MOLECULAR AND SEROLOGICAL IDENTIFICATION OF FOOT-AND-MOUTH DISEASE VIRUS SEROTYPES IN CATTLE OF BASRAH PROVINCE

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ABSTRACT

This study was conducted on serum from 6 different regions covering all parts of the Basrah province during the period from October 2012 to June 2013 in order to determine the prevalence of foot and mouth disease in bovine species. From the serology results, the overall prevalence of this disease was(72.7%.) . Of the 165 samples subjected to Nonstructural protein (NSP) enzyme linked immunosorbent assay (ELISA) screening test, 120 were interpreted as positive representing (72.7%.) while the other 45 samples were negative representing 27.3%. The seropositivity significantly associated with age groups (p<0.01) and sex (P < 0.05).

RT-PCR base detection of FMDV for primary and serotype specific diagnosis was used. Different types of clinically positive samples of FMD were collected from the cattle which includes samples from mouth vesicles, serum and saliva .RT-PCR based identification was done with(universal primer sets 1F / 1R, A- 1C562, O-1C272 and As1-1C505) with expected band of(328, 866, 639 and 911 bps.respectively) . Of eighty three samples collected, only 68(81.9%) were successfully amplified by RT-PCR, and of these, only 19(22.9%) samples were found to be serotype A-1C562 positive and 26(31.3%) were serotype O-1C272 positive and nil for serotype As1-1C505. The frequency of foot-and-mouth disease viral genome presence in cattle had no significant differences based on sex (P>0.05) while the difference among age groups was significant (P < 0.05)

INTRODUCTION

Foot-and-mouth disease (FMD) is a contagious viral disease of cloven-hoofed animals, including cattle, pigs, sheep and buffalo. The virus exists in seven distinct serotypes: O, A, C, Asia-1,SAT 1, SAT 2 and SAT 3(1). The disease can cause mortality in young stock, with high morbidity in adult animals, decreased productivity and failure to return to prime condition following infection. In addition, severe economic losses may be incurred due to sanctions against trade in livestock and animal products originating from infected regions. The aetiological agent, foot-and-mouth disease virus (FMDV), is a non-enveloped, RNA virus belonging to the genus Aphthovirus within the family Picornaviridae(2). The virus has a positive sense, ssRNA genome of about 8.3 kb, enclosed within a protein capsid. This capsid, ca.28 nm in diameter, is composed of 60 copies of four different structural polypeptides; the VP1, VP2 and VP3 proteins are surface exposed while VP4 is entirely internal. The coding sequence for the VP1 protein has been extensively used for molecular epidemiological studies (3, 4, 5). The VP1 protein is considered to be highly immunogenic but VP2 and VP3 also contribute to the antigenic properties of the virus (6). FMD is endemic in large parts of Africa, Asia and SouthAmerica. The virus can readily cross international boundaries and hence cause epidemics in previously free areas FMD is endemic and widespread; the serotypes O, A and Asia-1 are responsible for the disease outbreaks. In order to achieve better control of the disease in such countries, it is essential to monitor the circulating strains of the prevalent serotypes of FMDV in the field to ensure that the most appropriate vaccine strains are used(7). The infected animals can be discriminated from vaccinated by non-structural proteins which can be detected only in infected animals. There are several specific ELISA to detect non-structural proteins (8) For example, a MAb trapping (MAT) ELISA for detecting antibody to 3ABC and blocking ELISAs for detecting antibody to 3AB or 3ABC. Currently, the Enzyme-linked immunoelectrotransferblot assay (EITB) has been widely applied for sero-surveillance and risk assessment associated with animal movement. Another interesting method is a test strips containing the purified recombinant antigens of NS proteins (3A, 3B, 2C, 3D and 3ABC). These proteins are expressed in *E. coli* C600.A sample is positive if all four antigens (3ABC, 3A, 3B and 3D) reactive at equal to or higher than the cut-off value (9).

In addition to the classical techniques of virus isolation in tissue culture and antigen detection by enzyme-linked immunosorbent assay (ELISA),RT-PCR has become established as a reliable, fast and sensitive method of early FMD diagnosis (10). Rapid identification of the serotype of the virus that is responsible for an outbreak is essential to speed up diagnosis, for selection of an appropriate emergency vaccine. A rapid and sensitive RT-PCR method for FMDV detection, which differentiated FMDV from other genetically and/or symptomatically related viruses and, more importantly, comprised a multiplex-PCR method that differentiated between common serotypes, has been reported (11).

