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Characterization of G2P[4] rotavirus strains causing outbreaks of gastroenteritis in the Northern Territory, Australia, in 1999, 2004 and 2009 ☆

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ABSTRACT

Outbreaks of rotavirus diarrhea cause a large disease burden in the Alice Springs region of the Northern Territory, Australia. The introduction of the rotavirus vaccine Rotarix® has been associated with an increase in detection of G2P[4] strains in many countries. However, G2P[4] emergence has also been observed in vaccine-naive countries, suggesting a general global increase in the circulation of G2P[4] strains.

A G2P[4] rotavirus outbreak occurred in 2009, 28 months after the introduction of the Rotarix® vaccine and 43 children were hospitalized. Pre-vaccine introduction, G2P[4] strains were observed associated with large outbreaks in 1999 and 2004. To determine the genetic relationship between these strains whole genome sequence analysis was conducted on representative strains from each of the G2P[4] outbreaks, in 1999, 2004 and 2009. Phylogenetic analysis revealed the majority of genes from 2009 outbreak strain clustered with contemporary global strains, while the VP7 gene clustered with contemporary and older strains and was antigenically distinct to the majority of contemporary global G2P[4] strains; suggesting the strain was an intragenogroup reassortant. The 1999 and 2009 strains appear to share similar evolutionary origins, and both had a high degree of genetic identity to previously identified Australian and global strains. Conversely, the 2004 outbreak strain was more divergent in comparison to Australian and global strains.

The 1999 and 2004 outbreaks likely occurred due to the accumulation of immunologically naïve children in the population following low levels of G2P[4] rotavirus disease in the community in the years prior to each outbreak. The 2009 outbreak was associated with moderate vaccine coverage in the population and vaccine efficacy against the strain was low. The circulation of this unusual strain in the population combined with low vaccine coverage and diminished vaccine efficacy likely contributed to the outbreak occurring in this population.

1. Introduction

Group A rotaviruses are the predominant etiological agent of acute gastroenteritis in young children globally. Rotavirus infection is a significant cause of morbidity and mortality, causing 114 million episodes of diarrhea annually, resulting in 24 million clinic visits and 2.4 million hospitalizations in children under five years of age worldwide (Glass et al., 2006). The mortality rates associated with rotavirus disease are unevenly distributed between developed and developing countries; the overwhelming majority of the estimated 453,000 annual deaths occur in developing nations in Asia and sub-Saharan Africa (Tate et al., 2012). The World Health Organization has recommended the global introduction of rotavirus vaccines to combat the morbidity and mortality caused by rotavirus disease (WHO, 2009).

Rotavirus (*Reoviridae* virus family) has an 11 segment double-stranded RNA (dsRNA) genome encoding six structural viral proteins (VP1–4, VP6, VP7) and six non-structural proteins (NSP1–6) (Estes and Kapikian, 2007). The two outer capsid proteins VP4

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and VP7 elicit type-specific and cross-reactive neutralizing antibody responses, and are used to classify rotavirus strains into G (glycoprotein, VP7) and P (protease sensitive, VP4) genotypes respectively (Estes and Kapikian, 2007; Gentsch et al., 2005). To describe the complete genome constellation of rotavirus strains a genotyping classification system based on the open reading frame of each gene has been adopted; Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx. To date, 27 G (VP7), 37 P (VP4), 17 I (VP6), 9 R (VP1), 9 C (VP2), 8 M (VP3), 18 A (NSP1), 10 N (NSP2), 12 T (NSP3), 15 E (NSP4), and 11 H (NSP5) genotypes have been described (Guo et al., 2012; Jere et al., 2013; Matthijnssens et al., 2011; Papp et al., 2012; Trojnar et al., 2013).

The two live-oral vaccines are currently available on the global market; Rotarix® (GlaxoSmithKline Vaccines, Belgium) and RotaTeq® (Merck and Co., USA), and included in the routine vaccination programs of 53 countries including the USA, Brazil, Belgium and Australia (Dennehy, 2012). RotaTeq® is a live-attenuated pentavalent vaccine that contains five genetically distinct human-bovine reassortant virus strains. Each reassortant strain contains a human gene encoding one of the outer capsid proteins (VP7 encoding G1, G2, G3 or G4 and VP4 encoding P[8]) within a bovine WC3 strain backbone (G6P[5]) (Heaton et al., 2005). RotaTeq® is administered in a three dose schedule at, two, four and six months of age. Rotarix® is a live-attenuated monovalent vaccine, possessing a genotype G1P[8] strain and is administered in a two dose schedule at two and four months of age (Vesikari et al., 2007).

In early 2006, Rotarix® and RotaTeq® became commercially available in Australia and were subsequently introduced into the Australian National Immunisation Program in July 2007. Each state and territory independently evaluated which vaccine to implement. Victoria, Queensland, Western Australia and South Australia use RotaTeq®; while New South Wales, the Northern Territory, Tasmania and the Australian Capital Territory use Rotarix® (Buttery et al., 2011). Rotarix® was introduced in the Northern Territory in October 2006 due to the high burden of disease in the region (Macartney et al., 2011).

Over the last two decades there have been several outbreaks caused by G2P[4] strains around Australia. Prior to vaccine introduction, outbreaks occurred in Perth in 1993, Melbourne in 1994, Sydney in 2001, and Tasmania in 2003–2004 (Bishop et al., 2001; Kirkwood et al., 2004; Masendycz et al., 2001). The Northern Territory represents a unique setting in Australia with regards to rotavirus disease. Rotavirus has been notifiable since 1994 and a high burden of disease has been experienced in the region. Bi-annual outbreaks occur, particularly affecting the town of Alice Springs and surrounding communities, which place a high demand on healthcare facilities. Prior to vaccine introduction in the Northern Territory, G2P[4] outbreaks occurred in 1993/1994 (reassortant between group I and II), 1999 and 2004 (Kirkwood et al., 2009; Palombo et al., 1996). In 2009, 28 months after Rotarix was introduced in the Northern Territory, a G2P[4] outbreak occurred in the region and vaccine effectiveness against rotavirus hospitalization was found to be only 19% during the outbreak (Snelling et al., 2011).

