Some epidemiological features of BCoVs infection in Al-Qadisiya province by using real time-qPCR technique.

Khetam Qaid Mayee Mohsen Abid Nema Alrodhan
Coll. of Vet. Med./ Univ. of Al-Qadisiya
email: dr_khokha_86@yahoo.com
(Received 30 September 2013, Accepted 31 October 2013)

Abstract

This study was carried out to evaluate some epidemiological features of Bovine *Coronavirus* infection by using one-step real-time fluorogenic quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay based on SYBR Green I dye in detection. Coronaviruses detected by the same neocleocapsid (N) gene primers under 98% similarity with HECV-4408 (human enteric *Coronavirus*) in children according to NCBI with product size 124bp. 285 fecal samples has been examined by routine methods against pathogenic bacteria in the intestines (*E.coli*, *Salmonella* Spp.) and *Cryptosporidium parvum*, the samples positive for the parasite submitted to molecular testing because they may be mixed with *coronavirus* infections. 100 samples were screened for the presence of BCoVs antigens by using a immunochromatographic rapid test as a field fast test. Where 44% of samples showed positivity to BCoVs, out of 50 samples submitted to quantitative reverse transcription (RT-qPCR) assay. Out of 50 – 31 samples had been positive. We found, that distribution of BCoVs was significantly higher in rural areas 33.3%-87.5% as compare to cities 71.4%-75%, the infection in males reach to75% vas 53.5% in females, high infection rate 62.9% in < 1- 4 months age as compare with > 1 month age 62.9%. The results of infection rate showed high percentage during February 77.7% while the percentage on (January, December, March, and April) was (66.6%, 66.1%, 54.5% and 50%) respectively.

Key words : Bovine *coronavirus*, epidemiological features of BCoV, real time-qPCR based on SYBR Green I dye, HECV-4408, BCoVs immunochromatographic rapid test.

دراسة بعض الصفات الوبائية للفايروس التاجي البقري في محافظة القادسية باستخدام خطوة واحدة في الوقت الحقيقي الكمي للفلورة ـ تفاعل البلمرة العاكس الناسخ

ختام قايد مايع الحمداوي محسن عبد نعمة الروضان
جامعة القادسية /كلية الطب البيطري

الخلاصة

أجريت هذه الدراسة لتقييم بعض السمات الوبائية لعدوى الفايروس التاجي باستخدام خطوة واحدة في الوقت الحقيقي الكمي للفلورة- تفاعل البلمرة العاكس الناسخ Real Time RT-qPCR مقاس على أساس صبغة SYBR الخضراء في الكشف. تم كشف الفيروس من قبل نفس الجين التمهيدي تحت نسبة 98 % وفقا ل NCBI مع حجم المنتج 124 زوج قاعدي . كل العينات البرازية فحصت بالطرق الروتينية ضد البكتيريا الممرضة للأمعاء (الاشريشية القولونية، السالمونيلا) والابواغ الخبيئة، وقد تم استبعاد العينات الموجبة للنمو البكتيري في حين أن العينات إلموجبة للطفيلي قدمت إلى الاختبار الجزيئي لأنها قد تكون مختلطة مع الاصابة بالفايروس التاجي. تم فحص 100 عينة فقط لوجود مستضدات BCoVs باستخدام الاختبار الحقلي المناعي السريع . حيث أظهرت 44% من العينات إيجابية ل BCoVs، قدمت 50 عينة مفحوصة بالاختبار السريع للوقت الحقيقي لتفاعل سلسة البلمرة النسخ العكسي الكمي (Real Time RT- qPCR). حيث ظهر BCoVs في 31 من أصل 50 عينة برازية سريرية من العجول. وجدنا، أن توزيع BCoVs أعلى بكثير في المناطق الريفية 33.3 -87.5 % بالمقارنة مع المدن 71.4 -75 %، و الإصابة في الذكور تصل 75% بالمقارنة في الإناث 53.5 %. وزيادة الإصابة مع تقدم العمر < 1-4 62.9% مع انخفاض طفيف في عمر > 1 شهر 62.9 % ،

أظهرت النتائج أن معدل الإصابة العالية كانت خلال شهر شباط 77.7% في حين كانت النسبة في (يناير، كانون الأول وآذار، وإبريل) (66.6%، 66.1%، 54.5% و 50%) على التوالي.

الكلمات المفتاحية : الفايروس التاجي البقري ، الصفات الوبائية للفايروس التاجي، تقنية باستخدام خطوة واحدة في الوقت الحقيقي الكمي للفلورة ـ تفاعل البلمرة العاكس الناسخ المعتمدة على صبغة السايبر الخضراء، الفايروس التاجي المعوي المصيب للإنسان ، الاختبار المناعي السريع للكشف عن مستضد الفايروس التاجي البقري.

