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Original article

DETECTION AND CHARACTERISATION OF RABBIT HAEMORRHAGIC DISEASE VIRUS STRAINS CIRCULATING IN EGYPT

A. F. MAGOUZ[1], E. A. ELSAYED[1] & A. Y. METWALLY[2]

[1]Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt; [2]Department of Poultry Diseases, Animal Health Research Institute, Kafrelsheikh, Egypt

Summary

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Rabbit haemorrhagic disease (RHD) is a highly virulent viral disease of *Oryctolagus cuniculus* which threatens the rabbit population in Egypt and worldwide. The etiological agent is the rabbit haemorrhagic disease virus (RHDV), a member of the family *Caliciviridae*. The aim of this study was to identify the possible evolutionary changes of the currently circulating RHDV in Egypt. Twenty suspected cases were collected from outbreaks that occurred in non-vaccinated rabbit flocks during 2015 and 2016. Liver homogenate samples were investigated for detection and identification of circulating RHDV using haemagglutination (HA) and reverse transcriptase polymerase chain reaction. Further characterisation of selected five viral strains was performed by nucleotide sequencing of VP60 gene. All twenty tested samples were haemagglutinin positive and VP60 gene was amplified. Based on nucleotide sequence analysis, four isolates were identified as classical RHDV strains, while one isolate was assigned as RHDVa variant strain but with the same HA pattern. The new RHDVb variant was not identified.

Key words: phylogenetic analysis, RHDV, RT-PCR, VP60 gene

INTRODUCTION

Rabbit haemorrhagic disease (RHD), is a worldwide highly contagious and acute viral disease of rabbits (*Oryctolagus cuniculus*), leading to mortality rates of approximately 90% in both wild and domestic rabbits (Abrantes *et al.*, 2012**)**. The causative pathogen is the rabbit haemorrhagic disease virus (RHDV) which belongs to genus *Lagovirus* within the family *Caliciviridae*. The virus is a naked, icosahedral, single-stranded, positive-sense RNA virus (OIE, 2012; Ismail *et al.*, 2017).

Rabbit haemorrhagic disease virus was first reported in 1984 in the People's Republic of China killing about 140 million

domestic rabbits in the country within 1 year (Liu *et al.*, 1984). Subsequently, the disease rapidly extended to other parts of Asia, Europe, Africa and the Central and North America and spread to Europe, where it was first detected in Italy, then in several European countries to become a pandemic with many lethal outbreaks occurring in many countries (Gregg & House, 1989).

In Egypt, RHDV was first reported in 1991 in Sharkia Province, and then spread to other Egyptian provinces (Ghanem & Ismail, 1992). In spite of vaccination programs, RHD is still representing a threat in the rabbit production farms due to high morbidity and mortality rates as the rabbit meat is one of the main types of meat which are popularly consumed in Egypt (Alboghdady & Alashry, 2010).

The clinical picture of RHD is characterised by fever, depression, vocalisation, dyspnea, sudden death, loss of appetite, rapid respiration, coughing in addition to nervous manifestations (convulsions, ataxia, and paralysis of legs) which are recorded shortly before animal death after an incubation period which usually lasts for 1–4 days (Trzeciak-Ryczek *et al.*, 2015). Post mortem pathological changes include necrotising hepatitis, splenomegaly, pneumonia, frothy exudates in trachea, congestion of liver, haemothorax, subcutaneous abscesses, congestion and ulceration of nasal mucosa, haemorrhages and multiple abscesses in lungs and congested brain (Embury-Hyatt *et al.*, 2012).

The disease causes high mortality rates of 70–100% in adult rabbits while young kits are subclinically infected. This difference in disease susceptibility is not yet understood, but it may be due to the specific receptors that occur as young rabbits turn to adulthood (Dalton *et al.*, 2012).

The viral genome consists of a 7.5 kb RNA which encodes a 257 kD polyprotein and a subgenomic RNA of about 2.2 kb and they both encode for the capsid protein gene VP60 (Yang, 2015; Zhu *et al.*, 2015).

