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Morphological and Molecular Identification of Dengue Fever Vector *Aedes aegypti* (Diptera: Culicidae) in Jeddah Governorate-Saudi Arabia

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Mosquito-borne diseases are still a major human and animal health problem in the world. Identification of mosquito vectors is important in many respects including development of vector control strategies. Adults and immature stages of *Ae.aegypti* were identified based on the morphological characters using light and electron microscope and appropriate illustration (pictorial) keys. It is common knowledge that morphological ID is not as accurate as molecular ID. Recently DNA-based identification methods using molecular markers such as nuclear ribosomal internal transcribed spacer (ITS), cytochrome b (Cyt-b) and cytochrome c oxidase subunit 1 and 2 COI, COII which become an important method to differentiate between siblings and closely related species of mosquitoes. Adults of *Ae. aegypti* were collected from five areas in Jeddah city. Molecular identification by isolating both of DNA and total protein of *Ae. aegypti* were conducted. The results showed that DNA was isolated from mature and immature stages of *Ae.aegypti* using PCR technique from either the lab strain or from older and/or field specimens. This assay consisted of different primers reaction, which could amplify the DNA of both mature and immature stages producing fragment of three distinct sizes, ~1250 bp , 500 bp , ~ 400-600 bp and 300bp for CO1B , Cryb, ITS2 and ITS2-2 respectively Some of these loci were sequenced and submitted to the GenBank database NCBI Protein samples of (larva, pupa and adult) of the dengue fever vector, *Ae. aegypti* were isolated. The relative molecular weight of the detected bands was approximately in the range of 16.6 – 75.6 kDa.

Key words: Molecular identification, *Aedes aegypti* , Morphological, Protein profiles, Dengue fever, DNA fingerprinting.

Mosquito-borne diseases are still a major human and animal health problem in the world. (Gubler, 2002). Mosquitoes are the vectors of important human pathogens, including malaria, dengue, and yellow fever. Arbovirus disease surveillance requires accurate identification of mosquito vectors. Diseases transmitted by mosquitoes such as dengue fever which is the most significant mosquito-borne viral disease today. *Aedes* (*Stegomyia*) (Diptera: Culicidae) is a divers species group as regards morphology and distribution (Huang, 1979) contain over 40 species (Rai, *et al*., 1982). It is a peridomestic, active mosquito that prefers to breed in artificial containers near human habitation. *Ae. aegypti* is the principal vector for two medically important arboviruses, the causative agents of Dengue Fever (DF) and

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Yellow Fever (Mackenzie *et al.*, 2004). Correct and accurate identification of field- collected mosquitoes is essential for epidemiological and control efforts for arboviral diseases (Cook *et al.,* 2005). Morphology-based on taxonomic procedures are time consuming and not always sufficient for identification to the species level. It depends on morphological characters of adults and fourth instar larvae. (Krzywinski and Besansky, 2003). Identification adults of mosquitoes are commonly made with adult traps. During the process of collecting mosquitoes some characteristic patterns of scales such as white scales on the scutum and white bands on the legs, are very useful in distinguishing the individual species are frequently rubbed off that can often complicate the species identification process. Recently, the molecular approach has greatly improved the accuracy of species identification (Kumar *et al.,* 2007). Several studies were conducted to identify on insect species on the molecular aspect. (Byrd *et al*., 2011 Kumar *et al*., 2007, Xiang and Kochar 1991, Higa *et al.,* 2010 and McAvin *et al*., 2005). The Internal Transcribed Spacers (ITSs) of ribosomal DNA genes and Mitochondrial Cytochrome Oxidase C subunit I and II (COI and COII) most important marker technique in species identification. These molecular markers, used for mosquito taxonomy, for distinguishing among closely related species of various genera such as *Aedes* (Beebe *et al.,* 2007). Recently, several studies have been isolated and described total, specific protein and gene expression from mosquitoes such as *Aedes, Culex* and *Anopheles* from immature stages and specific parts of adult antenna, salivary glands and protein bindings with dengue virus (Ishida *et al.,* 2002, Chee and AbuBakar 2004, Hung *et al*., 2004 and Rohani, *et al*., 2005).