   The objective of this study was therefore to determine the sero-prevalence of FMD in the Basrah province and to use a RT-PCR for the detection of FMDV in clinical samples obtained from suspect animals and dtermination of the main serotypes in circulation in cattle

MATERIALS AND METHODS

Animals

This study included 165 cows of both sex and different age ,with typical clinical symptoms of FMD as lameness , high fever and presence of some vesicles in the oral cavity and hoof .These animals belonged to 6 different regions covering all parts of the Basrah province during the period from October 2012 to June 2013

Samples collection Blood

Five ml of blood were drawn from each animal for separating sera after clotting for 2 hours at room temperature, the sera were separated by centrifugation for 15 minutes at 6000-10000 rpm and stored at -20°C until the *in vitro* tests were performed. Serum samples have been analyzed by applying serotype-specific antibody ELISA

Tissue

The clinical samples(83)from suspect animals showing clinical symptoms were collected in a transport buffer and stored at -20 °C until needed. These samples consisted of sera, saliva and vesicles fluid

Measurement of the antibodies

ELISA CHEKIT FMD-3ABC Bo-Ov ELISA Test had been used to determine and measure the antibodies in the serum of cows against recombinant non-structural protein 3ABC of the FMDV which is present in ELISA reaction microplate according

to manufacturer instructions (Bommeli Diagnostics, Switzerland).Briefly**,** each sample and control were prediluted1:100 in a tube using CHEKIT-FMD-3ABC sample diluents, as 5 μl of sample and control were added to 495 μl CHEKIT-FMD-3ABC sample diluent. The CHEKIT-10x wash concentrate 1:10 was diluted with D.W (1 part concentrate with 9 parts of D.W under sterile conditions and stored at 4°C. One hundred μl of prediluted samples and controls were dispensed into the appropriate wells of the Microtiter plate. The Microtiter plate was covered with a lid and incubated for 60 minutes at 37°C in humid chamber.Each well was washed with approximately 300 μl CHEKIT wash solution three times. One hundred μl of the CHEKIT-FMD-3ABC-Anti-Ruminant-lgG conjugate was dispensed into each well. The microtiter plate was covered and incubated for 60 minutes at 37°C in humid chamber then step of washing was repeated. One hundred μl CHEKIT-TMB substrate was dispensed into each well. The substrate was incubated at room temperature for 15 minute. The color reaction was stopped by adding 100 μl CHEKIT-stop solution TMB per well. In the same order and at the same seed as the substrate. The results were read using a photometer at a wave length of 450 nm ,the OD of 83 the positive control (ODpos) and the OD of the samples (ODsample) were corrected by subtracting the OD of the negative control (OD neg)**.**

$$Value\% = \frac{OD\ sample - OD\ neg}{OD\ pos - OD\ neg} \times 100$$

Interpretation of the results: if a %OD of less than 20% is negative, 20-30% is ambiguous and greater than 30% is positive.

RNA extraction and RT-PCR

   Total RNA was extracted from sera ,saliva and vesicle fluid using the QIAmp RNA extraction kit (QiagenInc) following the mini spin protocol according to the manufacturer's instructions. The extracted RNA was reverse transcribed using the QIAGEN One Step RT-PCR Kit (USA) according to the manufacturer's instructions . To confirm the presence of FMDV cDNA, a standard diagnostic PCR targeting the cDNA corresponding to the 5' un translated region of the FMDV RNA was carried using one Universal Primer(328bp)(12)-1F:5'-GCCTGGTCTTTCCAGGTCT-3';1R:5'- CCAGTCCCCTTCTCAGATC-3' and three serotypes forward primers:

