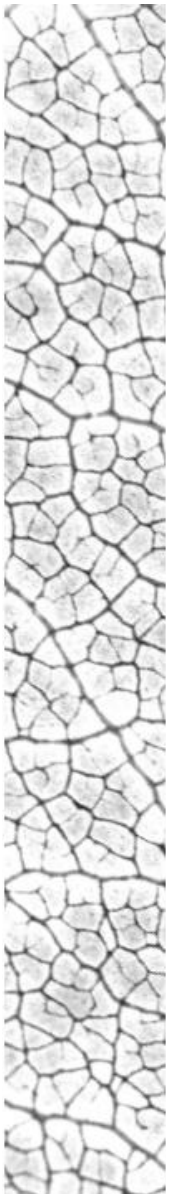

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Molecular study of Rota A viruses at many farm animals in Iraq

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Abstract

Background: The species A of Rotavirus is considered as of medical and veterinary important pathogen, worldwide distributed and responsible for acute gastroenteritis particularly at juvenile ages, the viruses explain marked genetic diversity which represented by numerous number genotypes infect different vertebrate hosts .

Methods: About 942 fecal samples had been taken directly from the rectum of 403 cattle, 236 sheep,174 goats and 129 of camels, their age younger than 6 months and suffering from diarrhea at different Iraqi Governorates; advanced specific molecular technique was implemented, Reverse Transcription -modern- Real Time PCR technique (rRT- PCR) with utilizing of a new generation thermo-cycler to obtain reliable result regarding exact viral genotypes in these animals at Iraq .

Results: The RVAs were observed in 29.61% of diarrheic animals the percentages of infection among these animals as followings: cattle, sheep, goats and camels were 31.51%, 29.23%, 27.58% and 27.13%, respectively, the distribution pattern of viral genotypes as following: in cattle: G6, G8, G10, P[1] and P[5] in addition to P[11]; in sheep: G3, G6, G10, P[1] and P[11]; in goats: G6 and also P[5]; in camels: G10, P[1] and P[11]. G10 was the most dominant among specific genotypes in all animals in this study.

Conclusions: According to the author's knowledge, the present study is the first study that recorded G3, G6, G8, G10, and also P [1], P [5] besides to P [11] in above animals at Iraq, which offers a valuable and reliable epidemiological information pertaining RAVs genotypes that were urgently needed for both human and animal necessary hygiene regards.

Keywords: Rota A viruses, G and P genotypes, Reverse transcription, Real Time PCR

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INTRODUCTION

The rotaviruses (RVs) in general are amongst the most important members of Reoviridae family; since discovering at year 1973 then hence far, it globally considered as a major cause of viral diarrhea that ensued from acute gastro-enteritis in both humans and animals, inducing a noticeable elevation in the mortality and morbidity rates in farm animals, especially young ages, concomitant with significant economic losses to the livestock industry enterprises (Clark et al. 2019, Hasso-Agopsowicz et al. 2019, Lu et al. 2019).

The mature virion particle of RV is characterized by a naked icosahedral configuration shape composed from initial triple-layered of capsid proteins, enclosing the viral genome of eleven (11) segments nucleic acid of double-stranded RNA that in turn encoding hexa structural (VP1 to VP6) besides to hexa nonstructural (NSP1 to NSP6) proteins (Suzuki et al. 2019; Pérez-et al, 2018).

The genome of RVs are markedly exhibits considerable large genetic range which involves and includes certain point mutations, deferent genetic re-assortment patterns, genetic re-arrangement, moreover a specified intragenic recombination. therefore, the aggregation of putative point mutations which may or can induce genetic/antigenic drift, whereas any re-assortment events may conducive to a particular genetic/antigenic shift (Arias et al.2015).

Until now, RVs have been classified into eight (8) groups (also termed as species): A to H according to their differences on the antigenicity of the certain middle capsid VP6 protein in addition to the consensus nucleotide sequence identities of the delineated VP6 encoding gene. The Rotavirus groups A, B, C in addition to group H are always almost infect both humans and animals, whilst the groups D-G have been detected only in animal and bird hosts. Rotavirus group A (RVAs) is the most common causes of viral diarrhea in humans and a wide variety of certain animal species and birds;

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their strains were only implemented in the commercial vaccine preparations at both human and animals (Taniguchi et al. 2012).