The present study aimed to identify the dengue virus vector *Ae. aegypti* based on morphological characters, molecular markers and total protein.

MATERIALS AND METHODS

Study sites

The presented study was conducted to identify collected mosquitoes from five sites of Jeddah province (western region) of Saudi Arabia. These locations were selected based on the number of reported dengue cases and the re-epidemics occurred during 2006-20011.

Mosquito samples

Laboratory and field-collection of *Ae.aegypti* mosquitoes were used in this study. Adult mosquitoes were frozen immediately at -80 to DNA extraction.

Morphological identification

In this study the different developmental stages (eggs, larvae and adults) were examined, using light and scanning electron microscopy. Morphological studies focused on features of developmental stages that purportedly differentiate various species and forms of *Aedes* species. Developmental stages were identified using the taxonomic keys of Huang (1977) and followed the Walter Read Biosystematics Unit (WRBU, 2010).

Light and scanning electron microscopy

The majority of taxonomic keys are based on the 4th larval instar. For that reason, this study describes the four larval instars of *A. aegypti* using scanning electron microscopy. The eggs 4th larval instar and adult stage of *A. aegypti* were studied for morphological features under light and scanning electron microscopy by focusing on the morphological characteristics of body segments including the head, thorax, and abdomen. Other organs such as the eyes and antennae were studied, then photographed, and compared with scanning electron microscopic studied attached to aluminum stubs with double stick tape.

Molecular method

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA extraction was obtained from adults and immature stages of *Ae. aegypti* using the DNeasy kit (QIAGEN, Valencia, USA) following the manufacturer's recommendations.: Each sample was homogenized in 180 µl Buffer ATL of DNA Extraction Solution. Twenty µl proteinase K was added and mixed thoroughly by vortexing. Then incubated at 56°C in a thermomixer, shaking water bath until the tissue was completely lysed. Vortex for 15 s and 200 µl Buffer AL was added to the sample, mixed thoroughly by vortexing and 200 µl ethanol (96–100%) was added. The mixture was pipetted into the DNeasy Mini spin then centrifuged at 8000 rpm for 1 min. DNeasy Mini spin was placed in a new collection tube. Five

hundred µl Buffer AW1 was added to a new collection tube, and centrifuged for 1 min at 8000 rpm. DNeasy Mini spin was transferred to a new collection tube and 500 µl Buffer of AW2 was added and centrifuged for 3 min at (14,000 rpm. The DNeasy Mini spin column was placed in 1.5 ml tube and 200 µl AE Buffer was added. Then Incubated at room temperature for 1 min and centrifuged for 1 min at 8000 rpm).

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a total volume of 25 µl containing 10 x reaction buffers, 25 mM $MgCl_2$, 10 mM dNTPs, 10 µM of each primer (Table 2), 5U Taq. polymerase and 2.5 µL of DNA. The reaction was completed to 25 µL using distilled water . The PCR program was as follows: 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min. After amplification the PCR products were analyzed on 1 % agarose gel in 0.5X TBE buffer. DNA ladder was used to determine the size of PCR products. The gel was stained with ethidium bromide and visualized by UV illumination using Gel Documentation System

Five primers were used for identification of *Ae. aegypti* .These primers were two ITS primers (ITS2 and ITS2-2), Three co-primers (CO1-A, CO1B) and Cytb).

Cloning PCR products

PCR products of *Ae. aegypti* was selected for cloning using pGEM®T-Easy vector obtained from PROMEGA Company**.** The ligation mixture containing 5 µl of 2X Rapid buffer, 1 µl of pGEM®T-Easy vector, 2 µl of PCR product, 1 µl of T_4 DNA ligase and the reaction was completed to 10 µl with ddH_2O. The reaction mixture was gently mixed by pipetting and incubated at 4°C overnight**.** The transformation protocol was done according to (Sambrook *et al*., 1989).