A- 1C562(863-866bp)F:5'- TACCAAATTACACACGGGAA-3 (12) and O-1C272(639bp)F: 5'-GCAGCAAAACACATGTCAAACACCTT-3(13)and:As1-1C505(911bp)F(5'-TACACTGCTTCTGACGTGGC-3) (14)'with one reverse primer EUR- 2B525'- GACATGTCCTCCTGCATCTGGTTGAT-3'(4). This PCR was carried out by using QIAGEN One Step RT-PCR assay . The reaction mixture composed of 1μL of one of the forward primers and reverse primers , RNase free water (Variable μl), 5x QIAGEN one step RT-PCR buffer (10.0 μl) , dNTP Mix (2.0 μl), QIAGEN One Step RT-PCR Enzyme Mix (2.0 μl) and Template RNA (Variable μl) in a 50 μL reaction volume. Amplification reaction was done under the following conditions one cycle of 50°C for 30 min for reverse transcription, : one cycle of 95°C for 15 minutes for pre denaturation followed by 30 cycles of 94 °C for 1 min,58 °C for 1 min for annealing of A- 1C562 or O-1C272 Primer while 54°C and 55°C were used as annealing temperature of (Universal and As1-1C505 Primer respectively), 72 °C for1.5 min for extension, and finally, one cycle of 72 °C for 10 min for Final extension and a subsequent hold temperature of 4°C using a (Technethermocycler UK). The PCR products were confirmed by 1.5 % agarose gel electrophoresis after ethidium bromide staining and viewing under UV light alongside a DNA weight markers (Bioneer,Korea) .

Statistical Analysis

Statistical analysis is done by using SPSS software version 11, the chi square was used to assess Statistical significance**.**

RESULTS

Antibodies detection

The IgG antibodies against FMDV antigen was detected by ELISA CHEKIT FMD-3ABC Bo-Ov ELISA Test , the result of this test was displayed in table- 1.Out of 165 cows serum samples ,120 samples were sero-positive with an overall rate of 72.7% . According to sex of these animals , the higher rate of sero-positivity was observed in females (78.6%) . In concern to age of tested cows the cows in the2[nd] group (**>4-8** years) appeared in higher rate (84.8 %) of sero-positivity followed by the rate (**51.7%**) of 1st age group cows.There was significant differences (p<0.01) between males and females and among different age groups of cows (p< 0.05) concerning the ELISA

sero-positivity results.

Table (1) ELISA results according to sex and age of Cattle.

Variables		Ex. No.(%)	(Cattle No.%)	
			seropositive	seronegative
Sex	Males	53 (32.1)	32 (60.4)	21(39.6)
	Females	112(67.9)	88(78.6)	24(21.4)
	Total	165(100)	120(72.7)	45(27.3)
p<0.01				
Age groups (years)	>1-4	60(36.4)	31(51.7)	29(48.3)
	>4-8	105(63.6)	89(84.8)	16(15.2)
	Total	165(100)	120(72.7)	45(27.3)
P < 0.05				

PCR Analysis

  The result of RT- PCR amplification which was performed on the reverse transcript
RNA confirmed by electrophoresis as the strands of the DNA   which are resulted
from successful binding between specific universal primer (1F – 1R) and A , O ,
Asia1 primers (A- 1C562 F , O-1C272 F , As1-1C505 F , EUR- 2B52 R respectively)
and the extracted RNA , appeared as single band on 2% agarose and under U.V.
illuminator using Ethidium bromide as specific DNA stain. Only bands with expected
size (328bp , 865bp , 635bp, 911bp) were observed while no band was observed in
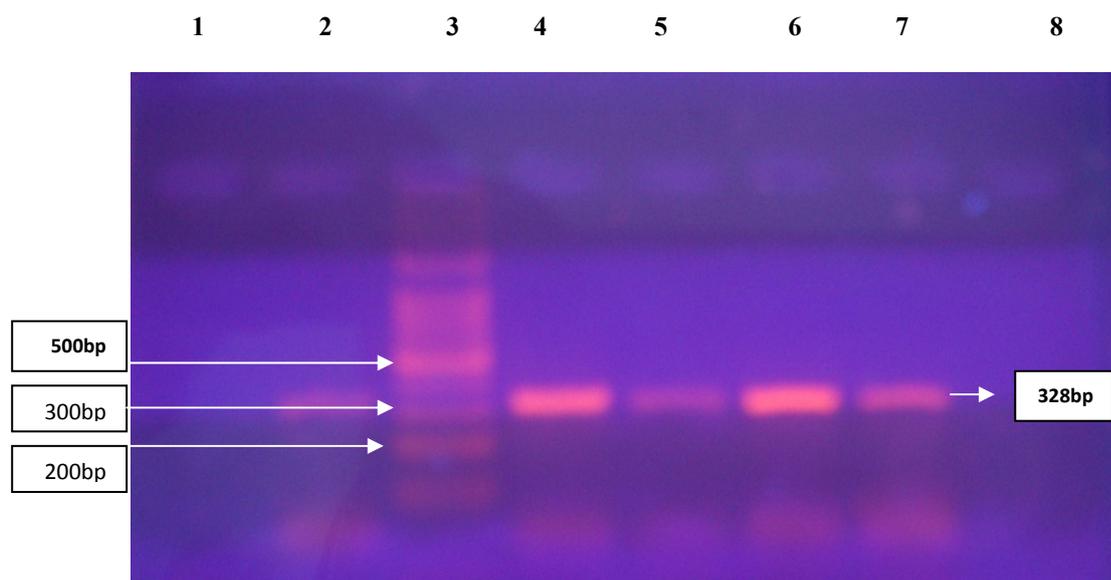 the negative control (Figure 1 ,2).