The VP60 capsid protein is the main structural protein of RHDV, which contains the type-specific antigenic epitope (Capucci *et al.*, 1998) that plays a critical role in inducing sufficient immunity against RHDV. Phylogenetic studies have been published to evaluate the genetic variations between RHDV based on partial and complete sequences of this gene (Tian *et al.*, 2007).

According to genetic analyses, RHDV species can be divided into two subtypes, classic RHDV and variant (RHDVa) and nucleotide sequence similarities between these subtypes show a 14% maximum difference which present clear genetic, antigenic and epidemiological differences (Li *et al.*, 2017). Moreover, atypical RHD outbreaks in vaccinated rabbits, which have resulted in high mortality rates in young rabbits, have been reported recently and the new variant was described as "RHDVb" or "RHDV2. This variant was reported for the first time in 2010 (Carvalho *et al.*, 2017) and the average nucleotide identity between RHDV2 and RHDV1 (RHDVa) can reach 82.4%, with amino acid similarity of about 89.2% (Kong *et al.*, 2016).

Diagnosis of RHD mainly depends on clinical signs observations, post-mortem examination, histological lesions, haemagglutination and haemagglutination inhibition tests and electron microscope examination. The virus cannot be cultivated in cell cultures therefore; genomic detection of the virus, antibody detection, and virus isolation in susceptible rabbits are required for virus detection and cha-

racterisation (OIE 2012; Ismail *et al.*, 2017). RT-PCR is considered a perfect and rapid diagnostic technique for RHD and is more sensitive than other serological methods (Soliman *et al.*, 2016).

The aim of this study was to identify the evolutionary changes of the currently circulating RHDV in Egypt during 2015 and 2016 with sequencing and phylogenetic analysis of the partial VP60 protein gene of five selected RHDV isolates.

MATERIALS AND METHODS

Sample collection and preparation

Infected rabbits were monitored for clinical signs and freshly dead rabbits were collected from twenty local farms located in different provinces in Egypt; Kafrelsheikh, El-Qalyubia, El-Gharbia, El-Behera and El-Menofia. The investigated rabbits were 2–3 months old except one case was a 6-weeks old rabbit. All rabbits were collected from non-vaccinated farms. Twenty pooled samples were collected and about 1 g of liver tissue was homogenised in phosphate-buffered saline (PBS; PH 7.2) to make 10% w/v suspension, repeatedly frozen and thawed three times, and then clarified by centrifugation at 5000 rpm for 15 min. Tissue supernatants were collected and stored at –80 ºC till being used in HA test, virus isolation and RT-PCR.

Virus isolation and identification

Eight susceptible crossbred rabbits at the age of 3 months weighting 1.5 to 2 kg were inoculated intramuscularly with 1–2 mL of 10% liver homogenate. Two rabbits were kept as non-infected controls. The rabbits were observed twice daily for clinical manifestations for one week (Ferreira *et al.*, 2004). All experimental procedures were approved by the Ethics and Animal Experiments Committee at the University of Kafrelsheikh, Egypt.

Haemagglutination test

Human type (O) blood was collected using 4% sodium citrate. An equivalent amount of PBS was added and the suspension was centrifuged at 1000 rpm for 5 min. The supernatant was decanted and the washing step was repeated twice. The RBCs sediment was re-suspended in PBS to prepare 1% RBCs suspension. The liver homogenates were subjected to a rapid haemagglutination (RHA) test followed by microtitre plate HA according to (OIE, 2012). The HA test was performed in V-bottomed micro-titration plates (Sigma). Two-fold serial dilution of the liver extract was made in phosphate buffered saline (PBS; pH 7.4). An equal volume of washed 1% human erythrocytes was added. The plate was incubated at 4 °C. After 1 h of incubation, the haemagglutination titre was recorded as the reciprocal of the the highest dilution showing complete agglutination of erythrocytes.

RNA extraction

Total RNA was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, Calif., USA) from liver homogenates of (highest HA titer) according to the manufacturer's instructions. Briefly, 140 µL of the liver homogenates were lysed in 560 µL of AVL buffer. After complete lysis 500 µL of 100% ethanol were added and mixed for 15 s by pulse vortexing. Aliquots of 630 µL were transferred to a spin column and centrifuged at 8000 rpm for 1 min. The binding RNAs were washed in 500 µL AW1 buffer and centrifuged at 8000 rpm for 1 min followed by the addition of 500 µL AW2 buffer then centrifuged at 14,000 rpm for 3 min. The RNAs

were eluted in 60 μL of buffer AVE and stored at −80 °C until used.