Phylogenetic study

The nucleotide sequencing was conducted under BigDye™ terminator cycling conditions and the reacted products was purified using ethanol precipitation and run using 3730XL automated DNA Sequencer (Macrogen Company, Seoul, North Korea)**.** These products used for sequencing after removal of unincorporated primers. Phylogenetic and molecular evolutionary analyses were conducted using MEGA5.1 Beta4 version 5 (Tamura *et al*., 2011) with those of *Ae. aegypti* isolates available in GenBank as showed in (Table3)

Protein extraction

Adults or immature stages of *Ae.* aegypti were finely homogenized together in eppendorf tubes containing 200 µl of the extraction buffer using a handle plastic homogenizer and left in refrigerator at 4°C over night, then followed by centrifugation at 12,000 rpm at 4°C for 15 minutes. The supernatants were transferred to new eppendorf tubes for electrophoresis analysis.

SDS-Gel Electrophoresis

Electrophoresis was performed in 12% polyacrylamide gels as described by Laemmli (1970)

Protein Determination

Protein concentrations were measured using a Bio-Rad protein assay kit based on the method of Bradford (1976), with BSA as standard and high, low standard protein.

RESULTS AND DISCUSSION

Morphological identification

Adults of *Ae. aegypti* were collected from five different localities of Jeddah governorate. Adults and larvae stages were identified by using standard taxonomic keys (Darsie and Ward, 2005). Briefly *Ae. aegypti* adults can be identified throughout the white scales on the dorsal (top) surface of the thorax that form the shape of a violin or lyre. The larvae stages can be identified using the comb scales on the eighth segment of the abdomen and the shape of the pectin teeth on the siphon. The eggs of *Ae.aegypti* can be identified by using the detailed observation on the outside surface Figs. 2-6.

Table 1. Coordinate of study locations in Jeddah governorate

Location	Coordinates	
	E	N
A	39.187563	21.48253853
B	39.20689261	21.4728754
C	39.20259096	21.45213269
D	39.25174507	21.47087433
E	39.2111804	21.58961988

A= Al-Balad , B= Al-Nazlah Al-Yamaneyyah, C= Ghuleel , D= Al-Jameiah and E= Al-Safa

Molecular identification based on DNA isolation

Among the molecular markers commonly used for mosquito taxonomy, the internal transcribed spacer 1 and 2 (ITS 2,1) of the ribosomal DNA and Cytochrome oxidase 1and 2 CO 1,2 of the Mitochondrial DNA (mtDNA or mDNA are present in all eukaryotes with high rate of sequence divergence. They are useful for distinguishing among closely-related species of mosquitoes. (Manonmani *et al.,* 2001)

The present study investigated the molecular identification of *Ae.aegypti* using DNA samples of three developmental stages and ITS and CO1 markers.

It is understandable that whole mosquito or its body parts contain cells that ultimately yield genomic DNA. The samples were first morphologically identified as mentioned above.

The results showed that the total purified DNA size differ significantly based on the insect developmental stages generally the adult and pupa stages produced DNA more than larvae in Fig. 7. These results totally agree with (Marrelli *et al.,* 2006 and Gupta and Preet 2012) who mentioned that DNA might be obtained from all developmental stages of the mosquito from both males and females either fresh, preserved in alcohol or even dried or frozen.