The intact outer capsid protein layer of RVs virion composed of VP7 and VP4 viral proteins which usually stimulate eliciting and producing of specific neutralizing antibodies of host immune response, Both of VP7 and VP4 encoding genes are generally explain a marked and intensive genetic diversity, therefore, RVAs had been sub-classified generally into at least 36 G (namely glycoprotein) and 51 P (namely protease sensitive) types in the bases regarding the divergences and differences in the consensus nucleotide sequences of their certain VP7 and VP4 genes, respectively (Desselberger et al. 2017).

The latest specific classification international system for the RVAs that devoted by the official and international Rotavirus Classification Working Group (abbreviated as RCWG) for classification of this virus, so far, includes all viral capsid besides to non-structural consensus protein genes, as followings:VP7_VP4_VP6_VP1_VP2_VP3 and NSP1_NSP2_NSP3_NSP4-NSP5/6 that of RTAs; therefore, under the aforementioned system, the following arrays of exact notations: Gx_P[x]_Ix_Rx_Cx_M_Ax_Nx_Tx_Ex and Hx has been drastically used for representing of the complete genotype constellation of above genes, respectively; x indicates the exact number of genotype and the number of P genotype which put inward a square brackets (P[x]) as showed in above (Rodríguez et al 2010).

The international RCWG genotyping system considered as a general discriminative uniform classification platform for the comparing the entire genomes of RVAs within in addition to amongst different host species. The viral strains of certain genotype molecular constellations, or VP7_VP4 (G_P) of the exact genotype combinations are usually found in different particular host species; for instance, G1 and P[8], G2 and P[4], G3 and P[8], G4 and P[8], G9 and P[8] and also to a lesser extent G12P[8] RVAs were usually infect humans. The genotypes G6, G8 and G10 RVAs in combination with P[1], P[5] or P[11] in cattle. Genotypes G3, G6,G10 in addition to P[1], P[11] and P[14] at sheep. Genotypes G6 and P[5] in goats. Whilst the genotypes G8, G10 and P[1], P[11] and P[15] infect the old-world camelids (Luchs et al. 2016, Ghosh et al. 2014).

The genetic re-assortment activities between all human infected RVA strains and animal strain were most likely ensued from a direct active zoonotic or anthropic transmissions; for instance, development of the bovine-like RVA strain, the genetic shift and drift in addition to a certain events of interspecies transmission have been most often shown at developing countries like Iraq, as a result of the vicinity and close proximity and vicinity between human and livestock farms which increases the probability of the occurrence of the bio-harms (Changotra et al. 2017, Suzuki et al. 2019).

There is a marked dearth and paucity of cosmopolitan data pertaining RVA genotypes at different animals (Boehm et al. 2019), in particular at our country, Iraq, according to author's knowledge, there is no previous study regarding viral genotypes in farm animal at Iraq; therefore, this study aims to pinpoint over RTAs genotypes in many farm animals, cattle, sheep goats and camels in order to set up or establish a reliable ground-work regarding these viruses in Iraq in order to abrogate or reduce any future putative hazards and offering a reliable information related future vaccine preparation.

MATERIAL AND METHODS

the present study was done and conducted from the beginning of October 2018 to the end of July month 2019, about 942 fecal samples had been taken by professional team of well sophisticated veterinarians from 403 cattle, 236 sheep,174 goats and 129 of camels, at different Iraqi Governorates: Baghdad, Basrah, Ninawa Babylon, Najaf, Thi-Qar, Muthana, Diwaniya, Myssan, Diyala, and Kut. all fecal specimens were exactly collected directly from the rectum of involved animals that suffering from diarrhea, their age younger than 6 months, the pooled fecal specimens were put inside specified sterile tubes (each sample divided into two tubes A and B tubes) without any delay, then sealed and kept insulated on ice boxes during collection and immediately preserved in a liquids nitrogen dry shipper for shipment to our laboratory, were preserved inside a specified deep freezer at -80°C till use.

The viral RNA was clarified from all sample tubes, about one hundred forty µl of fecal suspensions used for nucleic acid extraction and purification through implementation of a commercial QIAamp of Viral RNA Mini Kit (Qiagen Company, Hilden, Germany), which done in details according to the manufacturer's certain instructions.

Initially. After RNA purification, All samples were subjected to testing by one-step, of reverse transcription real time polymerase chain reaction (rRT-PCR) searching for RVA, according to previously mentioned and described procedure by (Jothikumar et al. 2009). After that, all negative specimens were excluded.

Reverse transcription step was done for all B tubes that contain positive samples, exactly under the certain application of manufacturer's instructions for all positive RVA samples through using of the QIAGEN®, One_step of the RT-PCR Kit (QIAGEN Company, Germany).