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Figure (1) positive and negative samples of vesicles fluid , serum and saliva according to RT- PCR amplification of (328bp) of universal primer gene .Lane (1 ,8)are negative , lane (2 ,4 , 5 , 6, 7) are positive , lane (3) is 100 bp DNA ladder marker.

Depending on the result of RT- PCR analysis which was performed on (83) head of cow. These animals showed positive RT- PCR results with variables rates of positivity , 81.9% , 31.3% and 22.9% of cattle were RT- PCR positive for universal , O-1C272 and A-1C562 respectively.. All tested cows were negative for Asia1 gene (Table-2).Concerning the effect of sex of cattle on RT- PCR positive results of all tested genes ,the females cows showed higher rate of RT- PCR positivity (universal: 87.5% and O-1C272 :33.9% and A-1C562 :23.9%) in comparison with males but the difference between males and females was not significant (P>0.05).On the other hand table (3) showed the significant effect (P < 0.05) of age on the FMDV genes distribution in the cows. Variable rates of RT- PCR positivity was observed in different age groups of cows . the second age group(**>4-8 year**) of cows showed higher rate of PCR positivity (universal: 8.9% ,O-1C272:37.03%andA-1C562:31.5%).
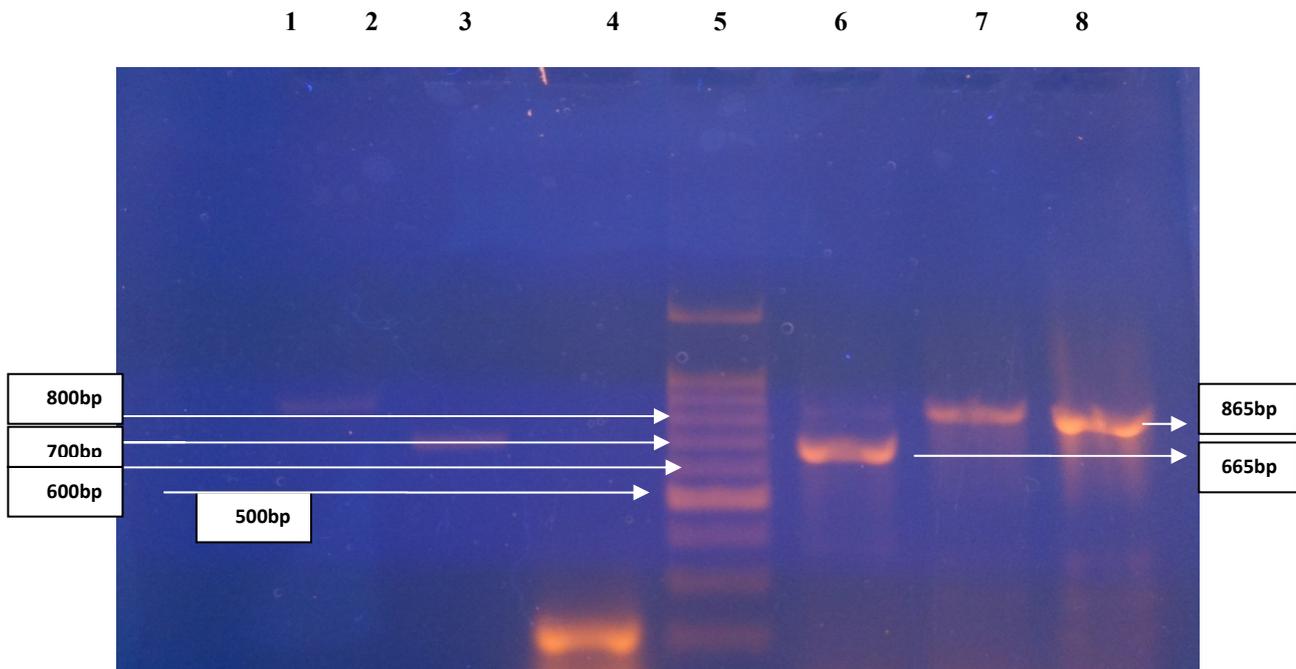
Figure (2) positive and negative samples of vesicles fluid , serum and, saliva according to RT- PCR amplification of (639bp , 865bp) of serotype O , A respectively. Lane (1,4) are negative, lane (3,6) are O serotype and lane(2,7,8) are A serotype, lane (5) is 100bp DNA ladder marker.