The results of PCR amplification demonstrated a fragment approximately ~1250 bp, ~500 bp, ~ 400-600 bp and ~300 bp in length, including the primers Fig. 8. The CO1A revealed no linkages with *Ae.aegypti* genomic sites, which reflect the un-complementary sequences between the insect genome and the primer. CO1B detected the insect genome at the loci of 1250 bp. Also Cytb identified a polymorphic pattern of ~500bp tightly linked with *Ae.aegypti*. The band produced by Cytb was more intensive than the other produced bands, which may be attributed to the size of purified DNA. The DNA sequence of CO1B and Cytb patterns are in registering process at NCBI GeneBank. The ITS2-2 identified the insect at two subsequent loci, these results may be due to the PCR reaction conditions and the DNA quality . DNA was extracted and purified using gel extraction method for each loci separately. The PCR result revealed that one clear pattern of 400 bp for both loci) Fig. 9. This result could be attributed to the lab sensitivity, PCR conditions and DNA quality as mentioned above. This findings were in agreement with (Wesson *et al.,* 1992 and Higa *et al.,* 2010).

Contrary ,the ITS2-2 primer provided single polymorphic band at~ 300 bp. The detected loci was tightly linked with *Ae.aegypti*. The DNA sequence of the ITS 2-2 was submitted to NCBI

Table 2. ITS and CO1 primers sequences used for detecting *Ae. aegypti*

Name of primer	Sequences	References
ITS2	5'- ATC ACT CGG CTC ATG GAT CG-35'- ATG CTT AAA TTT AGG GGG TAG TC-3	Porter & Collins, 1991)
CO1-A	5'-TATAGCATTCCCCGTTTA-3'5'-TCCTAATAAACCA ATTGC-3'	Modified from Simon *et al.* (1994)
CO1-B	5'-GGATCACCTGATATAGCATTCCC-3'5'-TCCAATGCA CTAATCTGCCATATTA-3'	O'meara (2001)
Cytb	5'-TATGTACTACCATGAGGACAAATATC-3' 5'- ATTACACCTCCTAATTTATTAGGAAT-3'	Simon *et al.* (1994)
ITS2-2	5'- GGG TCG ATG AAG AAC GCA GC-3'5'-ATA TGC TTA AAT TCA GCG GG-3'	Navajas *et al.* (1998)

Table 3. Six *Ae. aegypti*. ITS gene sequence used in homology analysis

No.	Country	Gene bank accession No
1	KSA (Riyadh)	GU980956.1
2	USA (Atlanta)	M95126.1
3	Peru	AY512672.1
4	Russia (Moscow)	HE820724.1
5	Japan (Nagasaki)	AB548800
6	KSA (Jeddah)	JX423805

Fig. 1. Location of the study area, Saudi Arabia

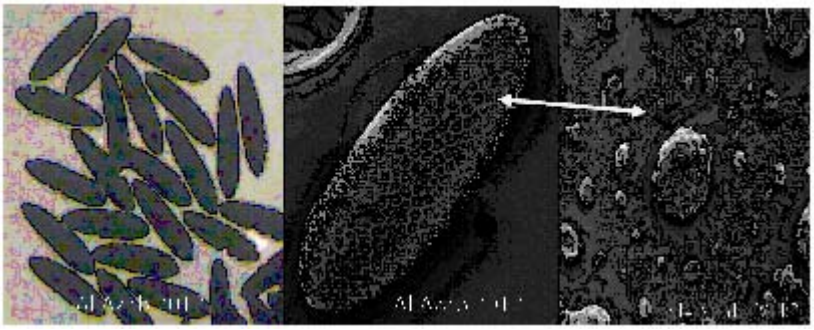


Fig. 2. Light and electronic photos of *Ae.aegypti* egg

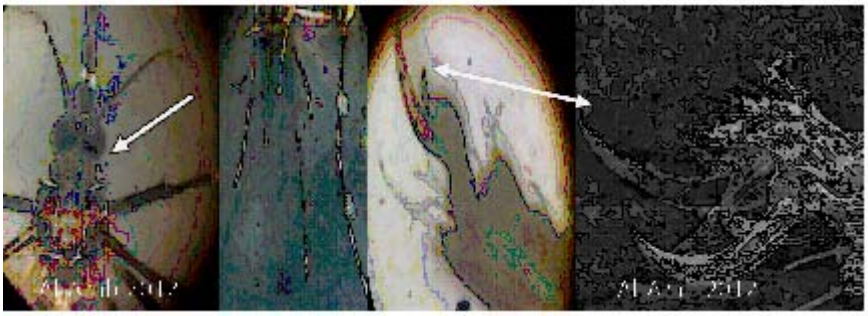


Fig. 3. Light and electronic photos of *Ae.aegypti* show lyre on the thorax and strip and claw of leg tarsus

