Molecular Detection and Phylogenetic Tree of Rotavirus from Human and Sheep, Iraq

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ABSTRACT
Group A rotaviruses (RVA) have been linked to acute gastroenteritis in children, as well as young domestic and wild animals. In Iraq few molecular studies involved the genotype profile of rota virus and this is the first study target VP1 gene. Data were collected from January to February 2018 and subsequently examined utilizing a PCR and because of low viral concentration we utilized Nested PCR. Sequenced PCR result at 327 bp fragment in 4 A rota virus-positive samples deposited in GenBank under accession number (MH11809.1, MH118097.1) for human and (MH118095.1, MH118094.1) to sheep. Children samples were identical to the other human A rota virus at (99-100%), with Japan feline-like human G6P and G6P[14] isolated from cattle as well as, animal rota virus (99-100%) similarity with cross relation with human Wa-Like and porcine, moreover Morocco characterized caprine, bovine rotavirus and Slovenian Roe deer ARV.

1. Introduction

Rotaviruses were rapidly recognized as a significant etiological factor in the occurrence of potentially fatal diarrhea among infants and children under the age of five on a global scale, as well as in the young populations of numerous animal and avian species (1)(2). The prevalence of group A rota viruses (RVAs) is extensive in the natural environment, as they have the ability to infect a wide range of mammalian and avian hosts (1). In human children and animals, these viruses are known to induce severe cases of gastroenteritis (2).

Rotaviruses are a type of virus that falls under the Reoviridae family. They are characterized by their segmented double-stranded RNA genome, the rotavirus genome is enclosed by a capsid that consists of three layers and has the shape of an icosahedron. The classification of RVAs involves the assignment of a genotype to each viral gene based on its nucleotide sequence. The composition consists of 11 segments, encoding for a total of six viral structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) (3)(4). Currently, a total of 22 genotypes have been documented for the gene responsible for generating the viral polymerase (VP1), whereas the gene encoding the core shell protein (VP2) has been associated with 20 genotypes (1).

There is a scarcity of information concerning the global diversity of enteric viruses. Several methods can be used to detect RVA in stool samples, such as virus isolation in cell culture, electron microscopy (EM), enzyme immunoassays (EIA), reverse transcription and PCR amplification (RT-PCR), and real-time reversetranscriptase-polymerase chain reactions (qRT-PCR) (5). The application of nested PCR assays involves the utilization of inner primers in conjunction with outside primers specifically designed for the study of clinical samples (6). The few copies of virus in stool samples benefit of using nested assays in this study. When contrasting nested RT-PCR with conventional RT-PCR, the latter demonstrates improved sensitivity and specificity. Enhanced specificity is attained in nested RT-PCR reactions through the utilization of two sets of primers (7). The application of a two-cycle PCR protocol enhances sensitivity. During a period of severe acute respiratory infection in the early 1990s identified a novel hanta virus using nested PCR reactions, thus increasing the profile of nested PCR. Before the present investigation, a number of viruses had been identified using nested PCR reactions, however,

no previous studies had focused on RVA (5).

The spread of RVA in domesticated animals also implies that this close proximity may aid in transmission. As previously demonstrated, the presence of environmental degradation in the studied areas and genetic material rearrangements were linked to transmission between a variety of species, including birds (23.6%), dogs (21.35%), chiropterans (17.98%), and cows (14.6%). According to the research, pigs make up (3.93%), cats make up (3.37%), small rodents make up (6.74%), and horses make up (8.43%) of the total rota virus infection (8)(9).

2. Material and Methods

RNA extraction and cDNA production

All stool specimens were collected between January and February 2018 from Women's and Children General Hospital in Al Diwaniyah province for children less than 5 years and a herd of sheep suffering from recurrent cases of diarrhea between lambs less than 4 months, all samples analyzed previously for bacterial isolation, RNA was extracted from (n = 123) stool samples divided to 90 from children and 33 lambs.

