

DETECTION AND GENOTYPIC CHARACTERIZATION OF ROTAVIRUS FROM BOVINE CALVES OF ASSAM, A NORTH EASTERN STATE OF INDIA

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ABSTRACT

Rotavirus (RV) has been considered as one of the most important cause of severe gastroenteritis among human infants and the neonates of most farm animal species worldwide. The present study was undertaken to detect rotavirus from faecal samples of bovine calves and to investigate genotypic distribution of rotavirus among bovine population during the period from June 2014 to May 2015. A total of 196 faecal samples were collected from both diarrhoeic and non-diarrhoeic calves with age group up to 4 months from different places of Assam, India. Screening of the 196 samples by RNA-PAGE revealed 26 (13.26%) to be positive for bovine RV with characteristic migration pattern of group A RV. The same number samples screened by RT-PCR revealed 71 (36.22%) to be positive for both VP7 and VP4 genes of group A bovine RV. RT-PCR was found to be more sensitive than RNA-PAGE. All non-diarrhoeic samples were negative for RV. Genotyping of RV was carried out by nested-multiplex PCR using type-specific primers of common genotypes. Study revealed that, the most common genotype circulating among bovine population of Assam is G10P[11]. Of 71 RV positive samples, 24 (33.80%) samples were found to be typeable for G10P[11] RV genotype while rest 47 samples were either non-typeable or mixed type.

Keywords: Bovine calves, group A rotavirus, RNA-PAGE, RT-PCR, Nested-multiplex PCR, G and P-genotyping.

INTRODUCTION

Rotavirus has been identified as an assured cause of severe gastroenteritis in the various mammalian species including calves and humans, and poultry species throughout the world (Dhama *et al.* 2009, Kapikian *et al.* 1986, Martella *et al.* 2010). RV belongs to the genus *Rotavirus* under the family Reoviridae is non-enveloped, icosahedral particles consisting of eleven segments of double stranded RNA (dsRNA) enclosed in a triple-layered protein capsid (Broor *et al.* 2003, Ciarlet and Estes, 1999). Although infectious serogroups of RV from A to H have been recognized as infecting man and various species of animals, group A RVs are the leading cause of diarrhoea in calves under one month of age throughout the world (Matthijnsens *et al.* 2012).

Group A RV possesses two outer capsid proteins *viz.* VP7 and VP4, both of which are independently responsible for virus neutralization (Estes, 2001). This virus was classified according to its combination of two kinds of serotype, namely G (glycoprotein) serotype determined by VP7 antigenicity and P (protease-sensitive protein) serotype associated with VP4 antigenicity (Dhama *et al.* 2009, Estes and Cohen 1989). Currently, 27 G-genotypes (from G1 to G27) and 35 P-genotypes (from P[1] to P[35]) have been identified in human and various species of animals (Matthijnsens *et al.* 2012).

Among these 27 G-genotypes and 35 P-genotypes, 11 G-types (G1-G8, G10, G11 and G15) and 6 P-Types (P[1], P[5], P[11], P[14], P[17] and P[21]) have been reported in bovine RV infection with G6, G8, G10, P[1], P[5] and P[11] predominating (Beg *et al.* 2010).

Genotyping has been preferred to serotyping due to its good correlation with serotypes, high sensitivity and use of synthetic reagents (Gouvea *et al.* 1990). RV is considered to be an important pathogen from ecological and public health points of view as certain animal RV strains have antigenic similarities to some human strains (Estes *et al.* 1983). The genotype G10P[11] is an important group A bovine RV because of its zoonotic transmission from human to cattle and also from cattle to human (Iturriza-Gomara *et al.* 2004). Since there are many combinations of G and P genotypes in group A RV, study on the genotyping of RV is very critical to develop effective measures and in formulating the control strategy to be applied against the disease.

The present report describes about detection as well as genotyping (G and P) of RV in bovine calves of Assam, India by using polymerase chain reaction (PCR) based typing assays. To the authors' knowledge, this is the first report on genotypic characterization of RV in bovine calves from this part of India.