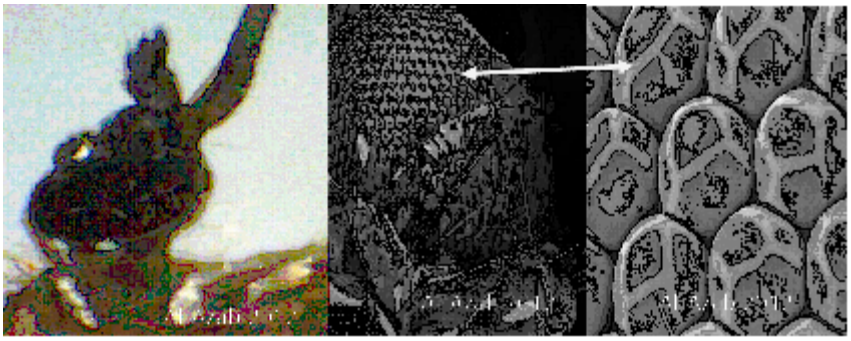


Fig. 4. Light and electronic photos show the compound eye of *Ae.aegypti*

GenBank database under accession numbers (JX423805, JX423806 and JX423807) Fig. 9. http://www.ncbi.nlm.nih.gov/nucleotide/407232545

The DNA sequence of the ITS 2-2 was submitted to NCBI GenBank database under accession numbers (JX423805, JX423806 and JX423807) Fig. 9. http://www.ncbi.nlm.nih.gov/nucleotide/407232545. Recent studies were conducted using different techniques to identify *Aedes* species based on DNA fingerprinting (Higa *et al.,* 2010, Hill *et al.,* 2008, Cywinska *et al.,* 2006, Dhananjeyan *et al.,* 2010, McAvin. *et al.*, 2005 and Toma *et al.*, 2002).

The result in agreement with Higa *et al.,* 2010 who reported that the lengths of ITS1 and ITS2 regions of *Ae. aegypti* were 425-435bp and 206-2-17bp respectively. Another researcher Dhananjeyan *et al.,* 2010 found that the PCR amplification of ITS2 region produced distinct fragment of 330 bp for *Ae.aegypti*. Wesson *et al.,* 1992 also determined the length of ITS1 region of *Ae.aegypti* was 419 bp.