Introduction

Coronaviruses are species in the genera of animal viruses that are members of subfamily *Coronavirinae* in the family *Coronaviridae* (1). They are enveloped viruses with a positive-sense single-stranded RNA genome and chiefly infect the upper respiratory and gastrointestinal tract of mammals and birds (1). And have an established potential for cross-species transmission that became broadly recognized with the emergence of a novel human coronavirus. Before the 2002 - 2003 severe acute respiratory syndrome (SARS) epidemic, coronaviruses have always been of considerable importance in animal health, they are infect a variety of livestock, poultry, and companion animals, in which they can cause serious and often fatal respiratory, enteric, cardiovascular, and neurologic diseases (2). Diarrhea among neonatal calves is a common disease. The form of the disease varies from calf to calf, some suffering acute dehydration and death whilst others suffer from sub-acute forms with malnutrition that lasts for several days (3). And the cost for the unwanted death of a calf was calculated to roughly $ 60 UD and a reduction in mortality in farms in Kuwait was seen to have a big positive impact on the gross margins (4).

Materials and methods

History of 285 fecal samples which was freshly collected from veterinary teaching hospital and dispensaries in districts and counties of Al-Qadissyia province between December 2012 to May 2013. All fecal specimens has been exanimate within 24h by routine methods against enteropathogenic bacteria (*E. coli, Salmonella* Spp.) and *Cryptosporidium* Spp. infection, the positive bacterial growth samples has been excluded while the positive *Cryptosporidium* samples submitted to molecular test as possible mix infection with *Coronavirus*.

FASTest® BCV Strip : out of 285 samples 100 samples has been examined by rapid immunochromatographic test, the test membrane contains two unique monoclonal antibodies. One of these monoclonal antibodies is bounded to colloidal gold particles, the second is immobilized in the test zone of the strip. If the stool sample extract contains bovine Coronavirus antigen these will form an antigen-antibody complex with the gold particles. 44 positive diarrheic samples matched with 6 negative bovine coronavirus samples and converted into confirmative molecular detection.

Molecular detection : RNA extraction Viral RNA was extracted from 50 samples by using AccuZol^{TM}Total RNA extraction kit (Bioneer, Korea) and done according to company instructions.

cDNA synthesis : Total RNA that extracted from 50 stool samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer Company, Korea and done according to company instructions. Then Real-Time PCR was performed for detection of bovine *Coronavirus* by using the primers specific for Neucleocapsidgene(N) BCoVs. Forward:

(ATTTGCAGAGGGACAAGGTG) & Reverse:

(TAGCAATTGACGCTGGTTGC) with 124bp product size. Real-Time PCR master mix was prepared by Real-Time-qPCR detection kit (AccuPower® GreenStar^{TM}qPCRPreMix, Bioneer. Korea), and done according to company instructions. Real time-qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive

amplification of Nucleocapsid gene in Real-time cycle number.

Statistical analysis : the results submitted to statistical study for the calculation of infection rate & significant differences ($p< 0.05$) among regions, sex, age, and months of year were studied to categorical variable by using Chi - square calculation method according to (5).

Results

By using immunocromatographic rapid test 44% out of 100 screened samples showed positivity to BCoVs antigen, whereas by using the molecular method as expressed in real time reverse transcription RT-qPCR cycle stages in the figures (1,2,3, & 4), the current study showed that the distribution of BCoVs was significantly higher in rural areas 87.5% as compare with cities 71.4 - 75%. BCoVs infections were showed clear disparity between districts were the positivity percentage reached to 75%, was no significant differences in this results, In subdistrictes, there was significant differences, the positivity percentage was 87.5 %. Table (1).

Table (1): Results of RT_ qPCR of BCoVs infection rate according to study regions.

Districts/sub districts	No. of diarrheic samples	No. of positive samples	Positivity Percentage
City center	2	-	0% a
Hamza	4	3	75% b
Afak	7	5	71.4 % b
Shamyia	2	-	0% a
Mehanawia	4	3	75% b
Shafeyia	4	-	0% a
Albdeer	8	7	87.5%b
Nuffer	3	2	66.6%b
Salahyia	3	1	33.3%c
Daghara	3	2	66.6% b
Sumer	10	8	80% b
Total	50	31	62%

Similar letters refers to the non-significant differences among cities while different letters refers to significant differences at ($p< 0.05$).

BCoVs infection according to sex appeared no significant differences between male and females even the high rate indicated in males were 75% and in females 53.3%. Table (2).