MATERIALS AND METHODS

Faecal samples: A total of 196 faecal samples from apparently healthy as well as diarrhoeic bovine calves with age group up to 4 months were collected aseptically from both organized and un-organized cattle farms of different places of Kamrup district, Assam, India from June 2014 to May 2015. In organized farms, intensive system of farming was followed where animals were kept in concrete floors, supplied with clean feed and water. In un-organized farms, they were rearing in semi-intensive system. All collected faecal samples were stored at -20°C until further processing. Approximately 1 g of faecal sample in case of non-diarrhoeic samples and for diarrhoeic samples, sufficient volume (1 ml) of the samples was vortexed with 4 volumes of 0.1M PBS (pH=7.4) and centrifuged in a refrigerated centrifuge machine (Sigma, Germany) at 12000 x g for 20 minutes at 4°C. The supernatant was collected and stored at -20°C until further use.

Detection of rotavirus in fecal samples: Initial screening of the processed fecal samples for rotavirus was done by RNA PAGE followed by RT PCR of VP4 and VP7 genes. To be added

Ribonucleic acid polyacrylamide gel electrophoresis (RNA PAGE): Viral RNA was extracted from the fecal suspensions by phenol chloroform mixture (Herring *et al.* 1982) and 0.1 M sodium acetate buffer (pH 5.0) containing 1% (w/v) sodium dodecyl sulphate (SDS). RNA concentration was assessed by nanodrop spectrophotometer (ND-1000; Thermo Scientific, USA). The extracted RNA was then subjected to native RNA PAGE. The electrophoretic run was carried out at 100-120volts for 2-3 hours using vertical gel electrophoresis apparatus (Biorad, USA). The genomic migration pattern was detected by silver-staining of the gel (Herring *et al.* 1982).

PCR for amplification of VP4 and VP7 gene of RV: For amplification of VP4 and VP7 genes of RV, the viral RNA was extracted from collected faecal samples using RNA Sure® Virus Kit (Genetix, Asia Biotech Pvt. Ltd., New Delhi) following the manufacturer's protocol. The extracted RNA was stored at -20°C until further use.

Primers: For PCR amplification of VP4 and VP7 genes of RV, previously published primers were used (Gentsch *et al.* 1992, Isegawa *et al.* 1993). For genotyping of RV, type specific primers reported for most common genotypes were selected based on available literatures (Gouvea *et al.* 1994, Hardy *et al.* 1992). The sequences of the primers, their positions in the genomic segment and the prototype viruses from which the sequence taken are presented in Table 1.

RT-PCR for VP4 and VP7 gene amplification: A reverse transcription PCR (RT-PCR) was used for

amplification of full length VP4 and VP7 genes of RV as per method of Falcone *et al.* 1999. Briefly, for VP4 amplification, in RT-1 reaction, denaturation of template and primers (Con3 and Con2) was done at 95°C for 5 min. in a thermal cycler (VERITI 96 wells, Applied Biosystems) and immediately chilled on ice. In RT-2 reaction, denatured dsRNA was then added to the reaction mixture consisting of 10 µl of the 5X reaction buffer, 1 µl of 10 mM deoxynucleoside triphosphate (10mM dNTP) mixture, 2 µl of 25 mM MgSO₄, 1 µl of MuLV Reverse Transcriptase enzyme (5U/µl), 1 µl of Taq Polymerase (5U/µl) and 3 µl of dimethyl sulfoxide in a final volume of 50 µl and then preincubated at 48°C for 45 min followed by 35 cycles of 94°C for 1 min., 52°C for 2 min., and 68°C for 3 min. and a final extension of 68°C for 10 min. and wind up by hold at 4°C. For VP7 gene amplification, in RT-1 reaction, denaturation of template and primers [Bov9Com3 (Forward) and Bov9Com5 (Reverse)] was done at 97°C for 5 min. in a thermal cycler (VERITI 96 wells, Applied Biosystems) and immediately chilled on ice. In RT-2 reaction, denatured dsRNA was then added to the reaction mixture consisting of 10 µl of the 5X reaction buffer, 1 µl of 10 mM deoxynucleoside triphosphate (10mM dNTP) mixture, 2 µl of 25 mM MgSO₄, 1 µl of MuLV Reverse Transcriptase enzyme (5U/µl), 1 µl of Taq Polymerase (5U/µl) and 3 µl of dimethyl sulfoxide in a final volume of 50 µl and then preincubated at 48°C for 45 min followed by 39 cycles of 94°C for 1 min., 46°C for 2 min., and 68°C for 3 min. and a final extension of 68°C for 10 min. and wind up by hold at 4°C.