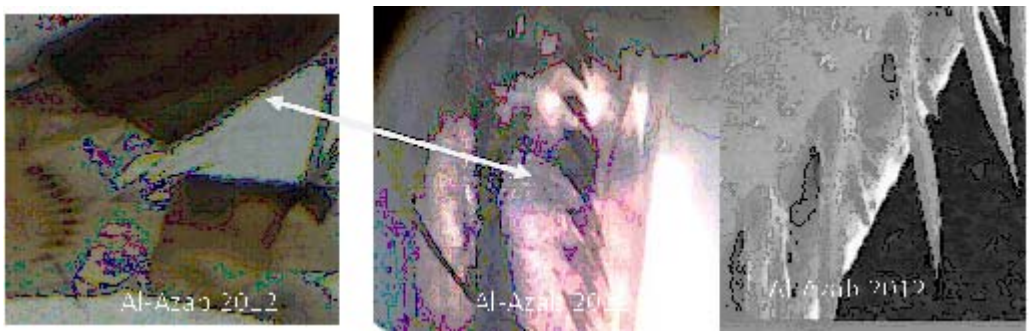


Fig. 5. Light and electronic photos show the pectin of *Ae.aegypti* larva

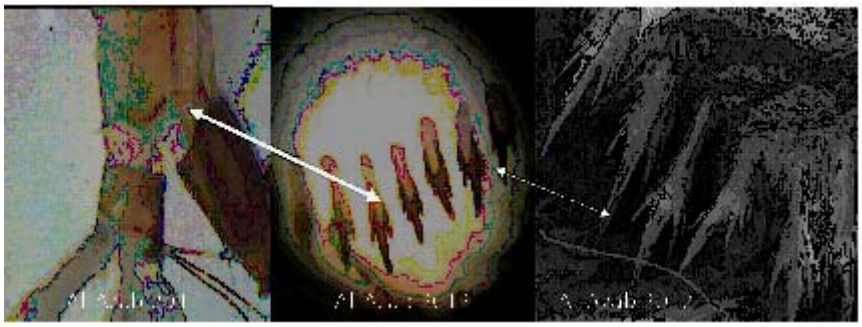


Fig. 6. Light and electronic photos show the comb scale of *Ae.aegypti* larva

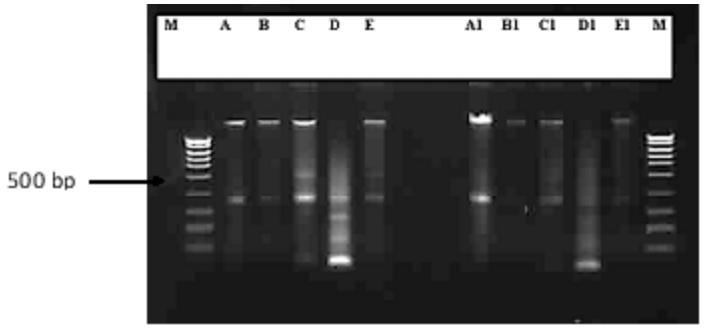
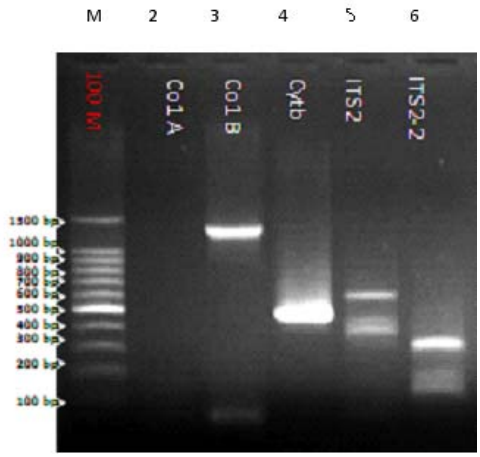


Fig. 7. DNA quantification using agarose gel electrophoresis for three *Aedes aegypti* developing stages. M is 1kb ladder. A, A1 are male B ,E ,B1, and E1 are female adult insect,C and C1 are Pupae stage and D and D1 are larval stages



M: 100 bp DNA ladder, L1: CO1A, L2:l Co1B, L4 Cytb,L5 ITS2 and L6: ITS2-2

Fig. 8. Agarose gel electrophoresis analysis for the PCR products of *Ae.aegypti*

