Phylogenetic Analysis of *MERSCoV* in Human and Camels in Iraq

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الخلاصة

ان فايروس *متلازمة الشرق الاوسط التنفسيه MERS-CoV* يصيب الانسان يقع ضمن التصنيف الوراثي لفايروس *MERS-CoV* المعزول من الجمال مما يؤكد الاهميه المشتركة والدور الذي تلعبة الجمال في نقل الاصابه الى الانسان .صممت الدراسة الحالية للتحري عن المرض لأول مرة في العراق ودراسة بعض المظاهر الوبائية له اضافة الى التوصيف الجزيئي وتحديد العلاقه الفيلوجينيه لهذا الفايروس وللفترة من تشرين الأول 2015 الى شباط 2016 .عن طريق جمع 100 نموذج 94 من المسحات الانفية وو9 من المسحات الفمية البلعومية في الجمال من مختلف الاعمار وفي مناطق مختلفه ,اذ تم التحري عن المستضدات الفيروس بأستخدام اختبار الترحيل المناعي السريع Immunochromatographic assay (ICA)

اظهرت النتائج ان 28% من العينا ت المفحوصة اعطت نتائج موجبة في حين لم تسجل فروق معوية بين الجنسين و سجلت الفئة العمرية من (5-10) سنه اعلى نسبة اصابة(36.84%) مع وجود فروق معنويه تحت مستوى احتماليه $p<0.05$ كما اعطت كلا النوعيين من العينات نتائج موجبة مع عدم وجود فروق معنوية احصائيا بين المسحة الانفية والفموية البلعومية .

كما اظهرت النتائج طبقا الى المناطق الدراسة المختلفة ان الشنافية كانت اعلى النسب (50%) مع وجود فروقات معنوية بين المناطق الدراسة تحت مستوى احتمالية $p<0.05$, في حين سجل شهر تشرين الاول اعلى نسبة اصابة (40%) بالمقارنة مع اشهر الدراسة مع وجود فرق معنوية تحت مستوى احتمالية $p<0.05$.

تم في هذه الدراسة استخدام التقنية الجزيئية للتحقق من النتائج وذلك عن طريق استخلاص الحامض النووي الرايبي RNA لفيروس *MERS-CoV* وقياس تركيزه ونقاوته بواسطة جهاز Nanodrop بعد ذلك تم تحويل RNA الى cDNA عن طريق استخدام الاستنساخ العكسي Reverse transcriptase ليتسنى التحري عن قطعة الجين المشفرة لبروتين الغلاف النووي *Nucleocapsid gene* وذلك بأستخدام بادئات primers ,ومجس خاص Taq man pobe وتطبيقها تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي (RT-qPCR) . كانت النسبة الكلية للأصابة 15% في حين تراوحت دورات العتبة Threshold cycles (CT) من (12-22) دورة .على اساس الجنس لم تكن هناك فروقات معنوية بين الجنسين بينما سجلت الفئة العمرية اكثر من عشرة سنوات اعلى نسبة اصابة(25%) مقارنة بالفئات العمرية الاخرى مع وجود فروق معنويه تحت مستوى احتمالية $p<0.05$.وقد سجلت شهر تشرين الاول اعلى النسب (40%) مقارنة بأشهر الدراسة الاخرى مع وجود فروق المعنوية تحت مستوى احتمال $p<0.05$.

كما اظهرت النتائج تفاعل سلسلة البلمرة في الوقت الحقيقي لعينات الجمال في المناطق المختلفة ان اعلى النسب وهي مطابقة لما تم تسجيلها لهذة العينات عن طريق الفحص السريع تم تسجيلها في المنطقة الشنافية (35%) بين مناطق الدراسة المختلفة .$p<0.05$) مع وجود فروقات معنويه تحت مستوى احتماليه ICA(

لقد تم في هذه الدراسة جمع 100 عينة من الحجاج وغير الحجاج من المسحات الانفية والغسيل القصبي (Nasal swabs &Broncho alveolar lavage sample) وفحصها RT-qPCR للتحري عن قطعة الجين المشفر للبروتين الغلاف النووي (*Nucleocapsid gene*) لفايروس *MERS-CoV* اذ كانت النسب الكلية للأصابة % 5 وقد تراوحت دورات العتبة Cycle (17-13)CT ولم تسجل فروقات معنويه بين الجنسين كما سجلت الفئة العمرية اكثر من 50سنة اعلى نسبة اصابة (10.81%) مع وجود فروق معنوية تحت مستوى احتمالية $p<0.05$ بين المجاميع العمرية المختلفة . كما اظهرت نتائج الدراسة الحالية ان جميع العينات الموجبة كانت تعود الى المسحات الانفية (6.71%)ولم يسجل الغسيل القصبي اي نتائج موجبة, كما سجل شهر تشرين الاول اعلى نسبة اصابة (13.33%) مع وجود فروق معنويه بين اشهر الدراسة تحت مستوى احتمالية $p<0.05$.