Table(2) : Results of RT_ qPCR of BCoVs rate infection according to sex.

Sex	No .of diarrheic samples	No. of positive samples	Positivity Percentage
Male	20	15	75% a
Female	30	16	53.3% a
Total	50	31	62%

Similar letters refers to the non-significant differences between sex while different letters refers to significant differences at ($p< 0.05$).

The infection increased with age < 1- 4 months age 62.9% and a slight decline in > 1 month age 62.9%. There were no significant difference founded in calves during the first month of age, while the calves at<1_4 months were more susceptible to infection with BCoVs. Table (3). During February the peak of BCoVs reach to 77.7% in comparing with January 66.6%, December 66.1%, March 54.5% and April 50%. A months infection pattern in this study showed a significant differences between winter and spring season. Table (4).

Table (3): Results of RT_ qPCR of BCoVs infection rate according to ages groups.

Ages groups	No. of diarrheic samples	No. of positive samples	Positivity Percentage
1day_1months	23	14	60.8% a
< 1_4 months	27	17	62.9% a
Total	50	31	62%

Similar letters refers to the non-significant differences between ages while different letters refers to significant differences at ($p< 0.05$)

Table (4): Results of RT_ qPCR of BCoVs infection rate according to months of years.

Months	No .of diarrheic samples	No. of positive samples	Positivity Percentage
December	6	4	66.1% ab
January	12	8	66.6% ab
February	9	7	77.7% b
March	11	6	54.5% a
April	12	6	50% a
Total	50	31	62%

Similar letters refers to the non-significant differences among months while different letters refers to significant differences at ($p< 0.05$).

Collection of figures showing the reverse transcription RT-qPCR cycle stages: The figures (1,2,3, and 4) expressed the amplification plots represent 8 positive samples, the results showed different positive reaction cycles of threshold (Ct), they started reactions at 28, 29, and 30 respectively.

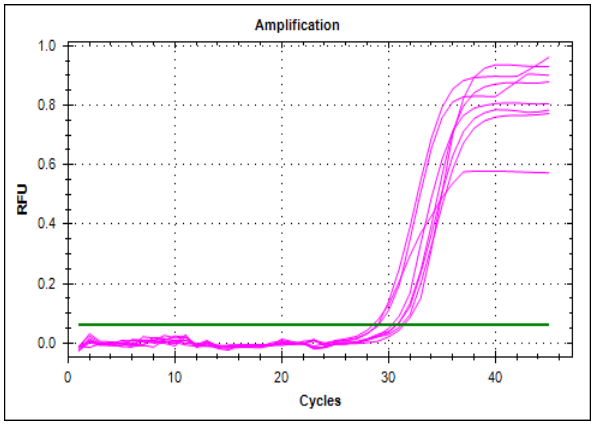

Fig. (1): Reverse Transcription Real-Time PCR amplification plot shown the positive results of Bovine enteric *Coronavirus* in bovine samples

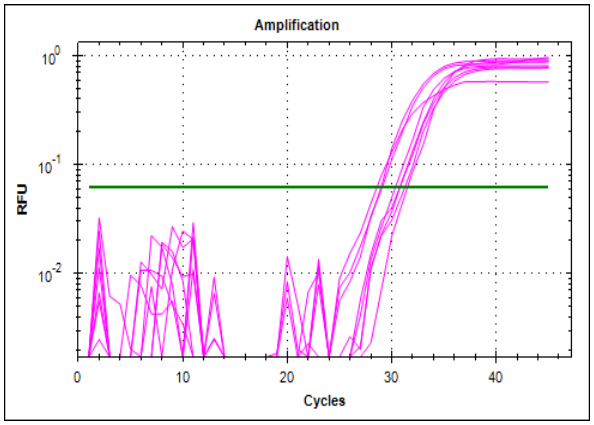

Fig. (2): Reverse Transcription Real-Time PCR amplification log plot shown the positive results of Bovine enteric *coronvirus* in bovine samples.

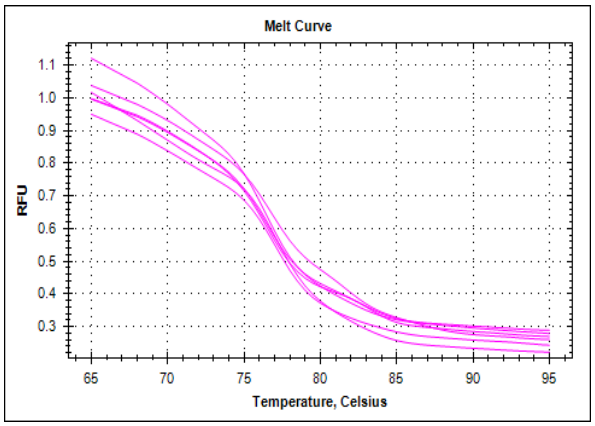

Fig. (3): Reverse Transcription Real-Time PCR melt curve shown the positive results of Bovine enteric *coronavirus* in bovine samples.