Ten µL from stool samples was suspended in 10% PBS before extracting RNA utilizing the AccuZolTM Total RNA extraction kit (Bioneer, Korea), RNA concentration was measured using a Nanodrop spectrophotometer and stored at -20C until employed in PCR amplification. cDNA synthesis: An AccuPower® RocktScript RT PreMix kit was utilized to synthesise cDNA from total RNA isolated from feces samples (10)(11).

Nested-PCR:

This study utilized RNA purification and Nested-PCR to amplify the gene encoding the viral polymerase (VP1) using (5) as a sources for primers and a rotavirus fragment design. The nested PCR product was tested using agarose gel electrophoresis. The nucleotide sequences of VP1 gene segments obtained were aligned using GeneDoc version. The primer sequences were assessed for specificity by utilizing BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers underwent evaluation for self-annealing sites, hairpin loop formation, 3′ complementary, and melting temperatures (Tm) using the IDT oligonucleotide calculator available at (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), Macrogene in the Korea produced all of the primers. The first round of the nested RT-PCR assay was performed using the OneTaq® One-Step RT-PCR Kit (New England Biolabs, Inc). Each 25 µL reaction mixture contained 19 µL of RNase-Free Water, 6 µL of 5× New England Biolabs OneStep RT-PCR Buffer, 300 µM dNTP Mix, 2U QIA-GEN OneStep RT-PCR Enzyme Mix, 2 µM each forward and reverse primer and 4 µL of RNA extract. For the reaction targeting VP1, the forward primer, VP1/F1 (5′-TGTGTCAAAACTATCAGAGCAGT-3′), and the reverse primer, VP1/R1, 5′-TGATAAGTTCTTGATCCTATACACTGA-3′, were used to amplify a 327 bp amplicon. For the VP1 reaction, the forward primer, N-VP F1, 5′-GGC TAT AAA ATG GYT TCN YT-3′, and the reverse primer, N-VP R1, 5′-ARY ADC CAR TAA TCR NYD RGT G-3′, were used to amplify a spanning 257 bp amplicon. After denaturing the RVA dsRNA and oligonucleotides for 5 min at 97 °C, the reaction mixture was added and reverse transcription and amplification were carried out on a PCR thermocycler (BioRad/USA). Thermocycling conditions consisted of a 30 min hold at 50 °C for reverse transcription, 15 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 50 °C and 45 s at 72 °C, with a

final hold of 7 min at 72 °C. The second round of the nested RT-PCR assay was performed using the AmpliTaq® DNA Polymerase with Buffer II (New England Biolabs, Inc). Each 50 µL reaction mixture contained 26.5 µL NuCLEANase® (c-LEcta GmbH, Germany), 5 µL of 10X PCR Buffer II, 2 mM MgCl2 Solution, 8 µL 10 mM GeneAmp® dNTP Blend, electrophoresis Bioneer/ Korea.

3. Results and Discussion

Due to the limited availability of stool samples with low virus loads, only a small number of genotypes for these samples were evaluated, which is a disadvantage of our investigation. Out of (123) stool samples only five samples showed limited viral concentration (3/90 children) and (2/33) lambs at 372 bp as illustrated in figure 1. and 20 samples amplified for nested VP1 gene at 257bp.

