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Phylogenetic Analysis of MERSCoV in Human and Camels in Iraq

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ABSTRACT

Most of Middle East Respiratory Syndrome cases have been reported in Arabian Peninsula in addition to document of sporadic cases in Europe and Asia. Genetic recombination implicated in the emergence of (MERSCoV), virulence host adaptation, transmission and other zoonotic and epidemiological features, this study was conducted to evaluate the genetic relationship among Middle East respiratory syndrome coronavirus (MERSCoV) of human and camels' origin at the period from October 2015 to February 2016. Hundred samples were collected from camel and 100 from human. Camel samples secerned by immunochromatographic assay (ICA) for detection of viral antigen. The total percentage of ICA positivity was 28%. Human and camel samples subjected to Revers transcription real time-PCR and carried out by RNA extraction by using specific primers (F-TGCAAGCTTTTGGTCTTCGC) (R-AGCAAGCTCAGCAATTTGGG) and Taq- Man-Probe (FAM-TCGGCACTGAGGACCCACGT-BHQ1) for detection of nucleocapsid gene (N gene) 113 bp. The total positive result in camels were 15%, there was no significant difference between sex and type of samples, in relation to the age group the results showed that age group more than ten years was the highest percent with significant difference at P<0.05. According to the months of the year, October recorded the highest infection rate with significant difference at p<0.05. While the result of RT-RT-PCR according to the regions of study showed that Al-shinafyah in western borders of Iraq-Saudi was the highest infection rate 35% with significant difference at P<0.05. On the other hand, 100 human nasal swaps and bronchial lavage samples were collected from pilgrims and non-pilgrims, the total positive result was 5%. The pilgrims recorded the highest infection rate. The result of conventional PCR by using specific primers (F-TGCAAGCTTTTGGTCTTCGC), (R- ATGGCTCCACTGTACCGAAG) for detection of Ngene (217 bp) of MERSCoV was confirmative, three humans and 11 camel positive samples were used in further sequencing and phylogenetic analysis by extraction and purification of the PCR products. Our clones sequence submitted in GenBank-NCPI and took their accession number.
(Camel- KX150506.1, KX150493.1, KX150494.1, KX150495.1, KX150496.1, KX150497.1, KX150498.1, KX150502.1, KX150503.1, KX150504.1, KX150505.1), (Human- KX150499.1, KX150500.1, KX150501.1) the phylogenetic tree construction and analysis results showed that most of Iraqi variants of camel and human were located in Clade-B in which Saudi Arabia strains were clustered. One of our clones (MERS-Iq.2Huh) of accession number KX150500.1 was located in clade-A in the same branch of Jordanian strain while bat corona virus, SARS corona and neoromica corona virus was out group clustered in separated branch.

Keywords: *MERSCoV, Real Time PCR, Phylogenetic analysis, camel*

INTRODUCTION

Middle East Respiratory Syndrome Corona Virus (MERSCoV) is widely spread in Arabian Peninsula and many other Middle East countries surrounded of Iraq [1, 2,3,4,5]. It has become one of the most important emerging human health threating virus [6]. *MERSCoV* is beta corona virus within *coronaviridae* family which are enveloped, positive sense RNA genome with nucleocapsid of helical symmetry infect human and variety of animal species [7]. The ability of high recombination, unique viral replication and low fidelity of corona virus polymerases allows for unexpected viral evolution to infect other host.[8,9] Phylogenetic analysis of African bat virus belonging to the same species of *MERSCoV* and indicate that the evolution of the virus in camels precede that in human suggesting the possible spreading from bats to camels took place in Africa and involved exchange of genetic materials among ancestral virus strain*s*[10,11] *Nucleocapsid gene (N gene)* is common target for cloning phylogenetic analysis and generation recombinants portions. N protein is highly immunogenic phosphoprotein and modulation of cell signaling method. The N *gene* has been used for corona virus genotyping and phylogenetic analysis which helped our knowledge of virus temporal geographic origins and evolution [12,13]. This study was conducted to evaluate the genetic relationship among local circulating *MERSCoV* variants in human and camel at the first time in Iraq.

