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Determination of bovine rotavirus G genotypes in Kashmir, India

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Summary
Rotavirus ribonucleic acid (RNA) was extracted from ten faecal samples of diarrhoeic calves positive for group A rotavirus by enzyme-linked immunosorbent assay (ELISA). A portion of the extracted RNA was run in polyacrylamide gel to determine the presence of rotaviral RNA and the rest subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) to generate the near full length VP7 gene. Only six samples yielded the desired product. The amplified products were subjected to G-typing by PCR using a cocktail of G6, G8 and G10 typing primers. All of the six samples were characterised as G10 and none of the samples revealed mixed infection by twin G types. Four samples, despite possessing sufficient rotavirus particles as revealed by ELISA and polyacrylamide gel electrophoresis, did not yield any amplified product on RT-PCR. This could be due to non-specific inhibitors of the PCR reaction, present in the faecal samples, being carried through the extraction procedures.

Keywords
Bovine – G-typing – India – Polymerase chain reaction – Rotavirus.

Introduction

Group A rotaviruses belonging to the family *Reoviridae* are important viral diarrhoeal agents in children and young animals, including calves, worldwide. These viruses possess eleven segments of double-stranded ribonucleic acid (dsRNA) and two outer capsid proteins, VP4 and VP7, both of which are independently responsible for virus neutralisation (7). Antigenic specificity carried by the VP4 and VP7 proteins is termed P and G genotype/serotype, respectively (8). To date, group A rotaviruses have been characterised as possessing 15 G and 21 P genotypes (5). To establish preventive measures against diarrhoea caused by group A rotaviruses, G and P type bovine rotavirus (BRV) distribution has been frequently investigated throughout the world (18, 12, 10). At least nine G types (G1-G4, G6-G8, G10 and G11) have been identified in bovine group A rotavirus strains (15). According to previous reports, G6 and G10 have been identified as the most common G types of BRV (10). In India, although the occurrence of BRV-related diarrhoea has been well documented, only one report is available on the distribution of G and P types (11). This paper describes the first attempt to G-type animal rotaviruses by nested polymerase chain reaction (PCR) in India.

Materials and methods

Field strains of bovine rotavirus

Ten diarrhoeic faecal samples from four to forty-five-day-old calves positive for group A rotavirus by enzyme-linked immunosorbent assay (ELISA) and maintained by the laboratory were selected for the study. Five samples originated from a military dairy farm (MDF) in Srinagar and five samples were from an exotic cattle breeding farm (ECBF) in Manasbal, Kashmir.

Extraction of double-stranded ribonucleic acid and electrophoresis

A 20% faecal suspension of each sample prepared in phosphate-buffered saline and clarified by centrifugation at 10,500 × g for 20 min. at 4°C was used as the basis for extraction of rotavirus ribonucleic acid (RNA). The RNA was extracted using the technique described by Chomczynski and Sacchi with some modifications (3). Briefly, 300 µl of the clarified 20% faecal suspension was vortexed with an equal volume of denaturating solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sodium dodecyl sulphate and 0.1 M β-mercaptoethanol). A 100 µl aliquot of 2 M sodium acetate was added and mixed in. After mixing, the solution was vortexed with an equal volume of a phenol:chloroform: isoamyl alcohol (25:24:1) mixture for 10 sec. and kept on ice for 15 min. The whole mixture was then centrifuged at 10,000 × g for 10 min. at 4°C. The upper aqueous layer was collected in a fresh tube and treated with two to three volumes of pre-chilled absolute ethanol and kept at –20°C overnight. On the following day, the RNA was sedimented at 10,000 × g for 15 min. at 4°C. The supernatant was discarded and the pellet washed with 1 ml of pre-chilled ethanol at 70%. The pellet was dried at room temperature and re-dissolved in 10 µl of distilled water. Extracted rotavirus RNA was analysed by polyacrylamide gel electrophoresis (PAGE) as previously described (20).