The aim of this study was to genetically characterize the G2P[4] strains isolated during the 1999, 2004 and 2009 rotavirus outbreaks in the Alice Springs region of the Northern Territory, Australia, to determine whether the reduced vaccine efficacy in 2009 was due to emergence of a unique G2P[4] strain. Whole genome characterization of the G2P[4] outbreak strains was performed to facilitate genetic comparison between these outbreak strains and coalescent analyses were also performed to elucidate the evolutionary relationship of these strains. Phylogenetic analysis was performed to compare the outbreak strains to global G2P[4] strains.

2. Materials and methods

2.1. Stool specimens

Rotavirus G2P[4] samples collected during outbreaks of gastroenteritis in the Alice Springs region of the Northern Territory in 1999, 2004 and 2009 were sent to the Australian Rotavirus Surveillance Program (ARSP), Melbourne, Victoria. All samples were frozen at −70 °C until analyzed. Patient information including date of birth, date of sample collection, and gender was routinely collected for samples collected during each outbreak. The length of hospitalization and immunization status with regard to the Rotarix® rotavirus vaccine was obtained where possible for samples collected during the 2009 outbreak. A total of 43 G2P[4] samples were collected from pediatric patients hospitalized with gastroenteritis during a rotavirus outbreak between February 21st and May 1st, 2009. Seventy-five G2P[4] samples were collected during an outbreak between January 3rd and February 28th, 2004. Seventy-four G2P[4] samples were collected during an outbreak between 16th March 16th and April 22nd, 1999.

An additional 17 G2P[4] samples collected during 2008 from the Northern Territory ($n = 8$), Western Australia ($n = 6$), New South Wales ($n = 2$) and Queensland ($n = 1$) were selected for comparison to the 2009 outbreak samples.

2.2. Nucleic acid extraction

Rotavirus dsRNA was extracted from 20% (w/v) fecal suspensions using a RNA extraction kit (QIAamp® Viral RNA mini kit (spin protocol), Qiagen, Inc., Hilden, Germany) in accordance with the manufacturer's instructions for use in RT-PCR.

2.3. Polyacrylamide gel electrophoresis

All samples with adequate volume were selected for analysis. The 11 segments of rotavirus dsRNA were separated on 10% (w/v) polyacrylamide gel with 3% (w/v) polyacrylamide stacking gel at 25 mA for 16 h. The genome migration patterns (electropherotypes) were visualized by silver staining according to the established protocol (Dyall-Smith and Holmes, 1984; Herring et al., 1982).

2.4. Amplification of rotavirus genes from G2P[4] strains

Each of the 11 rotavirus gene segments were individually reverse transcribed and amplified by PCR using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen, USA) as previously described (Cowley et al., 2013). The sense and antisense primers are listed in Supplementary data Table 1.

2.5. Nucleotide sequencing

PCR amplicons were electrophoresed in 1.2% (w/v) agarose gels containing ethidium bromide (10 mg/ml) and visualized under UV transilluminator. Excised amplicons were purified via gel extraction and spin column purification using the QIAquick Gel Extraction Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's protocol. Purified DNA together with oligonucleotide primers, were sent to the Australian Genome Research Facility, Melbourne for sequence analysis using an ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) in a Applied Biosystems 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA). Primer walking was employed to obtain the complete sequence of each gene.

2.6. Phylogenetic analysis

Electropherograms were visually analyzed and contiguous DNA sequences were created utilizing the Sequencher® Software program version 5.0.1 (Gene Codes Corp Inc., An Arbor, MI, USA) for each gene segment. Nucleotide similarity searches were performed using the BLAST (Basic Local Alignment Search Tool) server on the GenBank database at the National Center for Biotechnology Information, USA (www.ncbi.nlm.nih.gov). The nucleotide (nt) and amino acid (aa) sequences of each gene were compared with sequences available in the GenBank database that possessed the entire open reading frame (ORF). Multiple nucleotide and amino acid alignments were constructed using the MUSCLE algorithm in the MEGA5.20 program (Edgar, 2004; Tamura et al., 2011). The optimal evolutionary model was selected based upon the Akaike information criterion (corrected) (AICc) ranking implemented in jModelTest (Darriba et al., 2012; Guindon and Gascuel, 2003). Maximum likelihood phylogenetic trees were generated using the selected models of nucleotide substitution GTR + G_{G4} (VP1, VP2 and NSP2), GTR + G_{G4} + I (VP3, VP6 and NSP1), HKY + G_{G4} (NSP5), HKY + G_{G4} + I (VP4, VP7 and NSP4) and TrN + I (NSP3) using MEGA5.20 (Tamura et al., 2011). The robustness of branches was assessed by bootstrap analysis using 1000 pseudoreplicate runs and nodes with values >70% were considered to be strongly supported in the phylogenetic analysis. The strain RVA/Human-tc/USA/Wa/1974/G1P8 was used as an outgroup in all phylogenetic analysis. Nucleotide and amino acid distance matrixes were calculated using the *p*-distance algorithm in MEGA5.20 (Tamura et al., 2011).