G and P typing of RV by nested multiplex PCR: G-typing was carried out following the method described by Falcone *et al.* 1999 with the VP7 upstream primer and common G-type specific primers as described by Gouvea *et al.* 1994. Briefly, 5 µl of undiluted first RT-PCR product was added to a reaction mixture consisting of 3 µl of 25mM MgCl₂, 2 µl of 10mM dNTPs, 0.5 µl of Taq Polymerase (5U/µl), 5 µl of 10X Taq buffer, 1 µl of each type specific primers G5, G6, G8, G10 and G11 and an upstream primer Bov9Com3 and RNase-free water to final volume of 50 µl, and then subjected to nested multiplex PCR amplification with the following PCR cycles: Preincubation at 94°C for 10 min. and 25 cycles of 1 min. at 94°C, 2 min. 54°C and 1 min. at 72°C, followed by a final incubation at 72°C for 10 min. and wind up by hold at 4°C.

P-typing was carried out similarly with the VP4 upstream primer and P-type specific primers as reported by Hardy *et al.* 1992 and Gouvea *et al.* 1994. Briefly, 5 µl of undiluted first RT-PCR product was added to a reaction mixture consisting of 3 µl of 25mM MgCl₂, 2 µl of

10mM dNTPs, 0.5 μ l of *Taq* Polymerase (5U/ μ l), 5 μ l of 10X *Taq* buffer, 1 μ l of each type specific primers P[1], P[5] and P[11] and an upstream primer Con2 and RNase-free water to final volume of 50 μ l, and subjected to nested multiplex PCR amplification by following the PCR cycles as reported by Falcone *et al.* 1999 with slight modification of the annealing temperature, which was set at 55°C, briefly, preincubation at 94°C for 5 min. and 25 cycles of 1 min. at 94°C, 2 min. 55°C and 1 min. at 72°C, followed by a final incubation at 72°C for 5 min. and wind up by hold at 4°C.

Analysis of PCR products: PCR products were analyzed in 1.5 % agarose gel containing ethidium bromide (0.5 μ g/ml). The electrophoresis was carried out for 60 mins at 80 V. The amplicons were visualized under UV light using a UV transilluminator (DNR System, USA) to detect the presence of specific amplicons.

RESULTS AND DISCUSSION

Rotavirus has been a major cause of mortality and morbidity in young ones of most farm animal species as well as in human infants (Estes and Cohen 1989). In the present study, RV was detected in faecal samples of bovine calves from Assam, a north eastern state of India by RNA –PAGE and RT-PCR. The G and P genotype of RV was also determined by using multiplex nested PCR. Results showed that of 196 faecal specimens collected from both diarrhoeic and non-diarrhoeic calves, 26 (13.26%) samples from diarrhoeic calves were found to be positive for RV in standard PAGE assays. The freeze-dried RV maintained in the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati, Assam, India was used as positive control in the tests. No non-diarrhoeic samples were found to be positive for bovine RV. All positive RV nucleic acid segments were separated in a clustered arrangement of 4:2:3:2, with segments 2, 3 and 4 migrating close together, segments 7, 8 and 9 closely spaced, and segments 10 and 11 as found in long RNA migration patterns indicating typical of group A mammalian RV (Figure.1). In this study electropherotyping (RNA-PAGE) was selected as the primary method for detection of RV strains in faeces of diarrhoeic calves, because each RV strain reveals a single distinct electro-pherotype upon PAGE and 11 segments of dsRNA by PAGE yield a pattern which is both constant and characteristic for a particular RV isolate (Estes *et al.* 1984). Similar observations were also reported by earlier workers (Dash *et al.* 2011, Ghosh *et al.* 2007, Sravani *et al.* 2014).

