H9N2 infection, outbreak of HPAI H5N1 may go unnoticed while the virus is shed by protected birds and becomes more widespread. The situation in Egypt is more complicated as the masking of HPAI H5N1 in the field is not only due to the circulation of LPAI H9N2 in the field but also due to miss use and mass vaccination against HPAI. While non-vaccinated chickens had been inoculated with H9N2 and challenged with classical and variant H5N1 virus were not protected at 100% deaths but the death in non-vaccinated chickens delayed the death in positive control. In contrast to the result obtained by **Khalenkov *et al.*(2009);** where inoculation with A/ty/Israel/1567/04.

(H9N2) strain can protect against challenge with lethalA/Chicken/Egypt/1C/06 (H5N1) in non-vaccinated chickens. Some strains of H9N2 virus can modulate the severity of disease caused by HP H5N1 virus and provide partial protection against lethal challenge 127 under the conditions tested. Interestingly, even closely related H9N2 viruses may differ in their protective effect. This was explained in part by cross-protective cell-mediated immunity induced by the G1 genotype of the H9N2 virus **(Seo and Webster, 2001).**

AI virus isolation was conducted according to the standard protocols of **(OIE, 2008)** using SPF ECE vial intra allantoic route. The harvested allantoic fluid was tested using HI test for titration and the mean HI titer of isolated H9 viruses was ranging from 1 log2 to 11 log2 HAU after the passage in SPF ECE These results were also shown by **(Kilany, 2007 and Safwat, 2012).**Subtype H9N2 viruses, although of low pathogenicity, are correlated with increased severity because of co-infection with other poultry viruses; thus, they indirectly might lead to economic losses for the industry. On the public health side, our findings that AIVs are widespread throughout poultry sectors and geographic regions indicate that a large segment of the population of Egypt is at risk. Subtype H9N2 viruses also infect humans, thereby adding to the risk for infection with subtype H5N1 virus. Our results can be used to better focus and target animal health and public health policy in Egypt. Indeed, Egypt remains an epicenter for AIV circulation, and vigilant surveillance remains the single-most effective tool for keeping track of these viruses.

Here, we have studied the epidemiological situation of H9N2 AIV in Egypt from 2012 to 2014, in addition to the investigation of the molecular characterization of HA gene of seven viruses from different poultry species and regions. Geographical distribution of H9 cases in this study revealed that the infection was recorded in five governorates throughout Egypt without geographical selection where it was recorded in Delta region (Alexandria, Behaira, Gharbia, Marsa matrough and Kafr El-Sheikh) It suggested that H9N2 AIV became persisted with wide geographical distribution.

The majority of these cases were from apparently healthy chicken farms that may reflect the low pathogenic nature of the virus which permits the silent spread of such strains in commercial flocks. However, there were cases of respiratory distress and mortalities from chicken, all these cases were associated with other pathogens which aggravated the case.

The presence of H9N2 in commercial farms may indicate some defect in applying the biosecurity measures and that will threaten the poultry industry especially with the frequent presence of mortalities associated with other pathogen infection.

The positive H9N2 cases recorded during winter months was 58/81 cases with percentage of 71.6%, while it was 23/81 cases during summer months with percentage of 28.4%, that indicates the prevalence of the virus infection in the winter was more than that in the summer, that supports the theory of increasing the activity of H9N2 AIVs by low temperature. The

results mentioned here were agreed with **Naeem *et al.* (1999), (2003)** who found that AIV H9N2 caused lesions and mortalities during winter in northern Pakistan more than that occurred in summer season in southern Pakistan.

The phylogenetic relationship between the H9 HA gene of the tested isolates and those of selected H9N2 strains isolated in several other countries were analyzed. All the Egyptian isolates were closer to the viruses isolated in the Middle East (tab.5) which according to **Banks *et al.* (2000)** belonging to viruses from G1 lineage with more close relationship to the Israeli strains (A-Chicken- Israeli-1163-2011-H9N2) (average amino acid identity 88.7%-93.3%) that together formed a characteristic group among G1 like viruses.

Continuous surveillance with viral sequence comparison and phylogenetic analysis of current LPAI H9N2 are necessary to recognize newly emerging influenza variants and to monitor the global spread of these viruses ***(Velijkovic et al., 2009).*** So, that this study was planned for: Field survey for isolation of AI virus from chickens during 2012 to 2014.

Detection of Avian Influenza viruses by **RT-PCR**. Isolation of the virus on specific pathogen free egg **(SPF)** 9-11 day old by making two serial passages according to the international standard procedures gave negative result for isolation of AIV, this result agreed with results reported by ***(Al-Natour et al., 2005)*** as they mentioned presence of avian influenza antibodies without virus isolation**.** Confirmation by **HA** test. Genotyping of the isolates and mutation recognition by sequencing technique.

Clinical signs and postmortem examination of the examined flocks were similar to those described by ***(Swayne and Halvorson, 2003).***

The H9 isolates was clustered with recent Egyptian and the samples was G1-like group of HA gene sequencing **(Figure 1)**. The isolates were similar to the Egyptian strain of 2011(A/Quail/Egypt/113413v/2011) with about 92.3%-97.1% and with HongKong strain which was isolated at 1997(A/Quail/HongKong/G1/97) with 81.3%-86.1% **(Table 5).**

The presence of a new subtype of LPAI H9N2 may add another risk factor to the poultry industry in Egypt, especially with the endemic situation of HPAIH5N1 and the presence of other pathogens with low biosecurity level in some commercial sectors that permit easy virus transmission and adds more stress to the condensed poultry populations **(Arafa *et al.*, 2012).**

Finally, the percentage of AIV incidence in West Delta Region during 2012-2014 is 68% (102/150).With the total incidence for H9 was 54% (81/150)

In the respect of LPAI H9 as in table (1), the highest incidence of the virus was in broiler sectors 80/142 positive farms (56.3%) then layer sectors 1/6 positive farms (16.6%). In breeders, (0/2) no positive in examined farms during 2012- 2014.

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2/2/2015