2.7. Molecular dating analysis

Each gene dataset was comprised of strains analyzed in this study and additional Australian G2P[4] strains isolated between 1999 and 2011 available in GenBank (Accession numbers in Supplementary data Table 2). Prior to selection analysis each gene dataset was tested for evidence of intragenic recombination using the Recombination Detection Program version 3.44 (RPD3) using the recombination methods RDP, GENECONV, Bootscan, Chimaera, SiScan, MaxChi and 3Seq (Boni et al., 2007; Gibbs et al., 2000; Martin and Rybicki, 2000; Martin et al., 2010; Padidam et al., 1999; Posada and Crandall, 2001; Salminen et al., 1995; Smith, 1992). The time to Most Recent Common Ancestor (tMRCA) was calculated using the Bayesian Markov chain Monte Carlo (McMC) method in the BEAST package (v1.7.5) (Drummond et al., 2012). The data was analyzed utilizing an uncorrelated lognormal relaxed clock using the models TN93 + G_{G4} (VP7, VP4, NSP1, NSP2, NSP3, NSP4), TN93 + I (VP1, VP2, VP6 and NSP5) or HKY + G_{G4} (VP3) determined by AICc ranking in jModelTest (Darriba et al., 2012; Drummond et al., 2006). A coalescent Gaussian Markov random field (GMRF) Bayesian Skyride tree prior was used to describe the demographic history (Drummond et al., 2002; Minin et al., 2008). Three independent analyses were conducted with the McMC chain run for 100 million generations with sampling every 10,000 generations. Convergence was assessed using the program Tracer (v1.5.0) (http://tree.bio.ed.ac.uk/software/tracer/), with an effective sample size (ESS) of >200 after the first 10% of chain lengths were discarded as burn-in. The Maximum Clade Credibility (MCC) trees were obtained using TreeAnnotator (v1.7.5) removing the initial 10% of trees as burn-in. The time-ordered MCC trees were viewed in the FigTree (v1.4.0) (http://tree.bio.ed.ac.uk/software/figtree).

2.8. Assignment of genotypes

The genotypes of each of the 11 genome segments were determined using the online RotaC v2.0 rotavirus genotyping tool (http://rotac.regatools.be) in accordance with the recommendations of the Rotavirus Classification Working Group (Maes et al., 2009).

2.9. Accession numbers

The nucleotide sequences for genes described in this study have been deposited in GenBank under the accession numbers KC834635–KC834713.

3. Results

3.1. Sample characterization and patient demographics

During the 2009 G2P[4] rotavirus outbreak in Alice Springs, the average age of patients admitted to hospital was 5.5 months (1–63), 65.1% were male, and the average length of hospitalization was 5.1 days (2–9 days). Based on age, 34/43 patients were eligible to be vaccinated with at least the primary dose of Rotarix®, and vaccination status was known for 26 patients. Seven patients had received the primary dose, of these six patients were eligible for the second dose but had not received it. Ten children had received two doses of Rotarix®. The remaining 17 patients (2–28 months) had no vaccination history recorded and were assumed to have not been vaccinated. During the 2008–2009 surveillance period G2P[4] strains represented 76.4% of strains identified in Alice Springs and 50% of all strains Australia-wide; this was largely due to the large numbers of samples collected during the outbreak compared to the small number of samples in other regions of Australia. The age and gender was known for 66/80 patients from the 2004 outbreak, with an average age of 9.2 months (0–41) and 63% were male. In the Northern Territory in the 2003–2004 surveillance period G2P[4] strains accounted for 77.4% of strains identified in Alice Springs and 17.1% of strains Australia-wide. The age and gender was known for 50/74 and 71/74 patients respectively from the 1999 outbreak, the average age was 7 months (0–30) and 54% of patients were male. This outbreak occurred prior to routine surveillance and the proportion of G2P[4] strains in the region and nation-wide at the time are unknown.

3.2. VP7, VP8* and NSP4 sequence analysis of Australian G2P[4] strains

The electropherotype of 106 G2P[4] strains from the three outbreaks were determined (data not shown). For the 1999 outbreak 14/23 samples analyzed exhibited the same pattern. For the 2004 outbreak 60/73 samples analyzed had the same pattern. The minor electropherotypes identified in the 2004 and 1999 outbreaks were identical to the dominant patterns observed except for minor differences in the migration of gene segment 8. For the 2009 the outbreak 10 samples analyzed had an identical pattern.

Eight G2P[4] samples (from vaccinated and unvaccinated patients) with an identical electropherotype collected during the 2009 Alice Springs outbreak were selected for sequence analysis. The genes encoding VP7, VP8* and NSP4 were sequenced and genetic analysis revealed that all of the eight samples exhibited 100% nucleotide and amino acid similarity for each gene. Of the 17 G2P[4] strains identified circulating in Australia during 2008, two samples from Western Australia (PM178 and PM191) possessed an identical electropherotype to the 2009 outbreak strain and exhibited 99.6–100% nt and 100% aa similarity for the VP7, VP8* and NSP4 genes. Four strains sharing an identical electropherotype collected during the 2004 outbreak exhibited 99.6–100% nt and aa similarity for VP7, VP8* and NSP4 genes. Four strains sharing an identical electropherotype collected during the 1999

outbreak exhibited 99.1–100% nt and 98.3–100% aa similarity for the VP7, VP8* and NSP4 genes.

3.3. Sequence based whole genome classification of representative outbreak strains

A single representative strain from each outbreak was selected for whole genome sequence analysis; RVA/Human-wt/AUS/V233/1999/G2P[4], RVA/Human-wt/AUS/336190/2004/G2P[4] and RVA/Human-wt/AUS/V203/2009/G2P[4]. For simplicity, these strains will be subsequently referred to as V233-1999, 336190-2004 and V203-2009. The three strains possessed the archetypal DS-1-like genome constellation G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2.

3.4. Full genome comparison of outbreak strains

The 1999, 2004 and 2009 outbreaks were caused by distinct G2P[4] strains that shared varying degrees of genetic similarity to each other when the whole genomes were compared (Table 1). When each of the 11 genes were compared between the three strains, V203-2009 shared a higher degree of similarity to V233-1999 (98.8–99.7% nt similarity, with six genes sharing >99.0% nt similarity) than to 336190-2004 (96.2–98.8% nt similarity). The VP7 gene of V203-2009 was genetically divergent to both V233-1999 (96.3% nt and 97.9% aa similarity) and 336190-2004 (96.2% nt and 97.9% aa similarity), with numerous amino acid substitutions observed in hydrophobic regions 1 and 2, and antigenic region A (T87A), and in regions not associated with known biological functions (Fig. 1). The NSP4 gene of V203-2009 was genetically divergent to both V233-1999 (96.7% nt and 98.35 aa similarity) and 336190-2004 (96.4% nt and 97.5% aa similarity), with amino acid substitutions observed in hydrophobic domain 3, extracellular matrix (ECM) region, the overlapping ECM/VP4 binding/antigenic region II, the overlapping tubulin binding site/antigenic region 1 and the VP6 binding site. It is unknown if these changes had any effect on the pathogenicity of the strain.