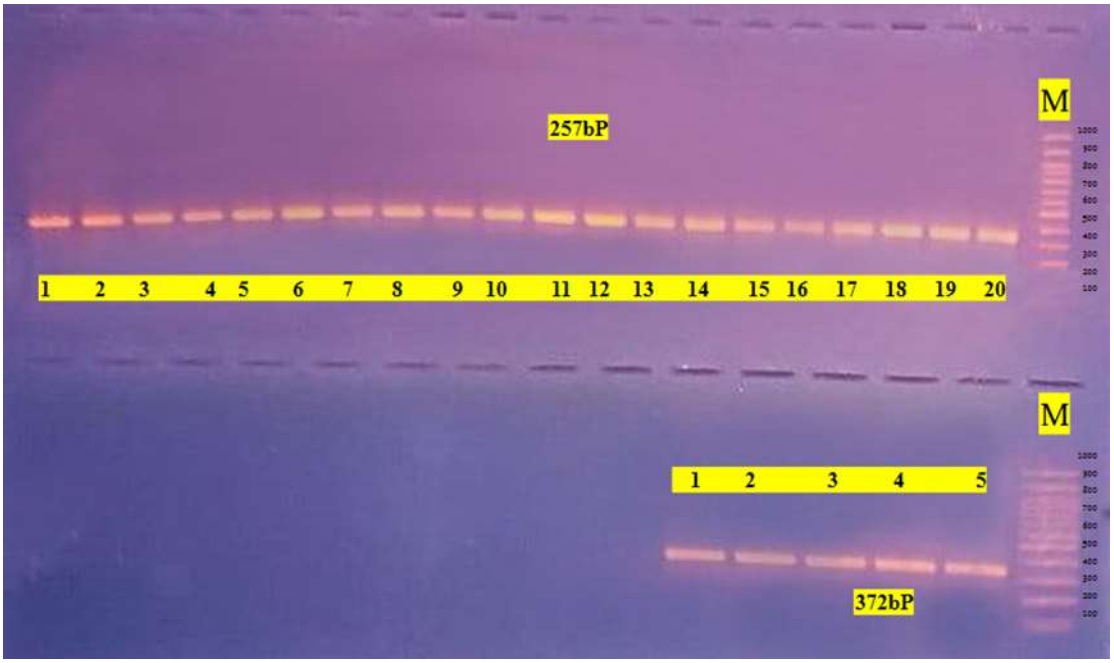

Figure 1: The detection limit of rotavirus concentration as analyzed by agarose gel electrophoresis of VP1 gene of rota virus. Lanes: represent positive PCR results (lane 1 positive control) (2-3) human samples, (4-5) sheep samples at (372)bp and positive nested PCR samples of VP1 gene human (1-2-3-4-5-6-7-8-9-10) and sheep (11-12-13-14-15-16-17-1-19-20) at (257)bp . M, 100-bp DNA ladder for molecular size standard.