ان النتائج الموجبة بين الحجاج كانت هي (13.33%) مقارنة مع غير الحجاج مع وجود فرق معنوي تحت مستوى احتمال $p<0.05$.وسجلت نتائج تفاعل سلسلة البلمرة التقليدي Conventional PCR لل cDNA للتحري عن قطعة جين الغلاف النووي (*Nucleocapsid gene*) 217b في جل الاكاروز المصبوغ بروميد الاثيديوم Ethidium bromide. وبأستخدام بادئات متخصصة وكانت احدى عشر عينة من عينات الجمال موجبة لهذا الاختبار ,في حين ثلاثة فقط عينات الانسان كانت موجبة وقد تم استخلاص وتنقية قطعة الجين لأستخدامها في قراءة تعاقب النيكلوتيدات (Gene sequence) والتحليل الفيلوجيني (Phylogenetic Analysis) وبعد الحصول على اربعة عشر نسيلة (Clones) قدمت في بنك الجينات في

المركز الوطني لمعلومات التقنات الاحيائية في الولايات المتحدة الامريكية (**GenBank /NCBI**) لغرض التسجيل والحصول على رقم الانضمام وقد اظهرت النتائج الرسالة الالكترونية لبنك الجينات أن اربعة عشر نسيلة قد سجلت وأعطيت رقم الانضمام الخاص في الانسان كما تم تسجيلها ايضا في ارشيف النيكلوتيدات الاوروبي (**ENA**) و بنك الجينات الياباني **(DDBJ).**

ان نتائج بناء الشجرة الوراثية والتحليل الفيلوجيني اظهرت ان جميع العتر العراقية وقعت في العرق(ب)(Clade B)الذي يضم العتر السعودية ويشمل عتر الجمال والانسان وفي نفس الفرع على الشجرة الفيلوجينية بينما كانت MERS_IQ.2Huh برقم تسلسل KX150501.1 تعود الى عتر الانسان في هذة الدراسة ضمن العرق (أ) (Clade A) والذي وقعت ضمن نفس الفرع المنفصل للعتر الاردنية والمصريه وEMC , بينما كانت عتر الخفافيش والسارس خارج مجموعة *MERS-CoV* ومن ضمن عائلة الكورونا خارج المجوعة تماما وبشكل منفصل في جذر خاص (Out group)

يمكن ان نستنتج ان فيروسات *MERS-CoV* منتشرة وبشكل واسع في الجمال وخصوصا في المناطق الغربية القريبة من الحدود السعودية في حين سجل عدد من الحالات الموجبة في الحجاج العائدين من الديار المقدسة في موسم الحج والعمرة وان الانواع الجينية السائدة كانت 100% مطابقة الى العتر المنتشرة في الجزيرة العربية .

ABSTRACT

The present 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.One hundred samples were collected from camel and 100 from human. Nighty four from nasal swabs and six from oropharyngeal swabs 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 and Taq- Man-Probe for detection of *nucleocapsid 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 of camel was the heights 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. the result of RT-qPCR according to the regions of study showed that Al-shinafyah in western borders of Iraq-Saudi was the highest infection rate 35% .On the other hand ,100 human 81 nasal swabs and 19 bronchial lavage samples were collected from pilgrims and non-pilgrims. The total positive result was 5%. The pilgrims recorded the highest infection rate. The results of conventional PCR by using specific primers for detection of *Nucleocapsid gene* (217 bp) of *MERSCoV*. The results were confirmative. Three human 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-NCBI for accession number.

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 widly 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 strains[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* have 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 human 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 to rapid immunochromatographic assay (ICA)for detection of *MERSCoV* antigen by using Rapid (ICA) MERS-COV Camel Strip kit Bionote Korea. [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. 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].