Phylogenetic analysis of the 11 genome segments was conducted to investigate the genetic relationships of the three outbreak strains to each other and to global strains (Fig. 2A–K). The 11 genes of V203-2009 all clustered with Australian strains collected in Melbourne in 2000, 2009 and 2011, within sub-lineages comprised of global G2P[4] strains isolated between 2000 and 2011. All genes except VP7 and VP4 shared the closest phylogenetic relationship to Australian strains. The VP7 gene clustered closest to a 2008 German strain (RVA/Human-wt/DEU/GER177-08/2008/G2P[4]) within a lineage comprised of both contemporary strains and strains isolated in the 1980s and early 1990s (Fig. 2A). The VP4 gene clustered closest to a 2009 Belgium strain (RVA/Human-wt/BEL/BE1248/2009/G2P[4]) within a lineage of contemporary G2P[4] strains (Fig. 2B).

The outbreak strain 336190-2004 exhibited a diverse genetic makeup. The VP1, VP2, VP3, VP6, VP7, NSP2, NSP4 and NSP5 genes clustered within sub-lineages comprised of global strains of varying genotypes predominantly isolated between 1997 and 2011 (Fig. 2A, D–F and H–K). The VP4, NSP1 and NSP3 genes formed discrete nodes in the phylogenetic trees, highlighting their divergence to the majority of G2P[4] strains identified globally (Fig. 2B, G, I). Compared to V203-2009 and V233-1999, 336190-2004 shared a lower degree of genetic similarity to previously characterized Australian G2P[4] strains.

The 11 genes of V233-1999 all clustered closely with Australian strains isolated in Melbourne between 2000 and 2011. The VP3, VP6, NSP1, NSP3 and NSP5 genes clustered closest to Melbourne G2P[4] strains isolated in 2000, 2008 and 2011 with the remaining genes clustering closest to strains isolated in USA, Belgium, Korea, Bangladesh and India between 1996 and 2009 (Fig. 2A–K).

Table 1
Percentage nucleotide and amino acid comparisons and tMRCA analysis between the 1999, 2004 and 2009 Alice Springs G2P[4] outbreak strains.

Gene	V203-2009 and V233-1999				V203-2009 and 336190-2004				V233-1999 and 336190-2004			
	nt and aa similarity	Comparison of tMRCA			nt and aa similarity	Comparison of tMRCA			nt and aa similarity	Comparison of tMRCA		
		tMRCA	HPD Low	HPD High		tMRCA	HPD Low	HPD High		tMRCA	HPD Low	HPD High
VP7	96.3/97.9	1994	1990	1996	96.2/97.9	1996	1993	1999	98.9/98.8	1994	1990	1997
VP4	99.5/99.7	1997	1999	1995	98.1/99.1	1991	1996	1984	98.5/99.1	1991	1996	1984
VP1	98.9/99.4	1995	1998	1991	98.0/99.2	1991	1996	1985	98.4/99.5	1991	1996	1985
VP2	99.0/99.9	1994	1997	1990	97.9/99.7	1987	1993	1983	98.7/99.8	1987	1993	1983
VP3	99.5/98.8[a]	ND	ND	ND	97.3/96.1	1993	1997	1988	97.2/95.7[a]	ND	ND	ND
VP6	98.9/99.2	1997	1998	1993	96.8/99.0	1988	1994	1982	97.2/99.7	1988	1994	1982
NSP1	98.8/98.8	1995	1997	1991	97.5/97.3	1989	1994	1984	98.1/98.1	1989	1994	1984
NSP2	99.1/99.1	1993	1997	1989	98.1/98.1	1992	1997	1987	98.6/98.4	1992	1997	1987
NSP3	99.0/98.4	1994	1997	1989	98.3/98.1	1992	1996	1987	98.6/98.4	1992	1996	1987
NSP4	96.7/98.3	1995	1998	1992	96.4/97.1	1996	1999	1993	97.3/96.6	1995	1998	1992
NSP5	99.7/100	1998	1999	1996	98.8/100	1994	1997	1990	99.2/100	1994	1997	1990

ND: The VP3 gene of V233-1999 was not included in the tMRCA analysis due to incomplete sequencing of the gene.
[a] The complete VP3 gene sequence could not be achieved for V233-1999 and the genetic similarity calculations based on nt 1–1713.

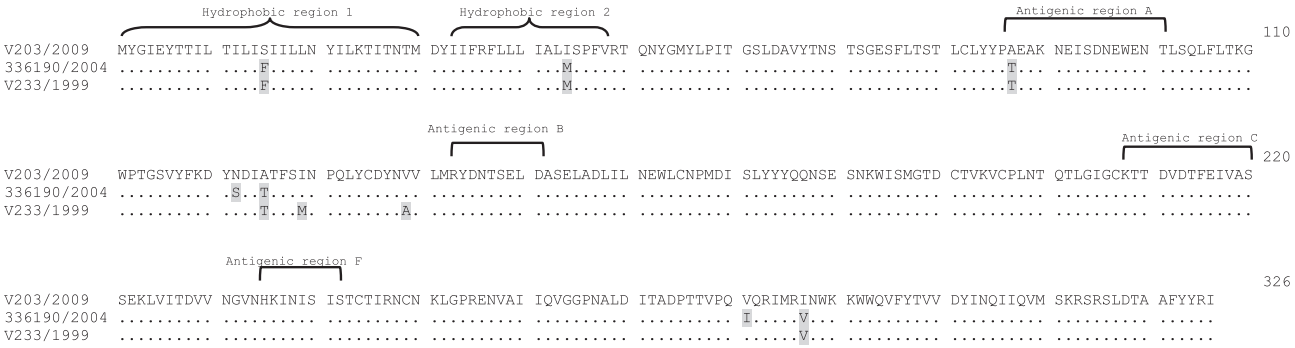


Fig. 1. Alignment of VP7 gene of the Alice Springs outbreak strains V203-2009, 336190-2004 and V233-1999. Amino acid differences between these strains are shaded. Residues comprising the antigenic regions are defined within in brackets.