Amplification of VP60 gene

One-step RT-PCR kit (QIAGEN) was used to amplify the capsid protein (VP60) gene of the RHDV. The RT-PCR reaction mix composed of 5 μL of total RNA, 10 μL 5× Qiagen one-step RT-PCR buffer, 10 μL Q buffer, 2 μL of dNTPs ,1 μL of each primers (Table 1), 1 μL of the enzyme mix (containing RT and PCR enzymes), and 10 μL of RNase free water. The RT-PCR reaction was performed at 50 °C for 30 min, then 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel in 1× Tris acetate EDTA (TAE) buffer containing 10 mg/mL ethidium bromide and visualised under UV light. For positive control, RNA extracted from a lyophilised local vaccine was used. A negative control containing only PCR master mix and primers was also included.

Sequencing and phylogenetic analysis

For gene sequencing, the target bands were excised from the agarose gel then purified by QIA quick Gel Extraction Kit (Qiagen, Valencia, CA) followed by sequencing using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) and Applied Biosystems 3130 genetic analyzer (ABI, USA). Sequence identities of five isolates were compared with previously published RHDV reference strains available in the public database (NCBI, USA) and analyzed using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of MegAlign module of Lasergene DNA Star software. Phylogenetic tree through a bootstrap of 1000 trials was constructed for the sequenced isolates with other reference strains available in the GenBank database using MEGA version 6 (Tamura *et al.*, 2013).

RESULTS

Clinical signs and gross pathology

The clinical examination of infected rabbits revealed fever, depression, cyanotic lips and nostrils, bloody foamy nasal discharge, convulsion, rapid respiration and sudden death (Fig. 1). Post mortem gross lesions showed bloody exudates in trachea, the lungs were edematous with punctuate haemorrhages. Liver was pale with focal necrosis, splenomegaly, and full-filled stomach with engorgement of blood vessels. Congestion of all visceral organs and intestinal tract was reported (Fig. 2 and 3).

Virus isolation and identification

The inoculated rabbits showed the same clinical picture of natural RHD infection with death occurring 3–4 days post infection with mortality rate 100%. The control non-infected rabbits were alive without any symptoms.

Table 1. Oligonucleotide primers used for amplification of VP60 gene (Embury-Hyatt *et al.,* 2012)

Primer	Oligonucleotide sequence	Position	Size, bp
Forward	CAACCTCCAGCCCACCACCAACAC	1158 nt of VP60 gene	332 bp
Reverse	TGGTTGGGAGCCTGTGCCGTACTG	1473 nt of VP60 gene	

Fig. 1. Clinically RHD affected rabbit showing bloody foamy nasal discharge.

Haemagglutination test

The liver homogenates were subjected first to a rapid slide haemagglutination test which shows positive HA reaction for all twenty examined samples. The microtitre plate HA test revealed positive reaction with all twenty samples with HA titers ranged from 2^4 to 2^7 .

RT-PCR

All twenty examined liver suspensions were RT-PCR positive and the partial fragment of capsid protein gene (VP60) was successfully amplified giving the specific size bands (332 bp) (Fig. 4).

Sequencing and genetic analysis

The amplified VP60 gene sequences of the isolated RHDVs were submitted to the Genbank database with the accession numbers F729-1-RHDV-2016 (KX781724), F729-2-RHDV-2016 (MF737351), F729-3-RHDV-2016 (MF737352), F729-4-RHDV-2016 (MF737353), and F729-5-RHDV-2016 (MF737354).