Table(2).RT- PC R results according to sex of Cattle

Sex	Positive No.(%)				
	Examined No	Universal gene	Serotype-A-1C562	Serotype-O-1C272	Serotype-As1-1C505
Male	27(32.5)	19(70.4)	6 (22.2)	7 (25.9)	0
Female	56(67.5)	49(87.5)	13(23.2)	19 (33.9)	0
Total	83(100)	68(81.9)	19(22.9)	26(31.3)	0

P>0.05

Table(3).RT- PC R results according to age of Cattle

Age groups (years)	Positive No.(%)				
	Examined	Univers al gene	Serotype-A	Serotype- O-1C272	Serotype-As1-1C505
>1-4	29(34.9)	19(65.5)	2 (6.9)	6 (20.7)	0
>4-8	54(65.1)	49(88.9)	17(31.5)	20(37.03)	0
Total	83(100)	68(81.9)	19(22.9)	26(31.3)	0

P < 0.05

DISCUSSION

Sero-prevalence of cattle FMDV

Antibody to structural proteins is produced following either infection or vaccination – severely impairing the recognition of infection in a vaccinated population. Therefore, areas or countries where emergency vaccination was performed and where vaccinated animals were not slaughtered, will suffer from severe trade estrictions(15). Some tests have become available, which make possible to distinguish between vaccinated or infected herds. Serological differentiation of postvaccinal and convalescent animals is based upon the humeral immune response to the non-structural proteins of the FMDV (NSPs). Over the last years several methods to detect antibodies to NSPs have been developed. The potential use of measuring antibody against NSP of FMD virus to differentiate infection from vaccination was first demonstrated by adioimmunoprecipitation (15). (16) described an electroimmunotransfer-blot (EITB) assay in which sera are examined for the presence of antibodies to several NSPs simultaneously by immunoblotting. However, ELISA is more suitable than immunoblotting for screening large numbers of sera, considerable effort has been focused on developing sensitive, specific and reproducible ELISA's for the detection of antibodies to NSPs. A number of such assays were also described (17, . 18, 19 , 20, 21). In animals seropositive for antibody to structural proteins, the detection of antibody to the polyprotein 3ABC is the most

reliable single index of infection (22, 23, 16 , 24). The high sensitivity of this assay was confirmed by using sets of sera from naive and vaccinated cattle as previously described by (17,18,**25**).

Nowadays the ELISA technology is widely applied in veterinary diagnostics.With the availability to be used routinely, it became a very reliable diagnostic tool. This study has been conducted in Basrah province for the documentation of serological diagnosis and monitoring of FMDV sero-prevalence.

Detection of specific antibodies against polyprotein 3ABC antigen in sera of different species of animals raised in a certain geographical area is considered a useful tool for epizootiological studies (26).