Detection of RV by RT-PCR showed a higher percentage of detection in comparison to RNA-PAGE. Out of 196 faecal samples collected, 71 [36.22%] samples from diarrhoeic calves were found to be positive

for bovine RV in standard RT-PCR. All non-diarrhoeic samples were negative for RV. In RT-PCR, full length amplification of both VP4 and VP7 gene of RV could be obtained with a desired band sizes of 876 bp and 1011 bp respectively (Figure 2 and 3) in all 71 samples. The freeze-dried RV maintained in the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati, Assam was used as positive control along with samples for full length amplification of both VP4 and VP7 gene of RV. Efficacy of RT-PCR over RNA-PAGE in detection of RV in diarrhoeic faecal samples has been reported earlier. Although RNA-PAGE has been considered as ‘gold standard’ for detection of RV (Ibrahim *et al.* 1990) it was observed that RT-PCR in the present study to be more effective and showed higher sensitivity than RNA-PAGE. This might be attributed to the nucleic acid based techniques such as RT-PCR which could detect a very minute concentration of the virus that may escape detection by RNA-PAGE.

As the molecular epidemiology of rotavirus developed recently, genetic typing based on VP7 gene and VP4 gene, G-genotype and P-genotype, respectively, were developed (Kobayashi *et al.* 1989). In the present study, genotyping PCR for G genotype using common type specific primers (G5, G6, G8, G10 and G11) revealed expected PCR products of 749 bp size (Figure 4) depicting G10 genotype in 24 of 71 RV positive samples while rest 47 samples had mixed or un-typeable G genotypes. However, no amplification was observed for other G genotypes.

Similarly, P-genotyping PCR using common P-typing primers (P[1], P[5] and P[11]), showed amplification of 314 bp in 29 of 71 positive samples (Figure 5) denoting the presence of P[11] genotype. Rest 42 samples showed a mixed amplification suggesting the presence of mixed P genotypes.

The G-P combination that could be identified in the present study was G10P[11] in all 24 samples that gave specific amplification for these genotypes. Epidemiological studies in calves from India showed predominance of group A RV strains *viz.* G6, G8 and G10 specificity (Nataraju *et al.* 2009) and P[1], P[5] and P[11] genotypes (Sravani *et al.* 2014, Suresh *et al.* 2012). In this study, nested-multiplex PCR for differentiating G-genotypes showed presence of G10 genotype, and occurrence of other mixed G-genotypes while differentiating P-genotypes, all typeable samples were found to be positive for P[11] genotype. The most prevalent G-P genotype combination of group A RV during the study was found to have G10P[11] (24 out of 71 rotavirus positive samples). Thus, the present study reveals that G10P[11] is predominant type of RV circulating in bovine population in Assam. Similar G-P genotype combinations were also recorded in different

parts of country in earlier studies (Beg *et al.* 2010, Gulati *et al.* 1999, Minakshi *et al.* 2005, Saravanan *et al.* 2006, Suresh *et al.* 2012). Circulation of RV with G10P[11] genotype has also been reported globally from different parts of the world (Alfieri *et al.* 2004, Ghosh *et al.* 2007, Fukai *et al.* 1998). The inability to type 47 samples for both G and P-genotypes suggest the occurrence of bovine RV G and P-genotypes other than those targeted in this study. The G10P[11] genotype strain was an important genotype of group A bovine RV because, this G-P

genotype combination has been attributed to cause asymptomatic as well as symptomatic infections in Indian children, which appeared to be acquired through zoonotic transmission from humans to cattle and also from cattle to humans (Iturriza-Gomara *et al.* 2004). Earlier to this, no report on bovine RV genotyping has been reported from Assam. In this study, G10P[11] genotype was found to be present in bovine population and this may be recognized as an important contributor to the diversity of RV found in human population.

Table 1. Details of the primers used in the study.

Primer	Type of PCR	Sequence (5' - 3')	Position	Reference
VP7 gene amplification				
Bov9Com3 (+)	RT-PCR	TCACATCATACTACTCTAATCT	1038-1060	Isegawa <i>et al.</i> (1993)
Bov9Com5 (-)		TGTATGGTATTGAATATACCA	50-71	
G-typing				
FT5-G5	Nested-multiplex PCR	CATGTACTIONGTTGTTACGTC	779-760	Gouvea <i>et al.</i> (1994)
DT6-G6		CTAGTTCCTGTGTAGAATC	499-481	
HT8-G8		CGGTTCCGGATTAGACAC	273-256	
ET 10-G10		TTCAGCCGTTGCGACTTC	714-697	
BT 11-G11		GTCATCAGCAATCTGAGTTGC	336-316	
VP4 gene amplification				
Con3 (+)	RT-PCR	TGGCTTCGCTCATTATAGACA	11-32	Gentsch <i>et al.</i> (1992)
Con2 (-)		ATTTCGGACCATTATATAACC	868-887	
P-typing				
pNCDV- P[1]	Nested-multiplex PCR	CGAACGCGGGGGTGGTAGTTG	269-289	Gouvea <i>et al.</i> (1994)
pUK- P[5]		GCCAGGTGTCGCATCAGAG	336-354	
pB223- P[11]		GGAACGTATTCTAATCCGGTG	574-594	