Materials and methods

This study was carried out by collection of 100 nasal and oropharyngeal swap samples from camels and 100 nasal swap and bronchial lavage samples from humans at the period from October 2015 to February 2016 from both sexes and different ages in many locations of Middle Euphrates/Iraq .Camel samples were subjected rapid immunochromatographic assay (ICA) for detection of *MERSCoV* antigen by using Rapid (ICA) MERS-COV Camel Strip kit Bionote Korea. According to [14]

Viral RNA has been extracted by Total RNA Extraction KitAccuZol™ kit bioneer Korea. The extracted RNA has been measured for concentration and purity by Nanodrop. Reverse transcription conducted by AccuPower® Rocket Script™ RT PreMix 96 plate kit bioneer Korea and Real Time PCR applied by AccuPower ® Dual star ™ qPCR PreMix 96 plate kit bioneer Korea using specific primers (F-TGCAAGCTTTTGGTCTTCGC) (R-AGCAAGCTCAGCAATTTGGG) and Taq- Man-Probe (FAM-TCGGCACTGAGGACCCACGT-BHQ1) for detection of *N gene* fragment 113 bp [15,16].

Conventional end point PCR for detection of *N gene* fragment 217 bp, the PCR products of positive samples were extracted and purified and sequenced by dye-terminator based sequenced illumina by AB. The genomic sequences were assembled and submitted in GenBank- NCBI then multiple sequence alignment was done by clustal Omega for phylogenetic tree construction and phylogenetic analysis [12, 17].

Statistical analysis using Chi square to assess statistical significance according to [18]

Results

The total positive results of *MERSCoV* antigen detection in camel by rapid test (ICA) was 28% and statistically there was no significant difference between sexes and type of samples (Table 1), while there was significant difference at $P<0.05$ among age groups, study locations and month of study (Table 2).

Table (1) the results of infection rate according to the sexes and type of sample in camel by using by ICA

Sex	No. of samples examined	No. of positive sample	Infection Percentage %
Female	**83**	**22**	**(26.5%)^{A}**
Male	**17**	**6**	**(35.29%)^{A}**
Type of sample collection			
Nasal swab	**94**	**27**	**(28.72%)^{A}**
Oropharyngeal swab	**6**	**1**	**(16.66%)^{A}**

- Similar letters refer to the non-significant differences

Table (2) The results of infection rate according to the age, region and month in camel by using ICA.

Age	No. of samples examined	No. of positive sample	Infection Percentage %
1month-1 year	**9**	**0**	**(0%)^{A}**
1-5year	**41**	**11**	**(26.82%)^{B}**
5-10	**38**	**14**	**(36.84%)^{B}**
>10	**12**	**3**	**(25%)^{B}**
Region			
Al-Diwanyah/Al- shnifyah	**20**	**10**	**(50) ^{A}**
Al-Diwanyah/Al-Shafayah	**20**	**8**	**(40%)^{A}**
Al-Diwanyah/ Afak	**15**	**3**	**(20%)^{B}**
Al-Diwanyah/ Sumer	**10**	**1**	**(10%)^{B}**
Babel/Hamza	**5**	**1**	**(20%)^{B}**
Al-Muthana/ Al-Rumetha	**10**	**1**	**(10%)^{B}**
Slaughterhouse	**20**	**4**	**(20%)^{B}**
Month of the year			
October	**20**	**8**	**(40%) ^{A}**
November	**24**	**6**	**(25%)^{A}**
December	**32**	**8**	**(25%)^{A}**
January	**8**	**0**	**(0%)^{B}**
February	**16**	**6**	**(37.5%)^{A}**

- Difference letters refer to the significant differences at P<0.05

The positive result of *MERSCoV* infection in camel by RT-RT-PCR in camel was 15% with different cycles of threshold (CT) ranging from 12-22. These result again statistically showed no significant difference in relation to the sex and type of samples (Table 3).

Table (3) the results of infection rate according to the sex and type of sample in camel by using RT-qPCR

Sex	*No. Sample*	*No. of positive sample*	*Infection percentage*
Female	**83**	**12**	**(14.45%)^{A}**
Male	**17**	**3**	**(17.64%)^{A}**
Type of sample			
Nasal swab	**94**	**14**	**(14.89%)^{A}**
Oropharyngeal swab	**6**	**1**	**(16.66%)^{A}**

- Similar letters refer to the non-significant differences

While there was significant difference at P<0.05 among age group, geographical location and months of the study Table (4).