The tested sera of cows 120 of 165 (72.7%) in Basrah gave positive results for the presence of specific antibodies.. The current result was supported by other local study **(27)** conducted on other animal species raised in the same regions in Basrah, this study indicated that 71.8% of the total ELISA tested sheep sera build specific antibodies against FMD virus-infection- associated antigen(VIA). The sero-positivity finding is higher than the overall sero-prevalence of 62, 61.4 ; 34.09%and (33.3 and 66.7 %) reported by (28 ; 29; 30;31) respectively in cattle in middle and south of Iraq. Sero-prevalence study in the neighbouring country of Saudi Arabia (32) also found   low prevalence of 16% compared with the current study. The sero-positivity in the present   study is higher than the overall sero-prevalence of 14.6,21,26.5 and 8.18% reported by. (31) , (33), (34) and (35) in other parts of world respectively.

On the contrary, (31) in his study to the seroprevalence of FMD among cattle in middle part of Iraq documented a higher rate of seroprevalence (100,75 and75%)in Karbala,Al-Najaf and Al-Diwaniyah respectively. A higher prevalence of FMD in middle and south of Iraq could be attributable to the nationwide prevalence of circulating FMD virus and fact that there has been no recorded comprehensive vaccination campaign.

The significantly (**P < 0.05**) higher sero-prevalence of FMD in adult cows than in calves observed in the current study is in agreement with the previous reports of . (36 ;31) ;.(37);. (35) ; (38) and (34) This may be attributable to the fact that young cattle are herded in homestead areas and hence have less chance of exposure. In addition, the herds of grazing cattle are usually composed of adult males, non-

lactating and non-pregnant female cows and hence more exposed to FMD than younger age group. On the other hand (28) ; (39) found no significant association between seropositivity of FMD and age of cattle.

Significant difference (p>0.05) was observed in the prevalence of FMD between female and male cattle in this study. This finding was consistent with the previous finding of (40) in their report on the incidence of FMD among dairy cattle documented a higher rate of incidence in female (16.63%) cattle than that of male(1.37%) cattle. On the contrary (30;31) in middle of Iraq documented no significant (p>0.05) association between sex of cattle and seroprevalence. The non significant (p>0.05) association between sex of cattle and seroprevalence also reported by many studies conducted in other parts of world (34 ; 39; 41)

RT-PCR genotyping

Early detection of infected animals prior to the appearance of clinical signs is essential for effective control of FMD viruses and requires a rapid and sensitive method of diagnosis. Recent advances in molecular biology have resulted in the development of a technique known as RT-PCR for the detection of FMD virus genomic RNA in cell culture fluids, oesophageal pharyngeal scrapings, epithelial or other tissues such as tonsils.

In the present study, sera ,saliva and vesicle fluid from the affected animals were collected and tested by RT-PCR using universal primers for FMD. The positive ones were amplified with specific primers, and their amplicons were confirmed by 1.5 % agarose gel electrophoresis after ethidium bromide staining and viewing under UV light alongside a DNA weight markers.

Local interpretation of agarose-based RT-PCR assays led to the detection of FMD virus serotypes O, A and universal gene but not serotype Asia 1.These results in agreement with indication of (42) Global Foot-and-Mouth Disease Situation report that FMD outbreaks In Iraq during 2012 are attributed to serotypes O, A. While during 2013, the serotype A has emerged as the most important cause of outbreaks instead of serotype O. Other Iraqi study also supported the present PCR result as the study of (31) who confirmed the presence of serotypes A and O. (30) in her study in middle part of Iraq also confirmed the presence of serotype A. In neighboring countries, (42) Global Foot-and-Mouth Disease Situation report confirmed the

presence of certain serotypes similar to the current studied serotypes, as a follow, only O serotype was present in KUWAIT and SAUDI ARABIA during 2011, 2012.While IRAN and TURKEY reported O, A, Asia 1 during 2011 , 2012.

Of eighty three samples collected, only 68(81.9%) were successfully amplified by RT-PCR.Being RNA in nature,FMD genome is very sensitive to degradation by RNAses and other degradative enzymes. If a sample is collected during the early phase of the infection (especially from vesicles), chances of viral amplification by RT-PCR are higher,but if the lesions get invaded by bacteria, or lesions start healing, the probability of obtaining the intact viruses from samples decreases drastically. Some samples were collected in the late phase of infection and the viral genome may

have been degraded by bacterial RNAses and other degradative enzymes resulting in either weak or no detectable signals by RT-PCR.