3.5. Determination of time of most recent common ancestor

Bayesian coalescent analysis of the tMRCA was performed for V233-1999, 336190-2004 and V203-2009, with Australian G2P[4] strains circulating between 1999 and 2011 (Table 1). Analysis indicated that the individual genes of V233-1999 and V203-2009 shared ancestral strains circulating between 1993 and 1998.

Analysis indicated that the individual genes of 336190-2004 and V203-2009 shared ancestral strains circulating between 1987 and 1996, and the individual genes of V233-1999 and 336190-2004 shared an ancestral strain circulating between 1987 and 1995. The broader tMRCA ranges observed in the analysis between 336190-2004 and the other outbreak strains reflected a more diverse evolutionary history for this strain. The more varied tMRCA values for the 336190-2004 genome, in particular the VP2, VP6 and NSP1 genes were supported by the maximum likelihood phylogenetic analysis where 336190-2004 did not exhibit strong cluster groupings with the Australian strains, and fell in different topographies with each gene (Fig. 2E,C,G).

4. Discussion

In the Northern Territory, Australia, rotavirus outbreaks occur bi-annually, however G2P[4] associated outbreaks have sporadically occurred over the past decade, being identified in 1993/4, 1999, 2004 and 2009. In 2009, 28 months after the introduction of the Rotarix® vaccine into the Northern Territory, a large rotavirus outbreak occurred in the Alice Springs region resulting in the hospitalization of 43 children. Based on PAGE and sequence analysis a single G2P[4] rotavirus strain was circulating during the outbreak. PAGE and sequence analysis indicated the 2009 outbreak strain was circulating in Western Australia prior to spreading to the Northern Territory. The G2P[4] outbreak strain continued to spread across the Northern Territory region of Australia, where it was identified as the dominant genotype in Darwin in the months following the outbreak (unpublished observations). G2P[4] was the dominant genotype in Western Australia and South Australia in the 2009–2010 surveillance period and during the 2010–2011 surveillance period during which G2P[4] strains were identified as the most common genotype across Australia (Kirkwood et al., 2010, 2011). However, based on PAGE and sequence analysis (unpublished observations) this increase does not appear to be due to the wider circulation of the 2009 Alice Springs outbreak strain, suggesting a diverse population of G2P[4] strains circulated in Australia. However, genetically similar strains to the 2009 outbreak strain continued to circulate in population slowly evolving predominantly by genetic drift. This is supported by the high degree of genetic similarity between the 1999 and 2009 outbreak strains and G2P[4] strains circulating in Melbourne (Victoria) between 2000 and 2011. However, the 2004 outbreak strain only shared moderate genetic similarity to Melbourne strains suggesting the strain may not have circulated widely around the country.

The Bayesian analyses estimating tMRCA for the 11 genes revealed the V233-1999 and the V203-2009 G2P[4] outbreak strains had a close evolutionary history sharing an ancestor strain or group of strains circulating between 1993 and 1998. These two strains shared a high degree of genetic similarity (98.8–99.7% nt and 97.9–100% aa similarity) with the observed genetic differences observed between the two strains predominantly the result of 16 years of genetic drift. The exception is the VP7 gene of V203-2009 which was more divergent in the phylogenetic analysis, suggesting the V203-2009 strain was an intragenogroup reassortment. Recent global phylodynamic analysis has shown that the periodic resurgence of G2 strains are due to the emergence of new VP7 gene lineages (Dennis et al., 2014). The tMRCA analysis suggests the VP7 genes of V233-1999, 336190-2004 and V203-2009 shared a common ancestor circulating within a similar time range highlighting the diversity of strains circulating in the population during this period. Detailed Bayesian analysis of global G2 strains may further elucidate the origins of the 2009 outbreak strain VP7 gene. The VP7 gene of V203-2009 exhibited the amino acid substitution in the antigenic region A (T87A) which suggests this strain may be antigenically distinct from the majority of G2 strains circulating globally over the last decade (Doan et al., 2011). The results of the Bayesian analysis revealed the 1999 and 2009 outbreak strain did not give rise to the dominant G2P[4] lineages circulating in Australia between 2009 and 2011. However, these strains probably persisted in the population in low numbers until 2011. The wider range of values obtained for the tMRCA for the outbreak strain 336190-2004 and lack of clustering in the phylogenetic analysis between 336190 and 2004, Australian G2P[4] strains isolated between 1992 and 2011 and the 1999 and 2009 outbreak strains indicated the strain has likely undergone reassortment over time and some genes were likely recently introduced from a global pool of circulating rotavirus strains while other parental strains had Australian origins.