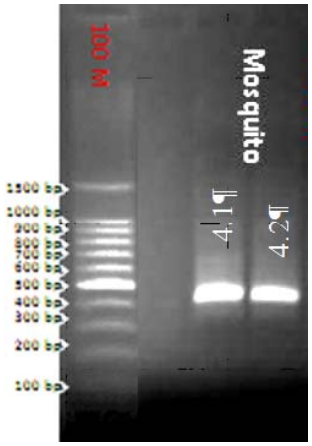


Fig. 9. Gel extraction of ITS2 identified PCR products of *Ae.aegypti*

JX423805

GGGTCGATGA AGAACGCAGC TAAATGCGCG TCAGAATGTG AACTGCAGGA
CACATGAACACCGACACGTT GAACGCATAT TGCACATCGT ACTACCAGTA
CGATGTACAC ATTTTTGAGTGCCTATATTT ATCCATTCAA CTATACGCGC
CGCCCGCCGG CGCGTATGCG TAGTGATGTTTTCCCGCCTT CAGTGCGCGG
TAAAACATTG AAGATAGTCA GACGTGGTGG TGACACACCGCGGTTGATGA
ATACATCCCA CTATGGCGCG CTCGCTCGCC TTGTGTTGTA TTCCATCATT
CACTAACTAA CTCCCTATAG TAGGCCTCAA ATAATGTGTG ACTACCCCCT
AAATTTAAGCATAT

JX423805

GGGTCGATGA AGAACGCAGC TAAATGCGCG TCAGAATGTG AACTGCAGGA
CACATGAACACCGACACGTT GAACGCATAT TGCACATCGT ACTACCAGTA
CGATGTACAC ATTTTTGAGTGCCTATATTT ATCCATTCAA CTATACGTGC
CGCCCGCCGG CGCGTATGCG TAGTGATGTTTTCCCGCCTT CAGTGCGCGG
TAAAACATTG AAGATAGTCAGACGTGGTGG TGGTGACACACCGCCGCGGT
TGATGAATAC ATCCCACTAT GGCGCGCTCG CTCGCCTTGT
GTTTTATTCCATCATTCACT AACTAACTAA CTAACGCTCT ATAGTAGGCC
TCAAATAATG TGTGACTACCCGCTGAATTT AAGCATAT

JX423805

GGGTCGATGA AGAACGCAGC TAAATGCGCG TCAGAATGTG AACTGCAGGA
CACATGAACACCGACACGTT GAACGCATAT TGCACATCGT ACTACCAGTA
CGATGTACAC ATTTTTGAGTGCCTATATTT ATCCATTCAA CTATACGCGC
CGCCCGCCGG CGCGTATGCG TAGTGATGTTTTCCCGCCTT CAGTGCGCGG
TAAAACATTG AAGATAGTCAGACGTGGTGG TGGTGACACACCGCCGCGGT
TGATGAATAC ATCCCACTAT GGCGCGCTCG CTCGCCTTGT
GTTGTATTCCATCATTCACTAACTAACTAACTAACGCTCTATAGTAGGCC
TCAAATAATG TGTGACTACCCGCTGAATTTAAGCATAT

Fig. 10. Alignment of ITS2 sequence type of *Ae. aegypti* -Jeddah

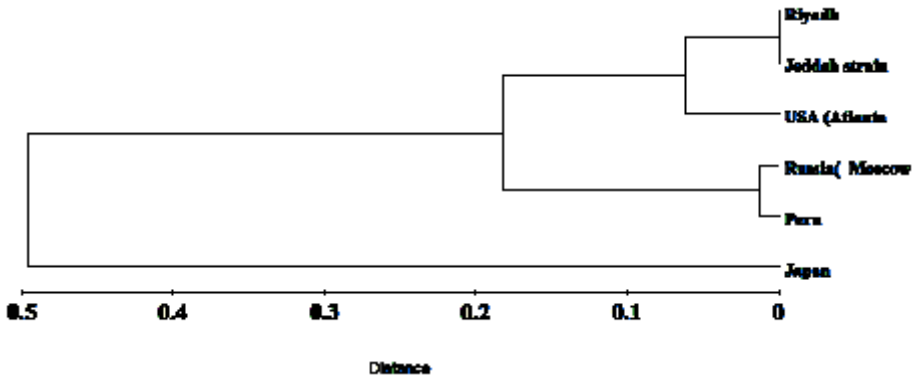


Fig. 11. Phylogenetic tree constructed based on analysis of *Ae. aegypti* ITS gene sequences of six *Ae. aegypti*