Reverse transcriptase-polymerase chain reaction of full length VP7

A pair of generic primers, Bov9Com5 and Bov9Com3, described by Isegawa *et al.* were used in this study (13). The nucleotide positions and the sequences of oligonucleotide primers are shown in Table I. The amplification procedure of this method comprised two steps, as follows:

a) reverse-transcription of the genomic dsRNA

b) amplification by PCR of the near full length VP7 gene segment.

An 8 µl sample of genomic RNA, extracted as above, was mixed with 100 ng each of the two primers Bov9Com5 and Bov9Com3 and denatured at 97°C for 5 min. and immediately chilled on ice. The denatured dsRNA was then reverse-transcribed at 42°C for 1 h with 5 U of AMV reverse transcriptase in a 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.6 mM $MgCl_2$, 10 mM NaCl, 0.25 mM DTT, 10 U of RNasin and 3 mM dNTP mix. Five millilitres of this reverse transcription product was amplified with 2 U of Taq deoxyribonucleic acid (DNA) polymerase in a 25 µl PCR mixture containing 10 × Taq buffer (150 mM Tris-HCl, pH 8.0, 500 mM KCl, 2.5 mM $MgCl_2$), 200 M of each dNTP and 0.2 µM each of Bov9Com3 and Bov9Com5 primers. The thermal cycling profile consisted of thirty cycles at 94°C for 1 min., 50°C for 1 min. and 72°C for 2 min., followed by final extension at 72°C for 7 min. The whole RT-PCR procedure was carried out in a thermal cycler. Production of a 1,011 bp band was considered a positive result.

Determination of the G type

Each RT-PCR product was subjected to a second round of amplification as described above with the generic primer Bov9Com5 and a cocktail of typing primers specific for G6, G8 and G10 (9). The nucleotide positions and the sequences of typing oligonucleotide primers are shown in Table I. Production of a 665 bp band was considered a positive result for G10.

Table I
Oligonucleotide primers used to amplify the near full length VP7 gene and for G-typing of bovine group A rotavirus

Primer name	Primer sequence	Location
Bov9Com5	5'>TGTATGGTATTGAATATACCA C< 3'	50-71
Bov9Com3	5'>TCACATCAT ACAACTCTAATC T< 3'	1,039-1,060
G6	5'>CTAGTTCCTGTGTAGAATC< 3'	499-481
G8	5'>CGGTTCCGGATTAGACAC< 3'	273-256
G10	5'>TTCAGCCGTTGCGACTTC< 3'	714-697

Analysis and detection of products

The PCR products were analysed on 1.0% agarose gel containing ethidium bromide (0.5 mg/ml) in Tris acetate ethylene diamine tetra acetic acid (EDTA) buffer (TAE) (0.04 M Tris-acetate, 0.001 M EDTA) (17). The fragments were visualised and photographed with a gel documentation system.

Results

Figure 1 shows the typical electrophoretic pattern of representatives of all ten isolates of BRVs. These electropherotypes were similar to and typical of long migrating group A rotaviruses. The VP7 genes of only six of the ten isolates (four from the MDF in Srinagar and two from the ECBF Manasbal) were successfully amplified using RT-PCR. The amplified genes were of the expected size (1,011 bp) and the G-typing assay was successfully applied to all the six specimens, each of which yielded a single band of 665 bp characteristic of the G10 serotype (Fig. 2).

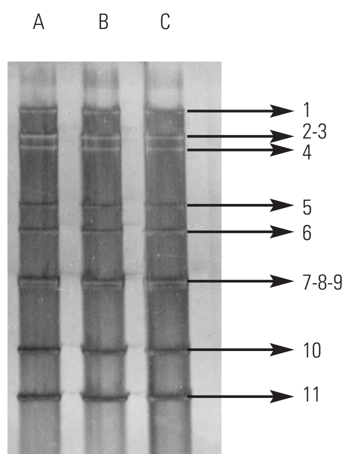
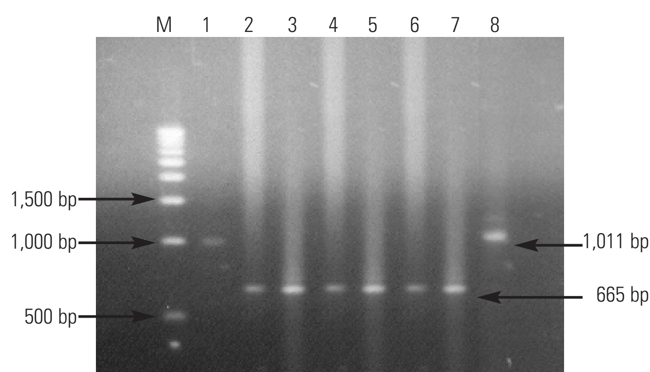