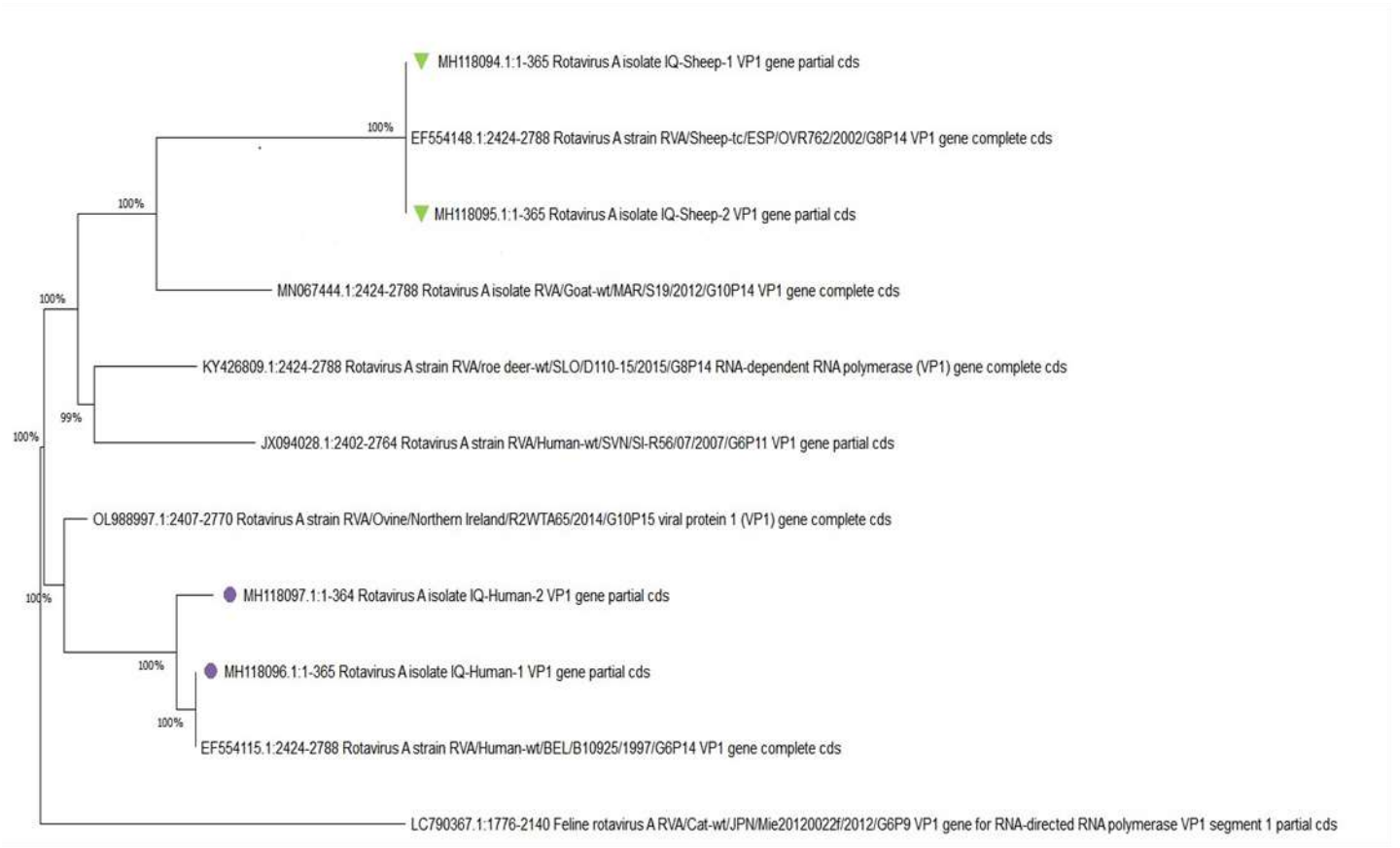

Figure 2 phylogenetic tree analysis of sequenced submitted Iraqi stool samples from human and sheep using MEGA X Maximum Likelihood method and Tamura-Nei model (12).

The species A of rota virus is a globally distributed pathogen that is of significant medical and veterinary importance. It is responsible for causing acute gastroenteritis, especially in young individuals. These viruses exhibit a high degree of genetic diversity, with numerous genotypes infecting various vertebrate hosts (5). The virus belonging to the Reoviridae family, possesses a capsid with three layers, housing 11 segments of double-stranded genomic RNA, (5-6) nonstructural proteins, and 6 structural proteins. Almost 35,000 strains have been found and divided into three preliminary serotype groups (F, G, and H), in addition to the five core serotype groups (A, B, C, D, and E). Given that at least 27 G and 35 P genotypes have been discovered thus far, a binary categorization approach for distinguishing "G" or "P" types has been proposed (13), this arises from the accumulation of point mutations, genetic reassortment, and intragenic recombination (14). The virus spreads mostly through the oral and faecal-oral pathways. Following attachment, there are three mechanisms for infiltrating the host cell: direct entry, fusion, or endocytosis. Rotaviruses primarily induce diarrhea by enterocyte loss, nutritional malabsorption, and disdigestion. Rotavirus infections can be asymptomatic or deadly, causing fast mortality.The mechanisms behind protective immunity against rotavirus infection remain incompletely elucidated, but it is widely accepted that stereotype-specific immunity plays an essential part (Rotavirus A − D, F – J) (15). Diarrhea is the most apparent symptom of the disease in calves, lambs, and children, as well as piglets and foals; the stools are white, yellow, or, in severe cases, blood-tinted or plain haemorrhagic (16). In dogs and cats, the infection typically results in self-limiting diarrhoea. Avian Rotavirus infections cause enteritis, growth depression, and/or retardation (16).