Compared to reference strains available in the GenBank database, the four isolates designed as F729-1-RHDV-2016, F729-3-RHDV-2016, F729-4-RHDV-2016 and F729-5-RHDV-2016 exhibited the highest nucleotide sequence identity with the Saudi Arabian RHDV isolates RHDV/1/SA/2012 (KJ949619) and RHDV/2/SA/2012 (KJ949620) (97–99%), while the other isolate F729-2-RHDV-2016 showed the highest nucleotide similarity with the Egyptian variant strains Menofia/2012 (KX133721); GIZA-2006 (JQ995154),

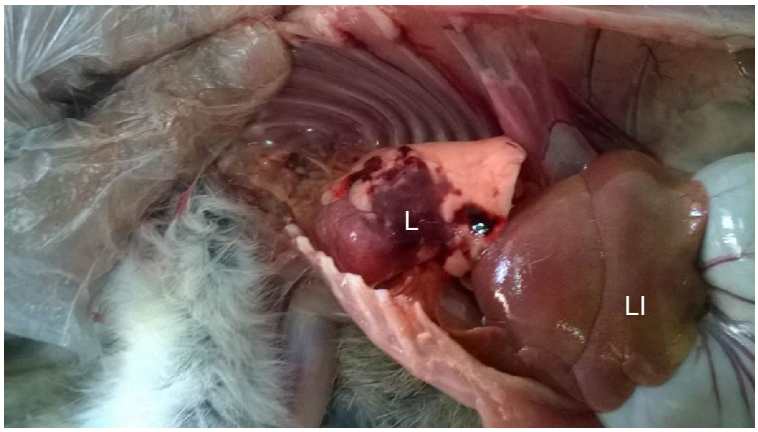

Fig. 2. Post mortem lesions of RHD: haemorrhages in lungs (L); pale and friable liver (LI).

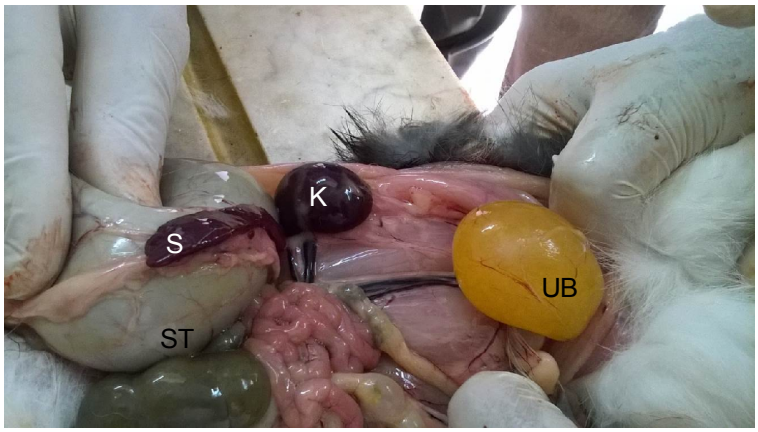

Fig. 3. Post mortem lesions of RHD: dark, congested spleen (S); full stomach with engorged peripheral blood vessels (ST); kidney showing severe congestion (K); urinary bladder impacted with discoloured urine(UB).

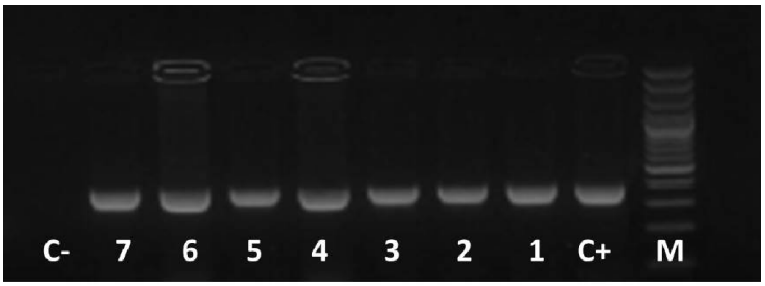

Fig. 4. Amplification of VP60 protein gene fragment (332 bp) by RT-PCR. M = molecular base marker (100 bp), C+ = positive control, lanes 1–7=RHDV field isolates, C- =negative control.