Fig. 1
Electropherotypic pattern of representative bovine rotavirus isolates from faecal samples of the diarrhoeic calves



Lane M: 500 bp molecular weight marker
Lane 1 and 8: full length VP7 gene fragment
Lane 2-7: 665 bp fragments specific to G10

Fig. 2
Polymerase chain reaction typing of six bovine rotavirus strains

Discussion

Although interpretation of this study is limited by the very small sample size and the fact that samples were collected from restricted regions in India, the findings are consistent with those of Gulati *et al.* who reported that the G10 serotype was the most predominant BRV (83%) in cows and buffalo calves aged less than one-month in India (11). These results contrast significantly with the distribution of BRV serotypes elsewhere in the world. Snodgrass *et al.* from the United Kingdom used a monoclonal antibody (Mab)-based ELISA for G-typing and reported that 66% of isolates were G6, while only 7.4% were G10 (18). Lucchelli *et al.* from the United States of America (USA) also used a Mab-based ELISA and demonstrated that 54% of isolates were G6 and only 14% were G10 (14). Parwani *et al.* used PCR-generated G type-specific complementary DNA probes and reported that 36.3% of the BRV strains tested were G6 and 19.8% were G10 (16). Chang *et al.* reported that 60.55% of BRV strains from the USA were of the G6 type while only 19.85% were G10 (2).

The findings in this study are particularly interesting in view of the fact that a rotavirus strain (1321) belonging to the G10 serotype was isolated from a child in Bangalore, India and was shown by RNA-RNA hybridisation and by sequence analysis to be related to BRVs (4, 6). Moreover, the incidence of asymptomatic infection of children with this 1321-like rotavirus in Bangalore was consistently high (about 34%) during a seven-year period from 1988 to 1994 (1). Interestingly, however, serotype G10 strains were not detected in symptomatically-infected children in the same area (1).

In the present study, naturally occurring mixed infection demonstrated by twin G types was not observed. However, in another study, Chang *et al.* reported having obtained mixed samples containing G6 and G10, G6 and G6s, and G6s and G10, accounting for 8.1%, 2.3% and 2.3% of the samples, respectively (2). The study reported in this paper is small and a comprehensive study on G and P types and G/P combinations in the field would be useful for understanding the epidemiology of BRV and designing an effective vaccine to control the disease.

Of ten selected samples, only six were identified by RT-PCR while four failed to produce an amplified viral band despite being known to contain sufficient rotaviral particles by RNA-PAGE and sandwich ELISA. The reason for this may be that non-specific inhibitors of the PCR reaction, present in the faecal samples, were carried through the extraction procedures (21). However, some studies report that incorporation of a purification procedure, based on selective adsorption of nucleic acids to glass in the presence of sodium iodide, into the extraction procedure could resolve the problem posed by the presence of non-specific inhibitors of the PCR reaction in faecal samples (19, 21).

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La détermination des génotypes G du rotavirus bovin au Cachemire (Inde)