On the other hand only 19(22.9%) samples were found to be serotype A-1C562 positive and 26(31.3%) were serotype O-1C272 positive and nil for serotype As1-1C505 these results were in contrast in part and agreed in other part with (31) who found that the total frequency of positive samples with real time PCR was found to be (20%) and serotype O was the predominant serotype in middle of Iraq while, serotype A was the second serotype and serotype Asia1 has normally been the cause of only a small and relatively stable proportion of cases compared with the proportion caused by serotypes O and A. Also he demonstrated that percentages of A, O and Asia1 serotypes in cattle under the study, in AI-Diwaniyah province were (11.1%, 66.6% and 22.2% respectively), in Baghdad (12.5%, 62.5% and 12.5%), in Babil (0%, 100% and 0%), in Al- Najaf (14.2%, 71.4% and 0%) and in Karbala (0%, 87.5% and 12.5%),

The primer pair 1F and 1R used by(12) and designed with reference to thec onserved sections of the 5 ′UTR of the FMD virus genome and was intended for the diagnosis of all seven serotypes. They found that the universal primer (IF/IR) located in the 5 UTR of the FMD virus genome successfully detected serotypes O, A, Asia-1 and C in clinical samples. The primers A-1C562 ,O-1C272 and As1-1C505.when used individually with the reverse primer EUR- 2B52 were designed for the detection of types A, O and Asia-1 respectively. In conclusion the sero-prevalence of FMD was found to be high at 72.7% and different levels of frequency of FMD virus serotypes

O, A and universal gene but not serotype Asia 1 were detected in the Basrah in south of Iraq.

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التشخيص الجزيئي والمصلي للانماط المصليه لفايروس مرض الحمى القلاعيه في ابقار محافظة البصره

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الخلاصه

اجريت هذه الدراسه في6 مناطق مختلفه ممثله لجميع اجزاء محافظة البصره خلال الفتره من شهر تشرين الاول من العام 2012الى شهر حزيران من عام 2013 على عينات من المصل وذلك لتحديد نسبة انتشار مرض الحمى القلاعيه في الابقار.من نتائج الفحص المصلي ان النسبه الكليه لانتشار هذا المرض كانت72.7%.من 165عينه خضعت لاختبار الممتز المناعي المرتبط بالانزيم المعتمد على مستضد البروتين اللا تركيبي تاكدت ايجابية 120 عينه وتمثل نسبه72.7% بينما كانت 45عينه سالبه وبنسبة .27.3%. ان ارتباط الايجابيه المصليه بالفئات العمريه كان عالي المعنويه من الناحيه الاحصائيه(p<0.01)وكذلك مع الجنس كان الارتباط معنويا(P > 0.05). استخدم اختبار سلسلة البلمره المنعكس للكشف عن فايروس مرض الحمى القلاعيه من حيث التشخيص الاولي والخاص بتحديد الانماط المصليه.جمعت عينات سريريه مختلفه ممثله لمرض الحمى القلاعيه من الابقار المريضه والتي تشمل الحويصلا ت الفمويه والمصل واللعاب. ان تشخيص الفايروس بالاعتماد على تفاعل سلسلة البلمره المنعكس تم باستخدام البادئات(1R / 1F و1C5 -A و-O 1C272وAs1-1C505)وبحزمه متوقعه (328و866و639على التوالي).من ثلاثه وثمانون عينه ضخمت(81.9%)68 عينه فقط بواسطة تفاعل سلسلة البلمره المنعكس ولوحظ ان (22.9%)19عينه منها كانت موجبه للنمط المصلي1C562 -A وان(31.3%)26 عينه منها كانت موجبه للنمط المصلي-O 1C272وكانت نتائج النمط المصلي As1-1C505 سالبه في جميع العينات.اعتمادا على الجنس لم تكن نسبة تكرار وجود الحامض النووي الفايروسي معنويه احصائيا (P>0.05) بينما كان الفرق بين الفئات العمريه كان معنويا (P < 0.05).

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