The level of immunity to G2P[4] rotavirus strains in the Northern Territory may have contributed to the occurrence of the three outbreaks. G2P[4] strains were circulating at very low levels in the years prior to each outbreak, suggesting that herd immunity to this genotype was low. Any prior exposure to G2P[4] strains may not have fully protected against the 2009 outbreak strain because it is an antigenic variant exhibiting amino acid changes in an antigenic region of the VP7 gene compared to contemporary strains. Exposure to non-G2P[4] strains may not have provided adequate

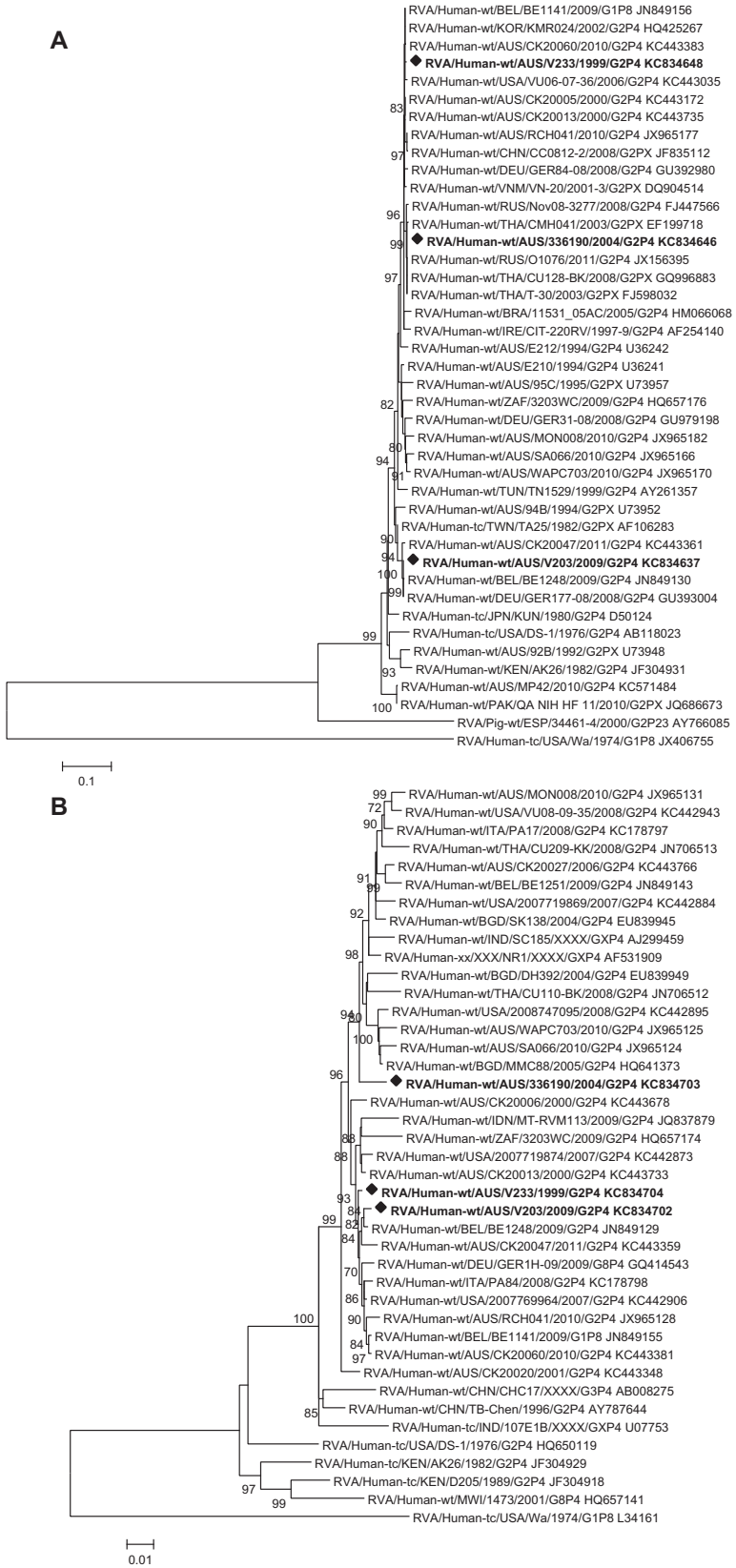


Fig. 2. Maximum likelihood phylogenetic tress constructed from the nucleotide sequences of VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5 genes of rotavirus strains V233-1999, 336190-2004 and V203-2009 with other group A rotavirus strains representing the G2, P[4], I2, R2, C2, M2, A2, N2, N2, T2, E2 and H2 genotypes respectively. The complete ORF of V233 could not be achieved and an alignment of nt 1–1713 of the ORF was used. In all trees the position of strains V233-1999, 336190-2004 and V203-2009 are indicated by an ♦ symbol. Bootstrap values ⩾70% are shown. Scale bar shows substitutions per nucleotide. The nomenclature of all the rotavirus strains indicates the rotavirus group, species isolated from, country of strain isolation, the common name, year of isolation, and the genotypes for genome segment 9 and 4 as proposed by the RCWG (Matthijnssens et al., 2011).