Table (4) **The results of infection rate in relation to age groups, region and month in camel by using RT-qPCR technique:**

AGE groups	*NO. Samples*	*No. of positive sample*	*Infection Percentage%*
1month-1 year	**9**	**0**	**(0%)^{B}**
1-5year	**41**	**6**	**(14.63%)^{A}**
5-10	**38**	**6**	**(15.78%)^{A}**
>10	**12**	**3**	**(25%)^{A}**
Region			
Aldiwanyah/Al shnifyah	**20**	**7**	**(35%)^{A}**
Aldiwanyah/Al shafayah	**20**	**5**	**(25%)^{A}**
Aldiwanyah/ Afak	**15**	**1**	**(6.66%)^{B}**
Aldiwanyah /Sumer	**10**	**0**	**(0%)^{C}**
Babel/Hamza	**5**	**0**	**(0%)^{C}**
A-lRumetha/Al sumawa	**10**	**0**	**(0%)^{C}**
Slaughterhouse	**20**	**2**	**(10%)^{B}**
Month of the year	***No. Sample***	***No. of positive sample***	***Infection Percentage***
October	**20**	**8**	**(40%)^{A}**
November	**24**	**3**	**(12.5%)^{B}**
December	**32**	**1**	**(3.12%)^{C}**
January	**8**	**0**	**(0%)^{D}**
February	**16**	**3**	**(18.75%)^{B}**

- Difference letterers refers to the significant differences at P<0.05

The positivity result of human *MERSCoV* infection by RT-RT-PCR was 5% with different cycles of threshold (CT) ranging from 13-17 there was no significant difference between gender (Table 5).

Table (5) The results of infection rate in sex groups in human by using RT-qPCR technique

Gender	No. Sample	No. of positive sample	Infection Percentage
Female	**30**	**2**	**(6.66%)^{A}**
Male	**70**	**3**	**(4.28%)^{A}**

- Similar letters refer to the non-significant differences

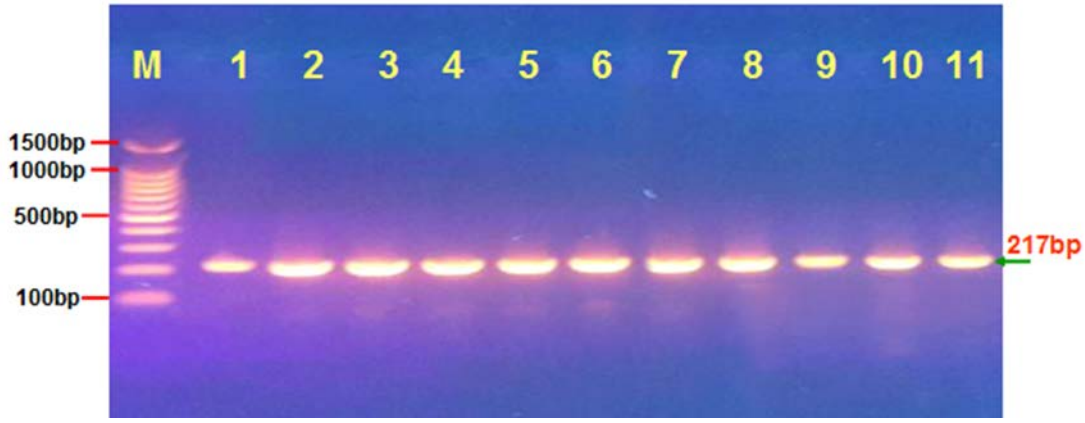
While there was significant difference at P<0.05 between type of sample, age group, months of the study and type of patients (Table 6).

Table (6) The results of infection rate in human in relation to age groups, type of sample, month and type of patient by using RT-qPCR technique:

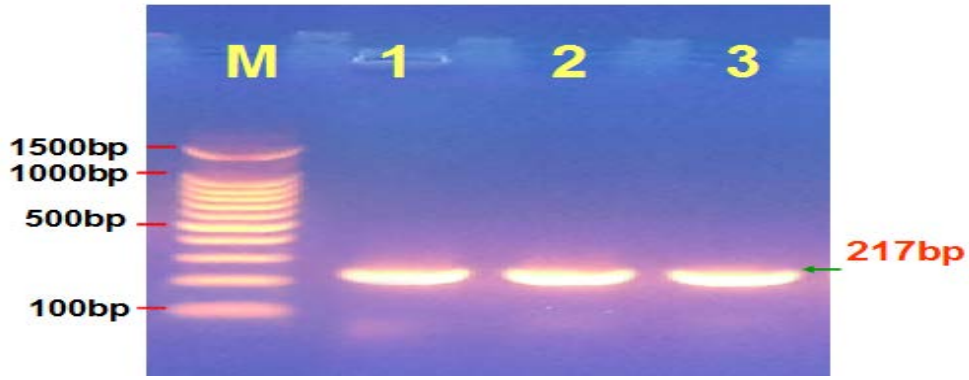
Age groups	*No. Sample*	*No. of positive sample*	*Infection Percentage*
<5year	**20**	**0**	**(0%)^{B}**
5-25	**18**	**0**	**(0%)^{B}**
25-50	**25**	**1**	**(4%)^{A}**
50-80	**37**	**4**	**(10.81%)^{A}**
Type of sample			
Nasal swab	**81**	***5***	***(6.17%)^{A}***
Broncho lavage sample	**19**	**0**	**(0%)^{B}**
Month			
October	**30**	**4**	**(13.33%)^{A}**
November	**34**	**1**	**(2.94%)^{B}**
December	**10**	**0**	**(0%)^{C}**
January	**10**	**0**	**(0%)^{C}**
February	**16**	**0**	**(0%)^{C}**
Type of patient			
pilgrim	**30**	**4**	**(13.33%)^{A}**
Non_ pilgrim	**70**	**1**	**(1.42%)^{B}**