Group A of Rotavirus genotypes G and P were detected by amplifying delineated primers of enrolled fragments of VP7 and VP4 genes as mentioned and described by (Gouvea et al. 1990, Chakraborty et

Table 1. The RTAs Infection Through the Months of Year

Months of Year	NO. of Collected Direct Fecal Samples	NO. of Certain Positive Fecal Samples	Percentages
October	87	19	21.83%
November	93	23	24.73%
December	99	31	31.31%
January	105	29	27.61%
February	93	34	36.55%
March	90	37	41.11%
April	89	38	42.69%
May	99	25	25.25%
Jun	85	21	24.70%
July	102	22	21.56%
Total	942	279	29.61%

Table 2. Geographical Distribution of RTAs infection throughout Iraq

Governorates	NO. of Directly Taken Fecal Samples	NO. of Certain Positive Fecal Samples	Rates of Infection
Baghdad	94	27	28.72%
Basra	87	36	41.37%
Ninawa	88	23	26.13%
Babylon	90	29	32.22%
Najaf	87	14	16.09%
Thi-Qar	82	27	32.92%
Muthana	91	13	14.28%
Diwaniya	79	22	27.84%
Myssan	78	26	33.33%
Diyala	85	29	34.11%
Kut	81	33	40.74%
Total	942	279	29.61%

al.2016 Fujii et al. 2019) through utilizing of conventional PCR and following sets of primers: VP7 foreword primer: 5′ **-**CTCCTTTTAATGTATGGTATTGAATATACC-3′ VP7 reverse primer: 5′- GTATAAAANACTTGCCACCATTTTTTCCA-3′ VP4 con 3 primer: 5′- TGGCTTCGCTCATTTATAGACA-3′ VP4 con 2 primer: 5′- ATTTCGGACCATTTATAACC-3′

The initial started step of denaturation was done at 94◦C for about 30 seconds, hereafter, followed by twenty runs of thermo-cycling: 30 seconds at temperature of 94◦C, 30 seconds at 50◦C, moreover, 60 seconds at 72◦C followed by final run of extension for 5 minutes at temperature of 72◦C. The PCR products were analyzed by gel electrophoresis step using 1.5% of agarose gels in addition to ethidium bromide (Heide et al.2005, Chakraborty et al.2016 Fujii et al. 2019), DNA Ladder of Promega Corporation, USA.

All RVA types G and P positive samples with conventional reverse transcription PCR were also subjected again for further examination assay with reverse transcription real time PCR (rRT-PCR) searching for the most common RVA genotypes, multiple consensus sets of specific array from numerous forward and reverse primers and probes were implemented according to the recommended method that originally designed and described by (Liu et al. 2015, Mirza et al 2018), through utilizing of Step One PlusTM Real-Time PCR System; Applied Bio-system. Massachusetts, USA.

Statistical analysis: the SPSS software of modern version 26 (IBM,NY,USA) was used through application of specified Chi-square (x2), in order to determine the parameter differences among the obtained data of current study.

RESULTS

The implementation of rRT-PCR technique for the collected samples from diarrheic animals was revealed that infection rate with RTAs infection was 29.61% (279/942); the percentages of infection at these animals (cattle, sheep, goats and camels) were, 31.51% (127/403), 29.23% (69/236), 27.58% (48/174) and 27.13% (35/129), respectively.

The spread of infections through the months at present study were explained that the highest infection rate had noticed during April 42.69% (38/89) followed by that in March which was 41.11% (37/90), whereas the lowest infection rate was recorded in July, 21.56% (22/102); statistical analysis don't explained any significant differences at the level of 0.05 among the infection rates throughout the months (**Table 1**).

The geographical distribution of RTAs infection throughout Iraq emphasized that the highest infection rate was recorded at Basra governorate which was 41.37% (36/87), whilst the lowest infection rate was reported in Muthana governorate, 14.28% (13/91), The statistical analysis were showed no significant difference among percentages of infection in relation to Iraqi governorates at level of 0.05 (**Table 2**). The genotyping by use of rRT-PCR revealed that G genotypes were the prevalent types in cattle, sheep and camels with percentages of 64.5% (82/127), 47.8% (33/69) and 65.7% (23/35), respectively; contrast result in goats explained that P genotypes were dominant in

Table 3. Infection with RTAs in cattle, sheep, goats and camels besides to genotyping results