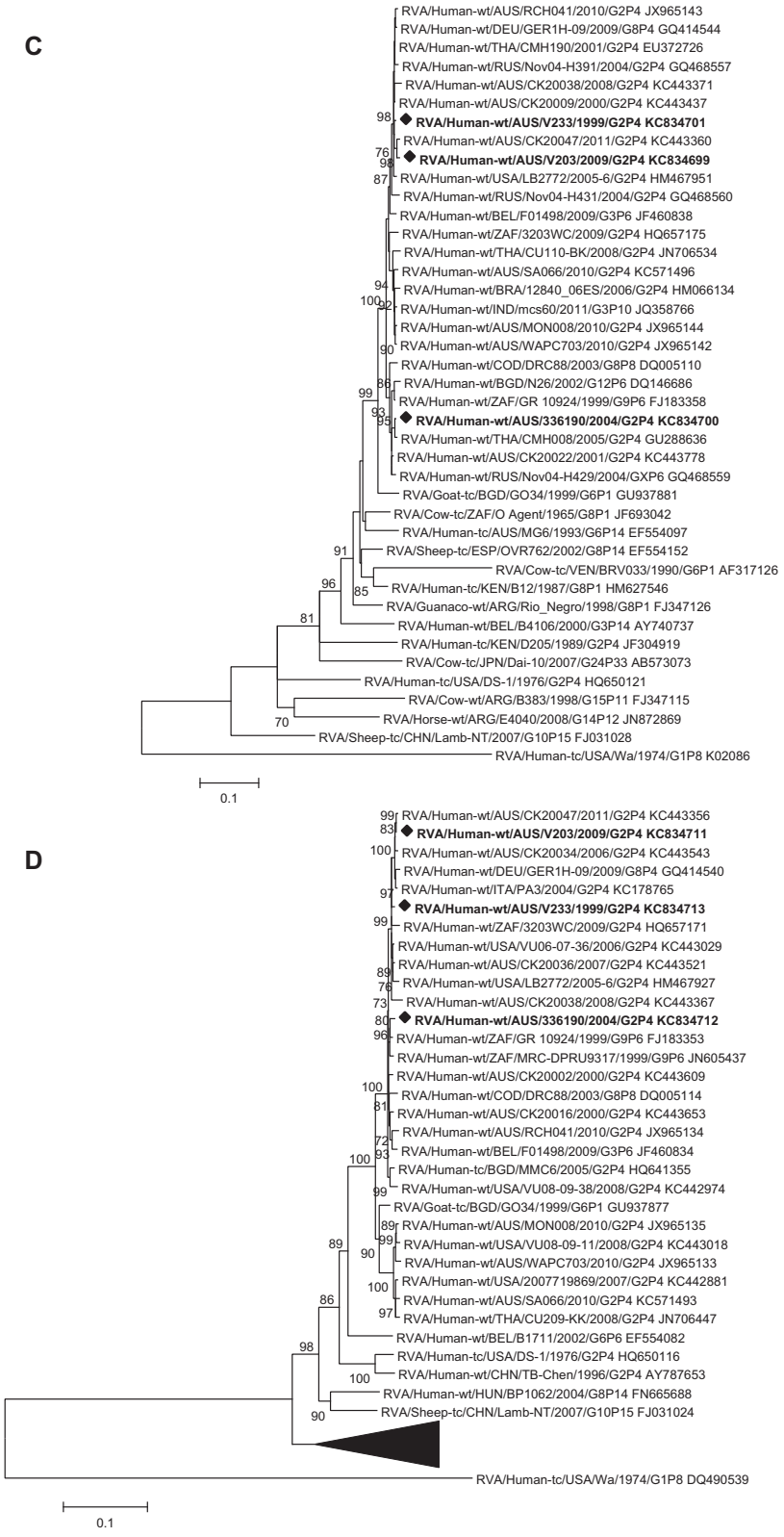


Fig. 2 (*continued*)

heterotypic protection. In addition, of the children hospitalized during the 2009 outbreak, 81.2% were eligible to be vaccinated however only 50% of these eligible children had received the full dose schedule. Thus, if these vaccination rates are indicative of the wider community then the level of vaccine induced herd protection would have been relatively low.

Both vaccinated and unvaccinated children were infected with the same strain during the 2009 outbreak. Vaccine induced