- Difference letterers refers to the significant differences at P<0.05

The result of conventional PCR of camel and human were confirmative Fig.-1, Fig.-2. Fourteen of our clones which were 11 of camel and 3 of human were took their accession number in GenBank-NCBI.



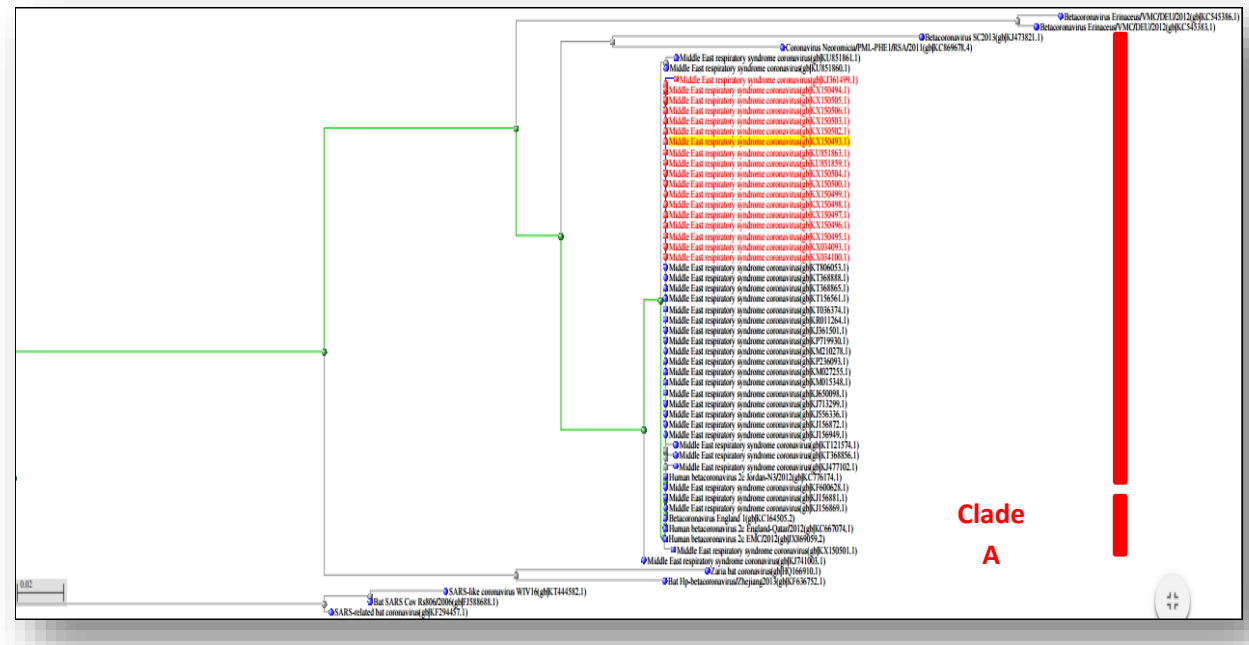
(Figure 1): Agarose gel electrophoresis image that show the PCR product analysis of *Nucleocapsid* -gene of *MERSCoV* positive Camel



(Figure 2): Agarose gel electrophoresis image that show the PCR product analysis of *Nucleocapsid* gene in *MERSCoV* positive Human samples.

The result of phylogenetic analysis showed most of Iraqi clones were grouped in clad B and clustered mainly with the same branch of Saudi Arabia and South Korea, only one clone in this study clustered in the same branch of Jordanian in clade A. while *bat corona virus*, *SARSV* and *neoromica corona virus* was out group clustered in separated branch Fig.-3, Fig.-4

Clade
B



Figure(4-14)phylogenetic tree of our clones MERS-CoV with references world *MERS-CoV* and Bat and *SARS-Coronavirus* and their accession No.