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Résumé
L'acide ribonucléique (ARN) du rotavirus a été extrait à partir de 10 prélèvements de fèces provenant de veaux atteints de diarrhée chez lesquels la présence d'anticorps contre les rotavirus de groupe A avait été établie par la méthode immuno-enzymatique (ELISA). Une partie de cet ARN a été séparée par électrophorèse en gel de polyacrylamide pour révéler la présence de l'ARN rotaviral, le reste ayant été amplifié par réaction de polymérisation en chaîne couplée à une transcription inverse (RT-PCR) pour régénérer la quasi-totalité du gène VP7. Seuls six échantillons ont permis d'obtenir le résultat escompté. Les produits amplifiés ont été soumis à un typage G par PCR au moyen d'un mélange d'amorces G6, G8 et G10. La caractérisation des six échantillons a permis de les identifier comme appartenant au type G10. Aucun des prélèvements n'a révélé l'existence d'une infection mixte par deux types G. Malgré une quantité suffisante de particules virales, comme en attestent l'épreuve ELISA et l'électrophorèse en gel de polyacrylamide, quatre prélèvements n'ont fourni aucun produit à l'issue de la RT-PCR. Cette absence de résultat s'explique probablement par la présence, dans les prélèvements fécaux, d'inhibiteurs non spécifiques de la PCR qui auraient résisté au processus d'extraction de l'ARN.

Mots-clés
Bovin – Inde – Réaction d'amplification en chaîne par polymérase – Rotavirus – Typage G.

Determinación de genotipos G del rotavirus bovino en Cachemira (India)

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Resumen
Los autores describen la extracción de ácido ribonucleico (ARN) de rotavirus a partir de diez muestras fecales de terneras aquejadas de diarrea, que previamente habían dado resultado positivo a un ensayo inmunoenzimático (ELISA) de detección de rotavirus del grupo A. Se hizo correr una parte del ARN extraído en un gel de poliacrilamida para determinar la presencia de ARN rotavírico, y se sometió el resto a transcripción inversa acoplada a la reacción en cadena de la polimerasa (RT-PCR) con el fin de obtener un fragmento casi completo del gen VP7. Sólo seis de las muestras dieron el resultado deseado. Después se procedió a una nueva PCR para determinar la posible correspondencia de los fragmentos obtenidos con el genotipo G, utilizando para ello un cóctel de cebadores de tipificación G6, G8 y G10. Las seis muestras resultaron pertenecer al tipo G10, y en ninguna de ellas se observó infección mixta por varios genotipos G. La amplificación por PCR no dio resultado alguno

con cuatro de las muestras, pese a que éstas contenían una cantidad suficiente de partículas de rotavirus (como se había comprobado por ELISA y electroforesis en gel de poliacrilamida). Ello podría deberse a la presencia en las muestras fecales de inhibidores inespecíficos de la reacción de PCR que los procedimientos de extracción hubieran arrastrado con el material genético.

Palabras clave
Bovino – India – Reacción en cadena de la polimerasa – Rotavirus – Tipificación del genotipo G.

References

1. Aijaz S., Gowda K., Jagallnath H.V., Reddy R.R., Maiya P.P., Ward R.L., Greenberg H.B., Raju M., Babn A. & Rao C.D. (1996). – Epidemiology of symptomatic human rotaviruses in Bangalore and Mysore, India, from 1998 to 1994 as determined by electropherotype, subgroup and serotype analysis. *Arch. Virol.*, **141** (3-4), 715-726.

2. Chang K.O., Parwani A.V. & Saif L.J. (1996). – The characterization of VP7 (G type) and VP4 (P type) genes of bovine group A rotaviruses from field samples using RT-PCR and RFLP analysis. *Arch. Virol.*, **141** (9), 1727-1739.

3. Chomczynski P. & Sacchi N. (1987). – Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.*, **162** (1), 156-159.

4. Das M., Dunn S.J., Woode G.N., Greenberg H.B. & Rao C.D. (1993). – Both surface proteins (VP4 and VP7) of an asymptomatic neonatal rotavirus strain (1321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. *Virology*, **194** (1), 374-379.

5. Das S., Sen A., Uma G., Varghese V., Chaudhuri S., Bhattacharya S.K., Krishnan T., Dutta P., Dutta D., Bhattacharya M.K., Mitra U., Kobayashi N. & Naik T.N. (2002). – Genomic diversity of group A rotavirus strains infecting humans in eastern India. *J. clin. Microbiol.*, **40** (1), 146-149.