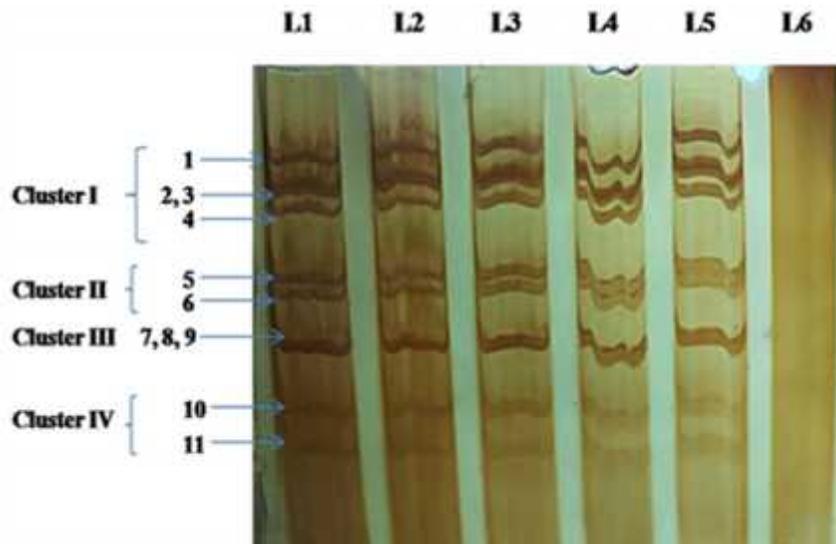


FIG. 1 RNA ELECTROPHORESIS OF BOVINE ROTAVIRUS RNA

L1: Positive Control, L2- L5: Positive Bovine faecal samples, L6: Negative Control

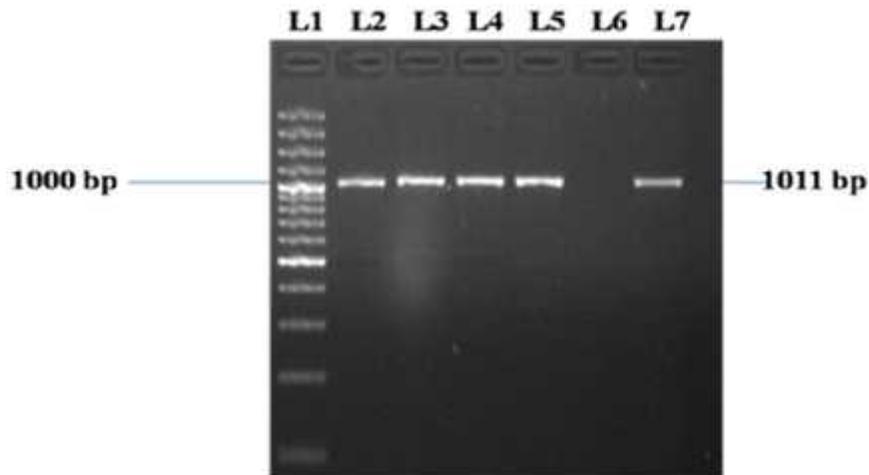


FIG. 2 AGROSE-GEL ELECTTROPHORESIS OF RT-PCR PRODUCT OF VP7 REGION OF BOVINE ROTAVIRUS

L1: 100 bp plus ladder. L2-L5: Positive Faecal samples, L6: Negative Control. L7: Positive Control (Bovine RV)