(96%), the American variant strains UT-01 (EU003582) and NY-01 (EU003581) (94%) and the Italian strain Vt97 (EU250331) (94%). The current RHDV isolates were closely related each to the other with similarity percentage ranging from 96 to 100% except for the isolate F729-2-RHDV-2016 which was found to be genetically different as it showed only 89–90% identity with the other isolates in this study. The phylogenetic analysis of partial sequences of the VP60 gene showed that the isolate F729-2-RHDV-2016 was clustered with the Egyptian variant strain Giza2006, while the 4 isolates F729-1-RHDV-2016, F729-3-RHDV-2016, F729-4-RHDV-2016 and F729-5-RHDV-2016 were clustered together with the classic RHDV strains and in close lineage with the Saudi Arabian strains RHDV/1/SA/2012 and RHDV/2/SA/2012 (Fig. 5).

DISCUSSION

Rabbit meat is one of the main types of meat which are popularly consumed by Egyptian peoples (Alboghdady & Alashry, 2010). In Egypt, RHDV is still representing a threat in the rabbit production farms in spite of vaccination pro-

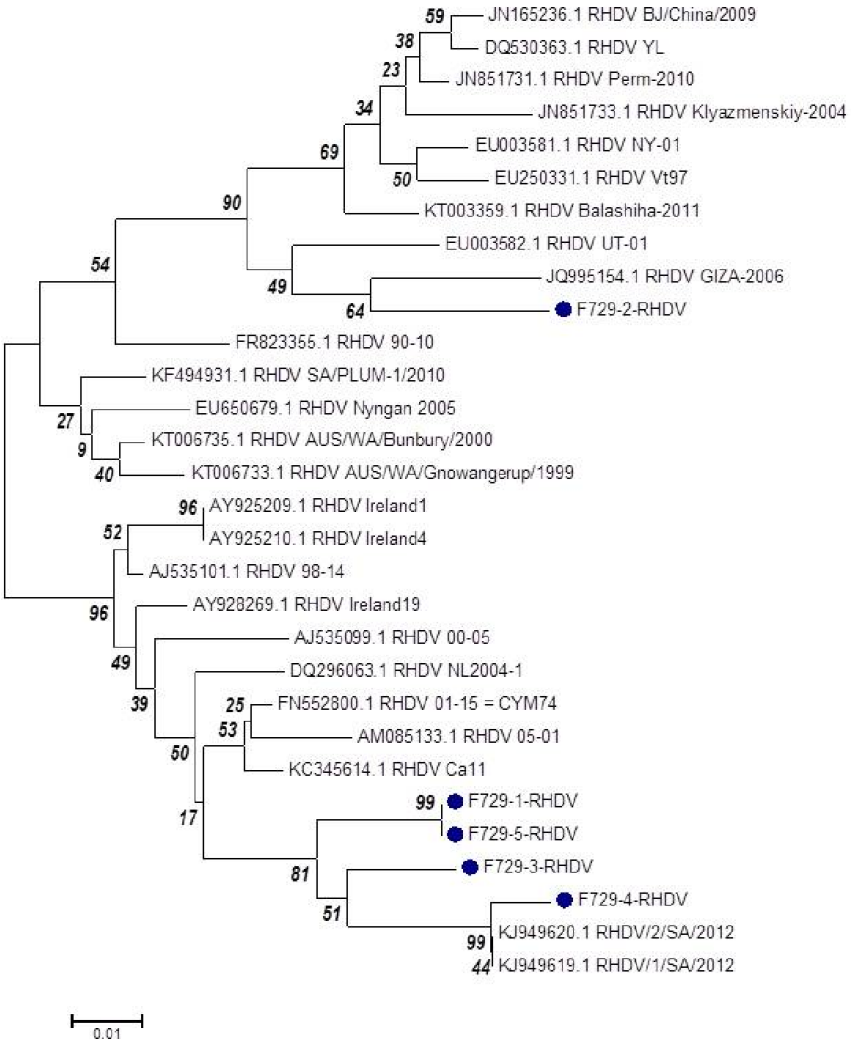

Fig. 5. Phylogenetic tree based on VP60 protein partial nucleotide sequence of selected isolates and previously published sequences. Marked strains (*) refer to the local isolates under study.

grams due to high fatality rates (Ewees, 2007; El-Bagoury *et al.*, 2014).