Results

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

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

Sex	No. of examined samples	No. of positive samples	infection Percentage (%)
Females	83	22	(26.5%)[A]

Males	17	6	(35.29%)[A]
Type of sample collection			
Nasal swabs	94	27	(28.72%)[A]
Oropharyngeal swabs	6	1	(16.66%)[A]

- Similar letters refers to the non-significant differences

Table (2) Iinfection rate according to the age, region and month in camel by using ICA.

Age	No. of examined samples	No. of positive samples	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 letterers refers 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) 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 refers to the non-significant differences

While there were 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) Infection rate in sex groups in human by using RT-qPCR technique

Sex	**No. Sample**	**No. of positive sample**	**Infection Percentage (%)**
Females	**30**	**2**	**(6.66%)[A]**
Males	**70**	**3**	**(4.28%)[A]**

- Similar letters refers to the non-significant differences

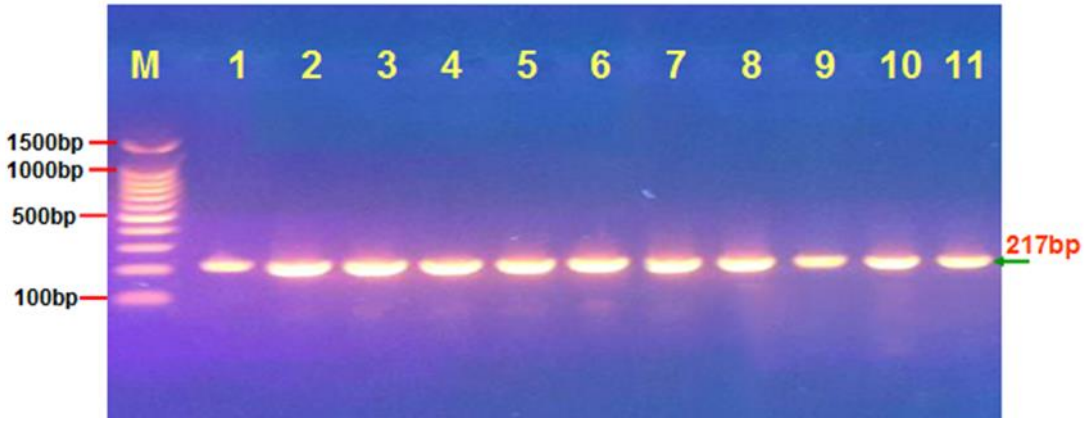
While there were significant difference at P<0.05 between type of sample, age group, months of the study and type of patients (Table 6).

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

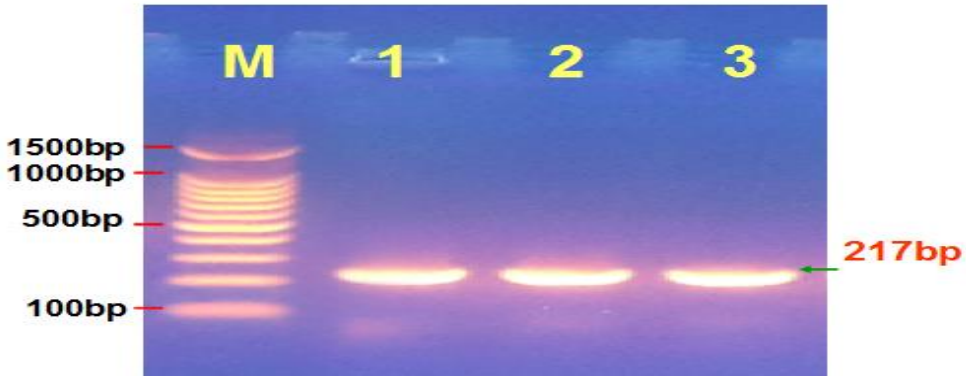
Age groups	No. Samples	No. of positive samples	Infection Percentage (%)
<5year	20	0	(0%)[B]
5-25	18	0	(0%)[B]
25-50	25	1	(4%)[A]
50-80	37	4	(10.81%)[A]
Type of samples			
Nasal swabs	81	5	(6.17%)[A]
Broncho lavage samples	19	0	(0%)[B]
Months			
October	30	4	(13.33%)[A]
November	34	1	(2.94%)[B]
December	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.