Animals	NO. of Positive Samples	G Genotypes				P Genotypes				Mixed Genotypes	
		NO.	%	Types	NO. (%)	NO.	%	Types	NO. (%)	NO.	Types
Cattle	127	82	64.5*	G6 G8 G10	20 (24.3) 27 (32.9) 35 (42.6)	41	32.2	P[1] P[5] P[11]	19 (46.3) 7 (17) 15 (36.5)	4	3 of G8 mixed with P[11]; 1 of G10 mixed with P[11]
Sheep	69	33	47.8	G3 G6 G10	10 (30.3) 9 (27.2) 14 (42.4)	28	40.5	P[1] P[11]	11 (39.2) 17 (60.7)	8	4 of G3 mixed with P[11] ; 3 of G10 mixed with P[11] ; 1 of G3 mixed with P[1]
Goats	48	9	18.7	G6	9 (100)	34	70.8*	P[5]	34 (100)	5	All of them G6 mixed with P[5]
Camels	35	23	65.7*	G10	23 (100)	9	25.7	P[1] P[11]	2 (22.2) 7 (77.7)	3	All of them G10 mixed with P[11]

Note: all percentages calculated after excluding of genotypes mixed infection.
*Significant at level of P < 0.05.

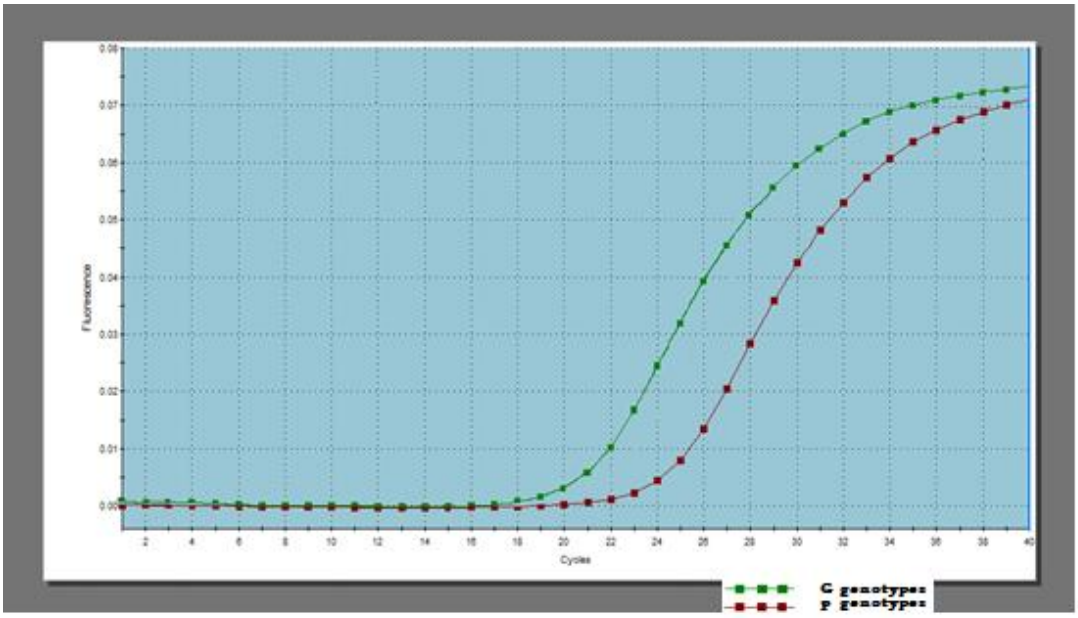

Fig. 1. The Initial Graphic Results for two RVAs Positive Cases; amplification of delineated fragments from VP4 and VP7 genes that Obtained by Real Time PCR Thermocycler

percentage of 70.8% (34/48); statistical analysis explain significant difference at level of 0.05 in percentage of infection with G genotypes in cattle and camels and P genotypes in goats (**Table 3**, **Fig. 1).**

The present study was recorded that specified G and P genotypes distribution pattern as followings: in cattle genotypes: G6, G8, G10, P[1] in addition to P[5] and P[11]; in sheep: G3, G6, G10, P[1] and P[11]; in goats: G6 and P[5]; in camels: G10, P[1] and P[11] (**Table 3**). After excluding of mixed infection, the study was pointed out that Genotype G10 was most dominant among specific genotypes in all animals in this study which recorded in 35, 14 and 23 of infected cattle sheep and camels cases, respectively. Significant differences not reported at level of 0.05 among Genotypes in the infected animals at the study areas.