6. Dunn S.J., Greenberg H.B., Ward R.L., Nakagomi O., Burns J.W., Vo P.T., Pax K.A., Das M., Gowda K. & Rao C.D. (1993). – Serotypic and genotypic characterization of human serotype 10 rotaviruses from asymptomatic neonates. *J. clin. Microbiol.*, **31** (1), 165-169.

7. Estes M.K. (2001). – Rotaviruses and their replication. *In* Fields virology (D.M. Knipe & Howley P.M., eds). Lippincott-Roven Publishers, Philadelphia, 1747-1785.

8. Estes M.K. & Cohen J. (1989). – Rotavirus gene structure and function. *Microbiol. Rev.*, **53** (4), 410-449.

9. Falcone E., Tarantino M., di Trani L., Cordioli P., Lavazza A. & Tollis M. (1999). – Determination of bovine rotavirus G and P serotypes in Italy by PCR. *J. clin. Microbiol.*, **37** (12), 3879-3882.

10. Fukai K., Maeda Y., Fujimoto K., Itou T. & Sakai T. (2002). – Changes in the prevalence of rotavirus G and P types in diarrhoeic calves from the Kagoshima prefacture in Japan. *Vet. Microbiol.*, **86** (4), 343-349.

11. Gulati B.R., Nakagomi O., Koshimura Y., Nakagomi T. & Pandey R. (1999). – Relative frequencies of G and P types among rotaviruses from Indian diarrhoeic cows and buffalo calves. *J. clin. Microbiol.*, **37** (6), 2074-2076.

12. Hussein H.A., Parwani A.V., Rosen B.I., Lucchelli A. & Saif L.J. (1993). – Detection of rotavirus serotypes Gl, G2, G3, and G11 in feces of diarrheic calves by using polymerase chain reaction-derived cDNA probes. *J. clin. Microbiol.*, **31** (9), 2491-2496.

13. Isegawa Y., Nakagomi O., Nakagomi T., Ishida S., Uesugi S. & Ueda S. (1993). – Determination of bovine rotavirus G and P serotypes by polymerase chain reaction. *Molec. Cell. Probes*, **7** (4), 277-284.

14. Lucchelli A., Kang S.Y., Jayasekera M.K., Parwani A.V., Zeman D.H. & Saif L.J. (1994). – A survey of G6 and G10 serotypes of group A bovine rotaviruses from diarrhoeic beef and dairy calves using monoclonal antibodies in ELISA. *J. vet. diagn. Invest.*, **6** (2), 175-181.

15. Okada N. & Matsumoto Y. (2002). – Bovine rotavirus G and P types and sequence analysis of the VP7 gene of two G8 bovine rotaviruses from Japan. *Vet. Microbiol.*, **84** (4), 297-305.

16. Parwani A.V., Hussein H.A., Rosen B.I., Lucchelli A., Navarro L. & Saif L.J. (1993). – Characterization of field isolates of group A bovine rotaviruses by using polymerase chain reaction-generated G and P type-specific cDNA probe. *J. clin. Microbiol.*, **31** (8), 2010-2015.

17. Sambrook J., Fritsch E.F. & Maniatis T. (1989). – Molecular cloning: a laboratory manual, 2nd Ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, 6.7.

18. Snodgrass D.R, Fitzgerald T., Campbell I., Scott F.M., Browning G.F., Miller D.L., Herring A.J. & Greenberg H.G. (1990). – Rotavirus serotypes 6 and 10 predominant in cattle. *J. clin. Microbiol.*, **28** (3), 504-507.

19. Vogelstein B. & Gillespie D. (1979). – Preparative and analytical purification of DNA from agarose. *Proc. natl Acad. Sci. USA*, **76** (2), 615-619.

20. Wani S.A., Bhat M.A., Ishaq S.M., Ashrafi M.A., Buchh A.S. & Haq M. (2003). – Detection of a mammalian-like group A rotavirus in diarrhoeic chicken. *Vet. Microbiol.*, **94** (1), 13-18.

21. Xu L., Harbour D. & McCrae M.A. (1990). – The application of polymerase chain reaction to the detection of rotaviruses in faeces. *J. virol. Meth.*, **27** (1), 29-38.