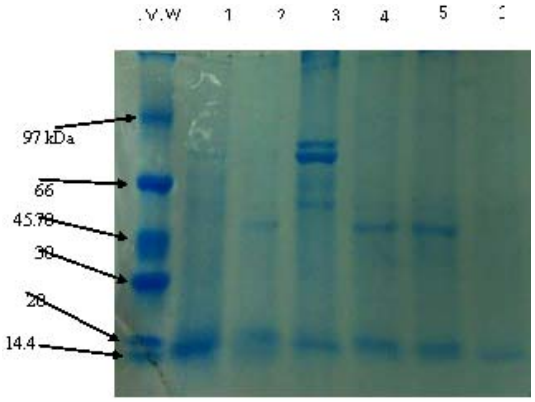


Fig. 12. Protein identification using SDS electrophoresis analysis of four development stages of *Ae. aegypti*

Phylogenetic analysis

Recently Phylogenetic analysis was conducted based on nuclear genes and morphology. (Reidenbach *et al.,* 2009 Shepard, *et al.,* 2006 and Reinert, *et al.,* 2008). Phylogenetic and molecular evolutionary analysis of ITS gene of *Ae. aegypti*, dengue fever vector isolates available in GenBank were conducted using *MEGA* version 5 (Tamura *et al.,* 2011) (Fig .11 and Table 3) The ITS2 region is more conserved than ITS1 and most widely sequenced DNA region in mosquito genera of *Aedes* (Wesson *et al.,* 1992 and Marrelli *et al.,* 2006). In this study, the ITS region of *Ae. aegypti* was sequenced.

The nucleotide sequencing of amplified conserved region of ITS genes of *Ae. aegypti* were Blasted using NCBI data base showed the high similarity rate (approximately 99%), compared to the closely related species *Ae. aegypti* that conducted in (Riyadh) and variation rate with other studies in USA, Peru, Russian and Japan. The results obtained from phylogenetic analysis revealed that KSA (Jeddah) and KSA (Riyadh) were 99% identical, whereas KSA (Jeddah) and / or KSA (Riyadh) were 94% identical to Sarthe identity between KSA (Jeddah) and / or KSA (Riyadh) and Peru , Russia and Japan were 81 % , 82% and 55%, respectively. The evidence of this results showed that the tested mosquitoes in (Jeddah) and (Riyadh) KSA belong to the same species.

Total protein

Two gels were obtained for different developmental stages of *Ae.aegypti* .The protein samples were quantified using high and low protein marker Fig. 12. The results revealed that six protein bands were identified ranged from 16.6 to 75kDa in molecular weight, Fig. 12. This data consistent with other data reported by Lee *et al*., 2009 who found similar protein bands, which fall in the range of approximately 200 kDa to less than 24 kDa. This result in agreement with Rohani, *et al*., 2005 who mentioned that the protein bands were within the range of 14 - 80 kDa. Another study described total and specific protein isolated from matures and immature stages of *Ae.aegypti*, Junsuo and Jianyong (2006).

CONCLUSION

Morphological identification is time consuming and not accuracy for identification to the species level**.** The results of this study demonstrated that DNA was obtained from specimens in three developmental stages (adults,

pupae and larvae). The gene fragment can be amplified from lab and the field strain of *Ae. aegypti*. The molecular marker of ITS gene provided reliable information for identifying species of mosquitoes. These markers are powerful with morphological taxonomic. These results provide basic information and an initial step for identification of differentially specific proteins of *Ae. aegypti*. Further studies are needed in molecular identification on dengue fever vector using advanced technique such as real time PCR and Microarry to differentiate between subspecies of *Aedes aegypti* mosquito. Proteomics study is necessary for identifying specific proteins and their function and biological roles in dengue fever infections leading to understand developing novel vector control strategies and parasite-vector interactions, gene expression that will be dominate post- research.

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