Clinical signs and post mortem lesions of the examined rabbits exhibited the typical signs of RHD as described in previous studies, which indicates that they play a significant role in the identification of the RHD viral infection (Metwally & Madbouly, 2005; Tian *et al.*, 2007; Embury-Hyatt *et al.*, 2012; Abd El-Moaty *et al.*, 2014; Trzeciak-Ryczek *et al.*, 2015; Soliman *et al.*, 2016; Ismail *et al.*, 2017).

Tissue collected from infected rabbits, was mainly the liver, as it contains the highest concentration of the viral particles (Ahmad *et al.*, 2011). The inability to cultivate this virus in cell cultures has necessitated using substitutional methods

for virus detection such as molecular techniques, antibody detection, and experimental infection in rabbits (Yang *et al.*, 2015; Ismail *et al.*, 2017).

The first test applied for RHDV diagnosis was the haemagglutination test using human type (O) and sheep erythrocytes and was then widely used by Chinese and European scientists as a screening test (Calvete *et al.*, 2002). In the current study, all tested 10% liver homogenates from the suspected cases were able to haemagglutinate human type (O) RBCs with HA titers ranging from 2^4 to 2^7 **.** On the opposite, Abd El-Moaty *et al.* (2014) reported the presence of RHDV isolates with changeable HA pattern in Egypt as the HA negative isolates have turned into positive after passage in susceptible rabbits. In accordance, Tian *et al.* (2007) reported that the HA test was not reliable for diagnosis or typing of RHDV field isolates as there is no correlativity between the HA activity and the genetic typing of either classical or variant RHDV strains. Our isolate (F729-2-RHDV-2016) which was confirmed by gene sequencing as a RHDV variant was able to haemagglutinate the human type (O) RBCs which was similar to that reported by Burmakina *et al.*, (2016) as they identified all classic and variant RHDV field isolates as haemagglutinating viruses suggesting that the genetic factor which plays the role in the HA activity among RHDV strains still remains unclear. Reversely, El Sissi & Gafer (2008) recorded that some RHD outbreaks in many Egyptian provinces caused by variant strains (RHDVa) with negative HA activity suggesting that the non-haemagglutinating RHDV strains are antigenic variants of the virus.

The collected liver suspensions were subjected to one step RT-PCR and results revealed successive amplification of partial fragment of the capsid protein gene (VP60) giving the specific size bands at 332 bp. The application of RT-PCR for the amplification of RHDV nucleic acid has been reported by several authors (Embury-Hyatt *et al.*, 2012; OIE, 2012; Soliman *et al.*, 2016). Yang *et al.* (2008) showed that the specificity and sensitivity of the RT-PCR method was significantly more than that of the hemagglutination test. In contrast, Carvalho *et al.* (2017) pointed out that the presence of vaccine virus in the tissues of recently vaccinated animals may lead to misdiagnosis of the disease.

The nucleotide sequences of the five local isolates were compared with RHDV sequences previously published in the Genbank database. The homology percentage results differentiated the 5 local isolates into 2 groups. The F729-2-RHDV-2016 isolate showed high homology percentage with the variant Giza2006 strain which has been characterised as a RHDV variant by sequence analysis of the VP60 gene (Ibrahim *et al.*, 2012), as well as the American variant strains UT-01, NY-01, Russian strain Balashiha-2011, Italian strain Vt97 and other published variant strains. The other 4 isolates, namely F729-1-RHDV-2016, F729-3-RHDV-2016, F729-4-RHDV-2016 and F729-5-RHDV-2016 showed high homology with the classical RHDV strains. Our classical and variant isolates showed genetic diversity among nucleotide sequences up to 10%. These results were similar to previous reports (El-Bagoury *et al.*, 2014).

Conclusively, classic and variant RHDVs are circulating in the Egyptian fields and the occurrence of outbreaks in farms where rabbits were previously vaccinated raised considerable concern about the efficacy of the current vaccines.

Therefore it is strongly advised to develop and apply bivalent RHDV vaccine including both classic and variant RHDVs to provide protection against infection with both types. In addition, continuous monitoring of the spread and emergence of new variants is critical in deciding the most suitable protective measures.

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Correspondence:

Asmaa Magouz
Department of Virology,
Faculty of Veterinary Medicine,
Kafrelsheikh University,
Kafrelsheikh, Egypt.
email: asmaamagouz@gmail.com