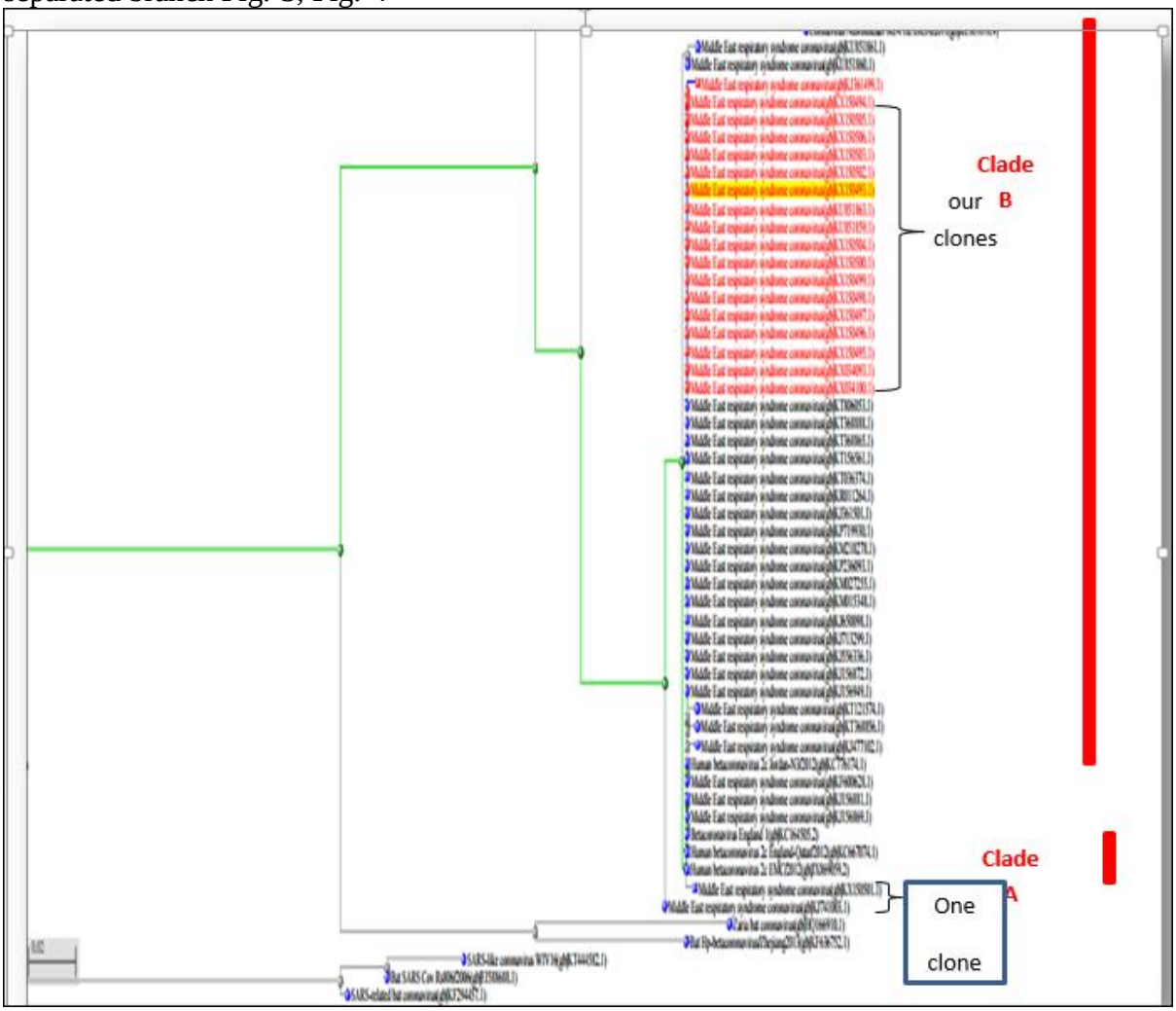


(Figure 1): Agarose gel electrophoresis image that show the PCR product analysis of *Nucleocapsid* -gene of *MERSCoV* positive Camel



(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



Figure(3)phylogenetic tree of our clones MERS-CoV with references world *MERS-CoV* and Bat and *SARS-Coronavirus* and their accession No.

Clade
our B
clones

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 considered 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 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 recorded any positive results , may cause by difficulty in transmission as lower respiratory tract shedding of the virus ,and presence of some degree of immunity ,actually there was lack in active surveillance programs and databases, so further seroepidemiological studies was 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 demonstrate 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 srrains 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.

Genotypicatlly 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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