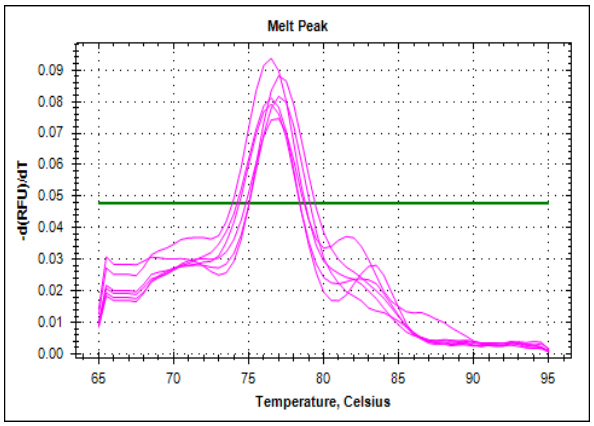

Fig. (4): Reverse Transcription Real-Time PCR melt peak shown the positive results of Bovine enteric *coronavirus* in bovine samples.

Discussion

immunocromatographic rapid test results which showed 44% out of 100 screened samples of positivity to BCoVs antigen. This results less than (6) Who confirmed that rapid tests for the detection of showed a high specificity 96.4%, but a relatively low sensitivity 60.0% (6) this may be related to the most clinical samples collected from subclinical or chronic infected calves. Rapid test has the advantage of not requiring special equipment or expertise, therefore, it is suitable for small laboratories and field research (6). whereas by using the molecular method, the current study showed that the distribution of BCoVs was significantly higher in rural areas in comparing with cities, the presented data give insight into the infection rate of BCoVs disagreements with (7) who proved that percentage in Al-Qadisiya province of BCoVs infections were

higher than the other province Babylon, Wassit, and Najaf and showed there was no significant difference at $p \geq 0.05$ in percentage of infection with BCoVs in those governorates and this related to using RT-PCR in detection. Because of the real-time PCR technique which was established in this study has been catalyzed wider acceptance of PCR because it is more rapid, sensitive and reproducible, while the risk of carry over contamination is minimized (8). BCoVs infections were showed clear disparity between districts, but the absent of virus in cities may due to less contact with the livestock in urban centers, small number of villages in the city, and raised of livestock flock. In subdistrictes, there was significant differences, this may be related to the geographical nature of the regions make it far from the veterinary services. Calves graze on crops in areas lacking agriculture suffers low level of food, thus be prone to skinny and susceptible to the risk of all diarrheic causes easily, and the movements of cattle population between different regions could be good reason to virus spreading. BCoVs infection according to sex appeared no significant differences between male and females even the high rate indicated in males. In fact, in the case of viral infection in general, this difference in susceptibility between males and females were shown for different viruses(9). For many years the concept of sex-based (or gender based) differences in host response to infection has been studied and appears to be highly related to differences in immunological capacities between males and females (10). Furthermore, it appears now that even though the relative importance of different factors may vary with the type of infection, X-linked genes, hormones, immunity and, at least in humans, societal context are among the factors that explain this sex-based difference (9,10). Even though it remains difficult to clarify evidence on how the different factors make a difference between genders, studies aimed to addressing the question appear to more and more link specific hormones such as androgens in males and estrogens in females (9, 11), host innate immunity (expression of cytokines and of pattern recognition receptors such as toll-like receptors (TLR) (12,13) as well as acquired immunity involving T and B lymphocytes (9) to explains. The infection increased with age < 1- 4 months age and a slight decline in > 1 month age, there were no significant difference founded in calves during the first month of age, while the calves at < 1_4 months were more susceptible to infection with BCoVs, and this may also due to the pathogen causing diarrhea in calves at an age when they have immature immune status, lacks specific antibodies illustrate high metabolism with added stresses imposed by weaning and sometimes deprivation of immune colostrum feeding (14). The results were agreement with (15) who exposed that BCoV is associated with enteric disease in cattle of all ages. The increase in the prevalence of coronavirus with age could be due to the fact that, as animals grow older, they were more likely to be exposed to the virus, as well more likely to come into contact with other animals that have recovered from the disease but remain as carriers. A months infection pattern in this study showed a significant differences between winter and spring season, and this results agreed with (15,16) were proved the winter season in which the prevalence of calf diarrhea caused by *Coronavirus* is higher than other seasons because BCoV is moderately sensitive to heat (17, 16), and in spring season the results were agreement with previous reports of enteric disease caused by BCoVs in higher tropical temperatures (18), (17) who expressed another factor were alteration of the biological properties of the BCoVs which makes the infection spread during the warm months.