Rota virus represent a significant public health issue in Iraq, between cities and based on

sociodemographic, clinical characteristics the rota virus infection vary between 18-57%, depending on location and other variables (17), Rotavirus gastroenteritis is a significant cause of diarrhea in Al-Ramadi city Furthermore, it seems like the infection can occur at any time of year, but it is most common in the summer (18). Vaccination alters genotype distribution, which may pose issues for the efficacy of rotavirus vaccines and future government development (19) while in Tikrit city group A rotavirus detected in children under five years with infection rate reach to 42.5%, and 42.45% in AL-Qadisiya province (20).

In Iraq, 29.61% of diarrhoeic animals had RVAs, cattle 29.23%, sheep 31.51%, goats 27.58%, and camels 27.13%, respectively illustrated giving essential RAV genotype epidemiology for human and animal hygiene (21).

Nested PCR assays exhibited adequate sensitivity to detect amplification in samples at low viral concentrations, to gain a more comprehensive understanding of the genetic diversity of RVA including that of human and animals (22). To understand the mechanisms that lead to rotavirus variation in nature, a comprehensive classification system that considers all 11 rotavirus gene segments is needed. This strategy might help discover specific genes involved in rotavirus pathogenicity, replication, and host range restriction. Researchers studying the evolution and epidemiology of rotaviruses might also benefit from it. To develop a classification strategy in Iraq, we analyzed the VP1 genome sequences of multiple human and animal rotavirus A strains with different G and P genotypes. Additionally, we utilized information from pre-existing databases. These sequences were used to establish the evolutionary relationships among all of the rotavirus genes. Belgium isolate EF554115.1 displaying (100%) identity with human Iraqi isolate MH118096.1 and MH118097, G6P[14] specific rotaviruses are primarily isolated from cattle and are known to be an uncommon cause of gastroenteritis in humans (23) The Iraqi isolates crossing with Japan LC790367.1 feline-origin G6 strains were the most likely source of these occasional G6 RVA viruses developing gastroenteritis in humans worldwide. Furthermore, a feline-like human G6P[8] strain was discovered circulating in Brazil in 2022, highlighting the significance of continued monitoring potentially global RVA outbreaks even feline RVAs rarely infect humans, producing gastroenteritis (24).

Depending upon VP1 gene nucleotide identity cutoff values 100% of sheep Iraqi isolates MH118094.1 and MH118095.1 with Belgium sheep isolate EF554148.1 shared origin is found between human Wa-Like and porcine rotavirus strains, as well as between human DS-1-like and bovine rotavirus strains, according to a full genome-based categorization of rotaviruses (25). as well as, the similarity with JX094028.1 Slovenia homo sapiens isolate recorded in a child who had gastroenteritis that was similar to the G6P[11] strain found in cattle. A full genome study proved that the human G6P[11] strain came from a cow this similarity come from different genome lines (26), linked with MN067444.1 Morocco two characterized caprine and bovine rotavirus strains, 99% identity with Slovenian Roe deer isolate, this roe deer strain is most closely linked to RVA strains seen in sheep, cattle, and humans, according to phylogenetic research (23).

4. Conclusions

Rotavirus is the most common cause of viral gastroenteritis worldwide. The nested RT-PCR test might identify virus in stool samples in low concentration, analyzed nucleotide percentage identity were 100% of defined genotypes for group A rotavirus with global isolate from human, cattle, sheep, goat, Reo deer and feline. There is an urgent need for studies dealing with genes other than those addressed

in our study for the purpose of drawing a genetic profile of the isolation that exists in Iraq, to organize strategies related to vaccination programs for livestock, and to reduce the level of spread of infection in the country.

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