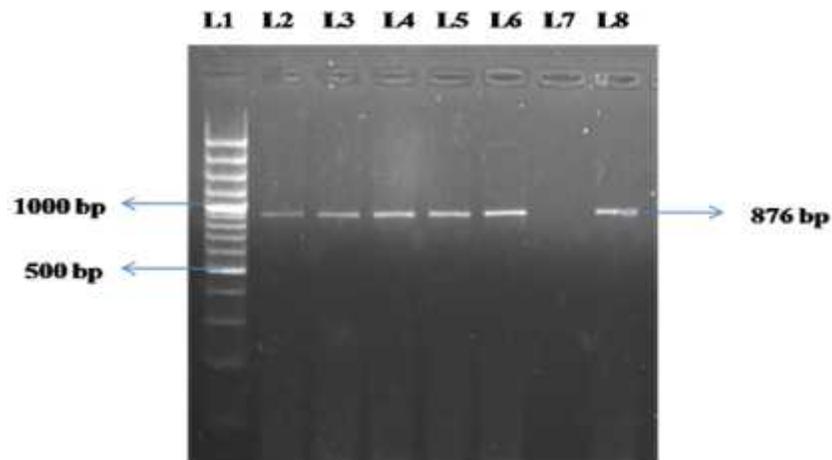


FIG. 3 AGAROSE-GEL ELECTROPHORESIS OF RT-PCR PRODUCT OF VP4 REGION OF BOVINE ROTAVIRUS

L1: 100 bp plus ladder, L2 – L6: Positive Faecal samples L7: NTC, L8: Positive Control (Bovine RV)

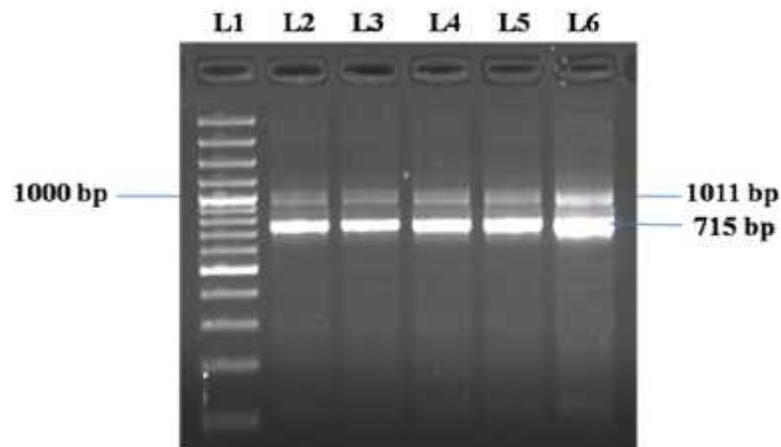


FIG. 4 AGAROSE GEL ELECTROPHORESIS OF NESTED-MULTIPLEX RT-PCR PRODUCT OF G GENOTYPE OF BOVINE ROTAVIRUS

L1: 100 bp plus ladder, L2 – L6: Amplified product of G10 (715 bp) genotype

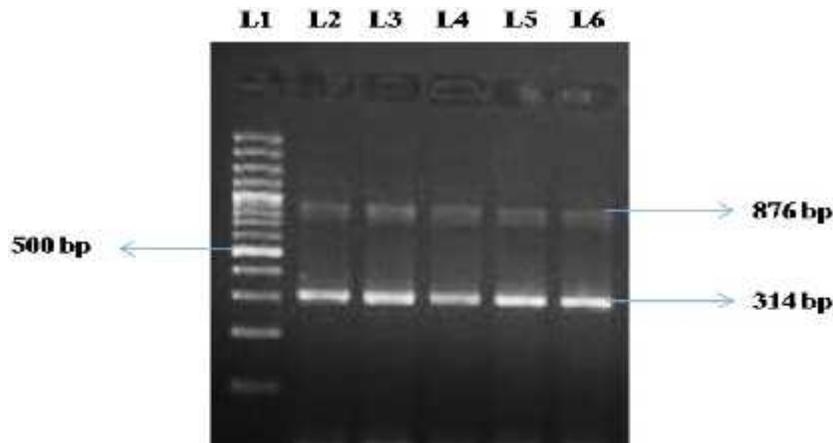


FIG. 5 AGAROSE GEL ELECTROPHORESIS OF NESTED-MULTIPLEX RT-PCR PRODUCT OF P GENOTYPE OF BOVINE ROTAVIRUS

L1: 100 bp plus ladder, L2 – L6: Amplified product of P [11] (314 bp) genotype

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