DISCUSSION

The Genus Rotavirus compromises a group of ubiquitous viruses that infect mammals and birds inflicting acute gastroenteritis associated with diarrhea which mostly seen in young animals that conducive to significant economic losses from neonatal malady, the susceptibility to infection most often decreases with the age progress, might be as a result of the changes in the particular animal physiology, and / or certain acquired host immunity from succumbing to the infection during neonatal period (Hasso-Agopsowicz et al.2019, Badur et al.2019)

In order to insure a reliable and accurate results besides to abrogate errors or prohibit any possible mistakes, we devoted and utilized a new generation of specified rRT-PCR assay in the present study because this assay is obviously highly sensitive and also highly specific .Our study had revealed that percentage of infection with RVAs in the diarrheic animals was 29.61% (279/942); the infection rates among these animals (cattle, sheep, goats and camels) were, 31.51% (127/403), 29.23% (69/236), 27.58% (48/174) and 27.13% (35/129), respectively.

The results of our study were slightly similar and imitate the results of Jindal et al, (2000) in India, which was 27.02% in diarrheic cow and buffaloes' calves

However, lower findings were obtained by Hossain et al. (2020) in Bangladesh, which was 11.7% in ruminants suffering from diarrhea and the study of Dash Dash et al. (2011) whom found that infection rate with RAVs was 16.83% in diarrheic calves at India, and study of Gazal et al. (2012) in diarrheic lamb which was 10.4%. Moreover, our result also higher than that reported by Al-Ruwaili et al. (2012) in diarrheic camels at Suadi Arabia which was 9.4% by ELISA test .

The exact potential reasons for the aforementioned variations might be obviously influenced by sample size, certain diagnostic procedures, hoist ages, clinical status animals and risk factors which may be varied from one country to another.

The present study was observed that no significant differences at the level of 0.05 among the infection rates throughout the months of the year and the highest infection rate was noticed during April, 42.69% (38/89) followed by that in March which was 41.11% (37/90), whereas the lowest infection rate was recorded in July, 21.56% (22/102)

Similar finding was pointed out by Hossain et al., (2020) in Bangladesh, they found generally higher frequencies of RTAs in certain ruminants at and during the warm months, Nevertheless, contrast result was manifested by Barua et al. (2019) whom reported the highest rate with RVAs at winter season .

Numerous enrolled reasons might stand behind such elevation of the prevalence of RVAs in the current study at April and March, for instance, increasing of exposure rate to the virus during spring due to grassing, increasing of flies besides to spreading of numerous variety of insects at this period.

The present study was recorded that specified G and P genotypes distribution pattern as followings: in cattle: G6, G8, G10 in addition to P[1],P[5] and P[11]; in sheep G3, G6, G10, P[1] and P[11]; in goats G6 and P[5]; in camels G10, P[1] and P[11]. Genotype G10 was most dominant among specific genotypes in all animals in this study which recorded in 35, 14 and 23 of infected cattle sheep and camels cases, respectively.

According to our Knowledge, all genotypes that observed in our study were recorded for first time at Iraq in these animals.

The domination of G10 Genotype came in zalignment with many of global studies, for instance: the study of Medeiros et al. (2019) in Brazil, they found that G10 genotype was the predominant type in cow followed by P[11]. Similar result was mentioned by Hossain et al. (2020) whom reported the domination of G10, G8, P[1] and P[11] in cattle and G6 in goats. Besides to the study of Gazal et al. (2012) in lamb whom found the dominancy of G10, G6 and P[11] genotypes .In old world camels similar finding was referred by Abo Hatab et al.(2009) in Egypt and Jere et al. (2014) in Sudan.

In spite of their importance as they considered as an epidemiological markers, there was a marked paucity regarding the studies pertain RVAs genotypes in our country. The present study pinpointed on the surveillance of RVAs genotypes pattern in above farm animals due to importance of their serious and potential viral contamination and sharing between domestic animals and human (Tamim et al. 2010)

Increasingly, The genetic diversity of RVAs substantially elevated by the accumulation and aggregation of peculiar point mutations and assortments of specific cognate genes in addition to the specified viral evolutionary mechanism of interspecies transmission; the molecular epidemiologic studies that tracking viral genotypes is frequently needed to assess the endemic and zoonotic transmission at our community besides to identify the occurrence and investigation the source origins of the viral outbreaks, moreover, preparation of future certain vaccines and also innovation of integrated modern control program to reduce prevalence intensity of the virus.

CONCLUSIONS

According to the author's knowledge, the present study is the first study that recorded G3, G6, G8, G10, and also P[1], P[5] besides to P[11] in above animals at Iraq, which offers a valuable and reliable epidemiological information pertaining RAVs genotypes that were urgently needed for both human and animal necessary hygiene regards.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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