References

1-Masters P S (2006). The molecular biology of coronaviruses. *Adv. Virus Res.* 66: 193–292.

2-Holmes K V, and Lai MM **(**1996). *Coronaviridae*: the viruses and their replication: in Fields virology,

3rd ed. Fields BN, Knipe DM, and Howley PM (ed.) *Raven Press, New York, N.Y.*, pp: 1075-1093.

3-Gay CC, Hodgson JC, Lofstedt J, and Bolin SR (2012). Diarrhea in Neonatal ruminants: intestinal diseases in ruminants: *merck veterinary manual.*

4-Razzaque MA, Bedair M, Abbas S and AL-Mutawa T (2009). Economic empact of calf mortality on dairy farms in Kuwait. *Pakistan. Vet. J*. 29(3):97-101.

5-Leech NL, Barrett K C, and Morgan G A (2011). IBM SPSS For Intermediate statistics.4th ed. Taylor and Francis Group. *LLC.USA*.

6-Klein D, Kern A, Lapan G, Benetka V, Möstl K, Hassl A, and Baumgartner W(2009). Evaulation of rapid assays for the detection of bovine *coronavirus, rotavirus* A and *Cryptosporidium parvum* in fecal samples of calves. *Vet. J*.182: 484-486.

7-Al-Kanany KA (2012). Molecular investigation of Bovine Coronavirus in diarrheic calves by using RT-PCR in some governorates of Iraq. MSc thesis in veterinary Medicine-Internal and Preventive Veterinary Medicine. University of Baghdad.

8-Mackay IM (2004). Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* 10:190e212.

9-Klein SL (2000). The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci. Biobehav. Rev*. 24, 627-638.

10-Fish EN (2008). The X-files in immunity: sex-based differences predispose immune responses. *Nat. Rev. Immunol.* 8: 737-744.

11-Wilson TG, Yerushalmi Y, Donnell DM, and Restifo LL (2006). Interaction between hormonal signaling pathways in Drosophila melanogaster as revealed by genetic interaction between Methoprene-tolerant and Broad-Complex. *Genetics.* 172(1): 253--264.

12-Hannah MF, Bajic VB, and Klein SL (2008). Sex differences in the recognition of an innate antiviral responses to Seoul virus in Norway rats. *Brain. Behav. Immun*. 22: 503-516.

13-Hill KE, Pigmans M, Fujinami RS, and Rose JW (1998). Gender variations in early Theiler's virus induced demyelinating disease: differential susceptibility and effects of IL-4, IL-10 and combined IL-4 with IL-10. *J. Neuroimmunol.* 85: 44-51.

14-Hansa A, Rai RB, Wani MY and Dhama K (2012). ELISA and RT-PCR based detection of bovine coronavirus in Northern India. *Asian J. Animal Vet. Adv.*, 7: 1120-1129.

15-Bidokhti MR, Tråvén M, Krishna NK, Munir M, Belák S, Alenius S and Cortey M (2013). Evolutionary dynamics of bovine coronaviruses: Natural selection pattern of the spike gene implies adaptive evolution of the strains. *J. Gen. Virol.* 94(Pt 9): 2036-49.

16-Radostits OM, Gay CC, Hinchcliff KW, and Constable PD (2007). Veterinary Medicine, A textbook of the diseases of cattle, horses, sheep, pigs, and goats, 10th edn. Saunders-Elsevier, Philadelphia, 1286–1296.

17-Saif L J (1990). A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? *Cornell. Vet.* 80(4):303–11.

18-Park SJ, Jeong C, Yoon SS, Choy HE, Saif LJ, Park SH, Kim YJ, Jeong JH, Park SI, Kim HH, Lee, BJ, Cho HS, Kim SK, Kang MI, and Cho KO (2006). Detection and characterization of bovine coronaviruses in fecal specimens of adult cattle with diarrhea during the warmer seasons. *J. Clin. Microbiol.* 44(9): 3178-88.

19-Martínez A, Caballero M, Silva S and Jiménez C (2002). Aislamiento ycaracterización de coronavirus bovino asociado a un brote de diarrea epizoótica (Disentería Bovina) en bovinos adultos en Costa Rica. *III Seminario Internacional de Sanidad Animal y I Seminario de Producción Animal*. ESPE, Sangolquí 12-15/11/2002.