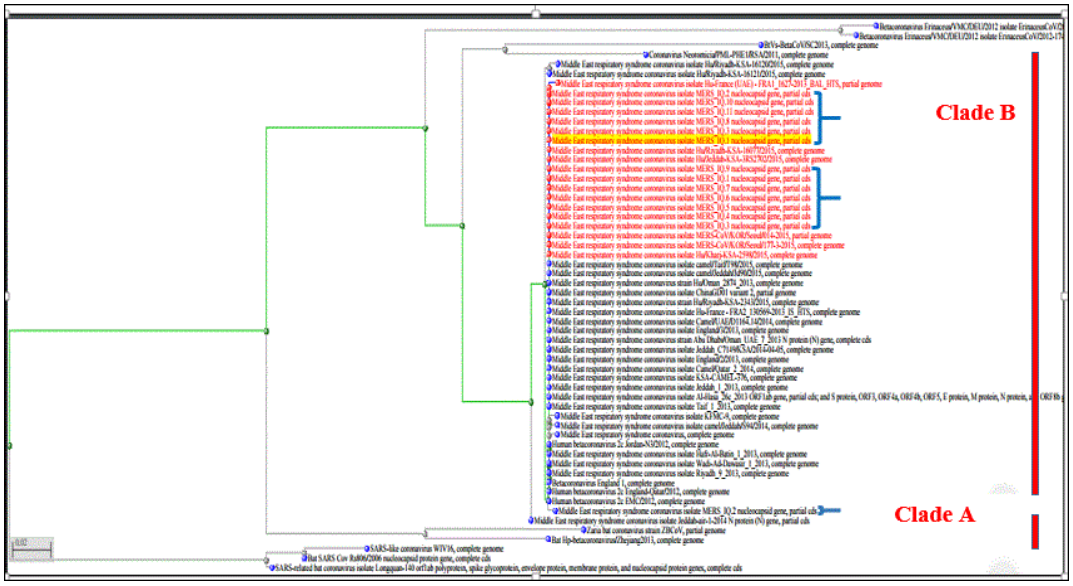


Figure (4) phylogenetic tree of our clones *MERSCoV* with references world *MERSCoV* and Bat and *SARS-Coronavirus* and the title of strains

Discussion

MERSCoV was first recorded in 2012 in Saudi Arabia, the virus associated with severe respiratory illness, renal failure and high rate of mortality 50% [18, 19]. The emergence of this important infectious pathogen has raised global concerns regarding the current epidemiological features and its future evolution. Camels may consider the potential source of human infection and act as reservoir transmit the virus to human. This study was designed for molecular characterization of the virus and to explain some epidemiological of MERS [20, 21].

High prevalence of infection in camels recorded by using rapid (ICA) and definitive molecular techniques although our results were lower than that revealed by many previous serological surveys [4, 22, 23, 24].The detection of *MERSCoV* antigen in combination with viral RNA demonstration indicate likely presence of infectious virus as compare with serological tests [14], in which the positive *MERSCoV* antibodies may be developed from past exposure without virus shedding, as well as most of those study conducted in endemic locations. The results of this study showed geographical variability in camels' infection, the highest rate was at Iraq-Saudi borders in which mixing of grazing camels in same pastures across these borders.

Despite of high infection rate of camels [25,26,27,28] and direct contact of camels' owners including consumption of animals' products, we did not record any positive results, which may be caused by difficulty in transmission as lower respiratory tract shedding of the virus, and presence of some degree of immunity. Actually there was lack of active surveillance programs and databases, so further seroepidemiological studies are recommended. On the other hand, there was positive results recorded among pilgrims whom they may becoming infected during period of pilgrimage in Mecca / Saudi Arabia [29,30] mentioned the risk of pilgrims returning with *MERSCoV* from human to human transmission in that large mass gathering of Hajj.

Phylogenetic analysis demonstrates that most Iraqi variants of *MERSCoV* of camel and human of this study fell in the Clade-B with Saudi strains as well as 2015 South Korea outbreak strains [31 ,32, 17] due to continuous mixing and introduction of camel across Saudi borders in addition to large number of travelers in Hajj season. Furthermore, recent recombination and emergence of that novel virus play a role in close relation and high identity among these strains of *MERSCoV*.

Conclusions

MERS was widely distributed in apparently healthy camels at the western borders of Iraq, the risk of pilgrims returning with *MERSCoV* during the large mass gathering of annual Hajj and minor Umrah must be considered. Genotypically, human and camel variants fell in the same branch of phylogenetic tree with about 100% identity that indicates the role of camel as a reservoir or intermediate host in zoonotic transmission. Application RT-RT-PCR screening technique in combination with wide seroepidemiological surveys for the disease in aiding in the strict surveillance.

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