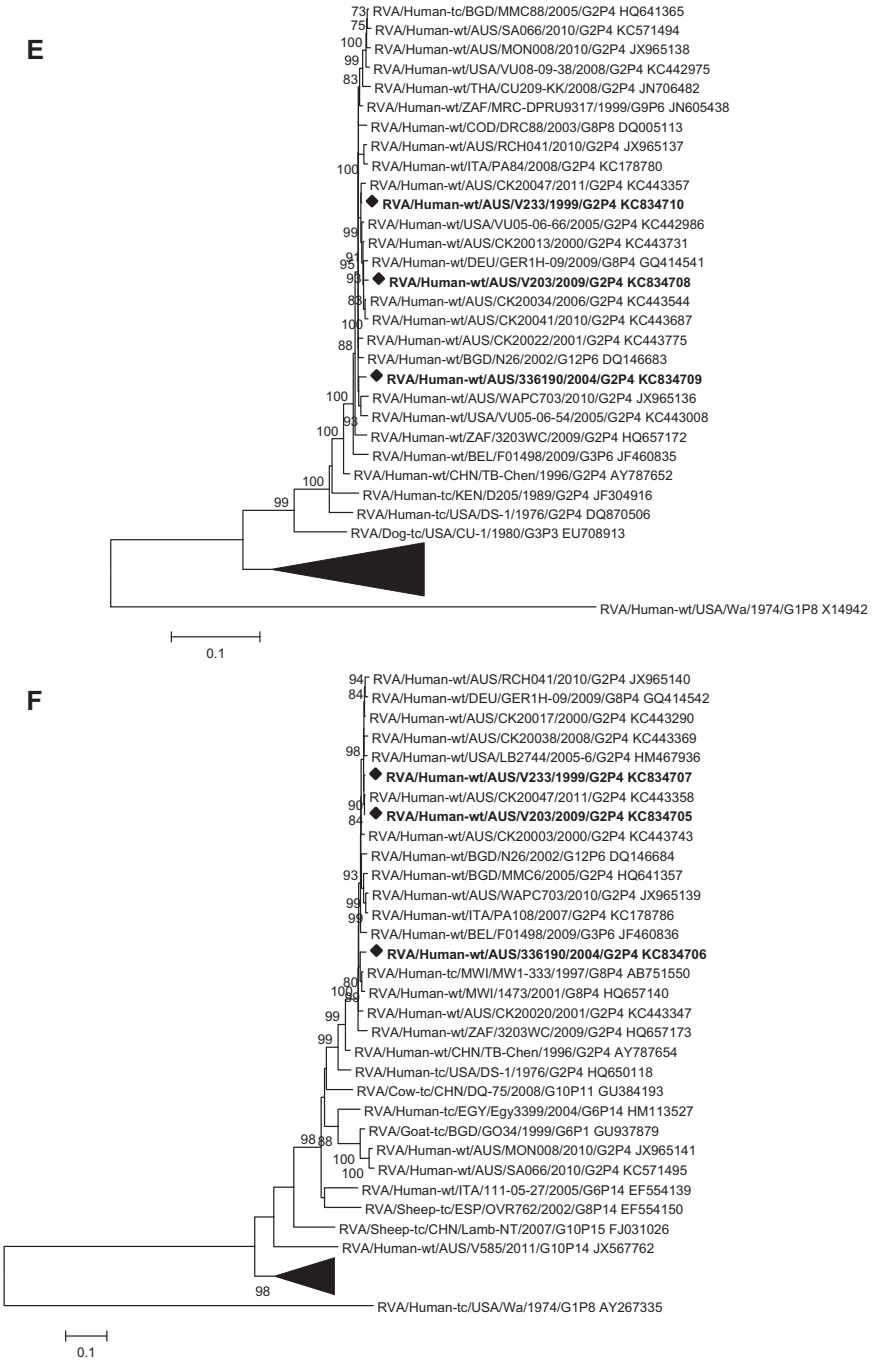
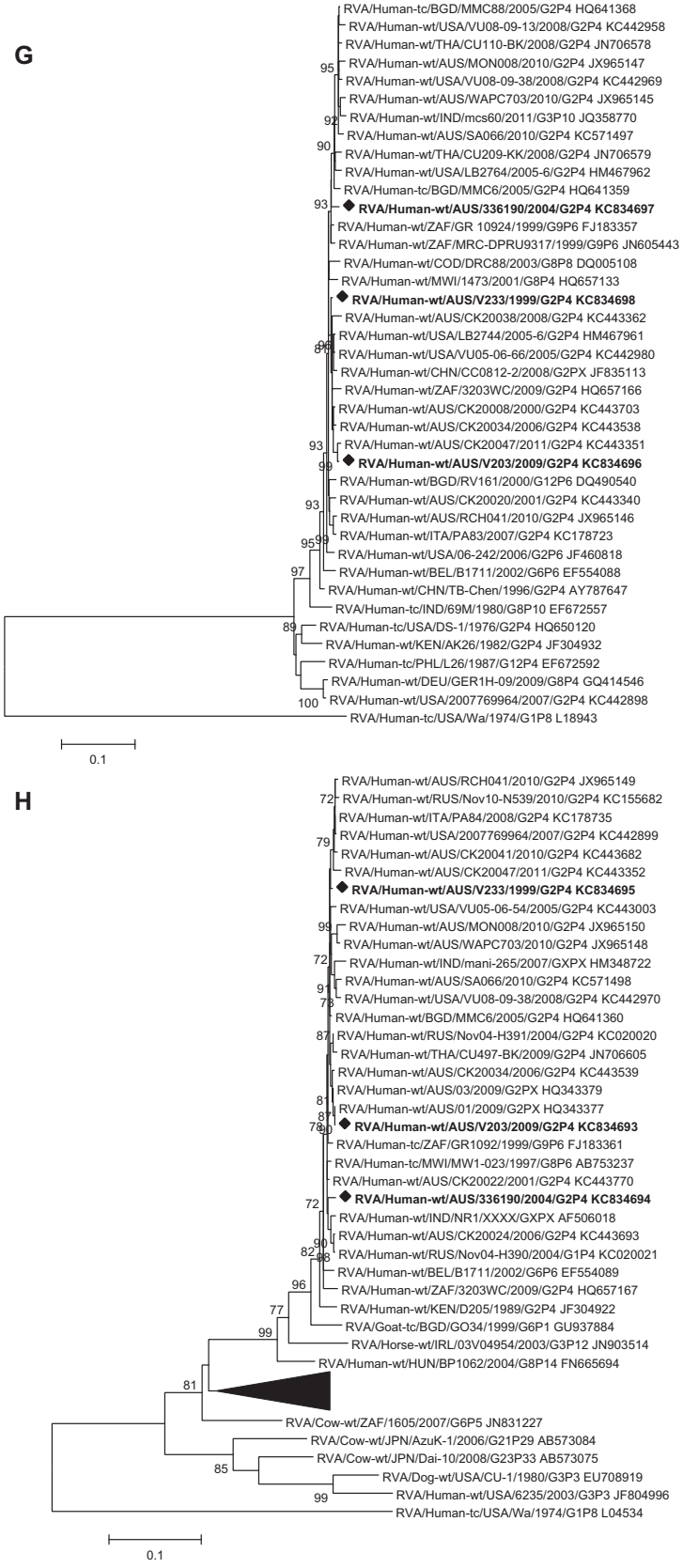


Fig. 2 (*continued*)

protection appeared to be less effective than predicted in this population. The vaccine effectiveness during the G2P[4] 2009 outbreak (in patients up to 36 months of age) against rotavirus hospitalization was 19% (95% CI, −105–68) for two doses of Rotarix® compared with none (Snelling et al., 2011). However, there was evidence that vaccination provided a protective effect against severe rotavirus disease complicated by acidosis in infants less than 12 months of age (Snelling et al., 2011). Previously, a study in Brazil found vaccine efficacy against G2P[4] strains for children aged 6–11 months was 77% (95% CI, 42–91%), however there was no evidence of protection for children older than 12 months of age (Correia et al., 2010). It is possible that the heterotypic immune response afforded by Rotarix® vaccination may wane over time in less developed settings, and this may contribute to a decrease in herd immunity in the population.

The overall efficacy of Rotarix® has been lower in developing country settings compared to developed country settings often correlating with a lower rate of anti-rotavirus seroconversion and IgA titer following vaccination (Patel et al., 2013). Similarly, lower homotypic and heterotypic immunity has also been observed following natural rotavirus infections in settings with a high disease burden (Gladstone et al., 2011). Several factors have been implicated in lower natural immunity and VE in developing county settings, including host characteristics such as poor nutritional status, underlying environmental enteropathy, and high maternal anti-rotavirus antibodies which might neutralize



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vaccine viruses. In the Northern Territory, admissions coded for enteric infections occur at a rate 10-fold higher in Indigenous infants than among non-Indigenous infants and the living conditions of these infants are typically crowded, with inadequate facilities for sanitation and food preparation (Li et al., 2006). It is possible that co-morbidities present in the Northern Territory

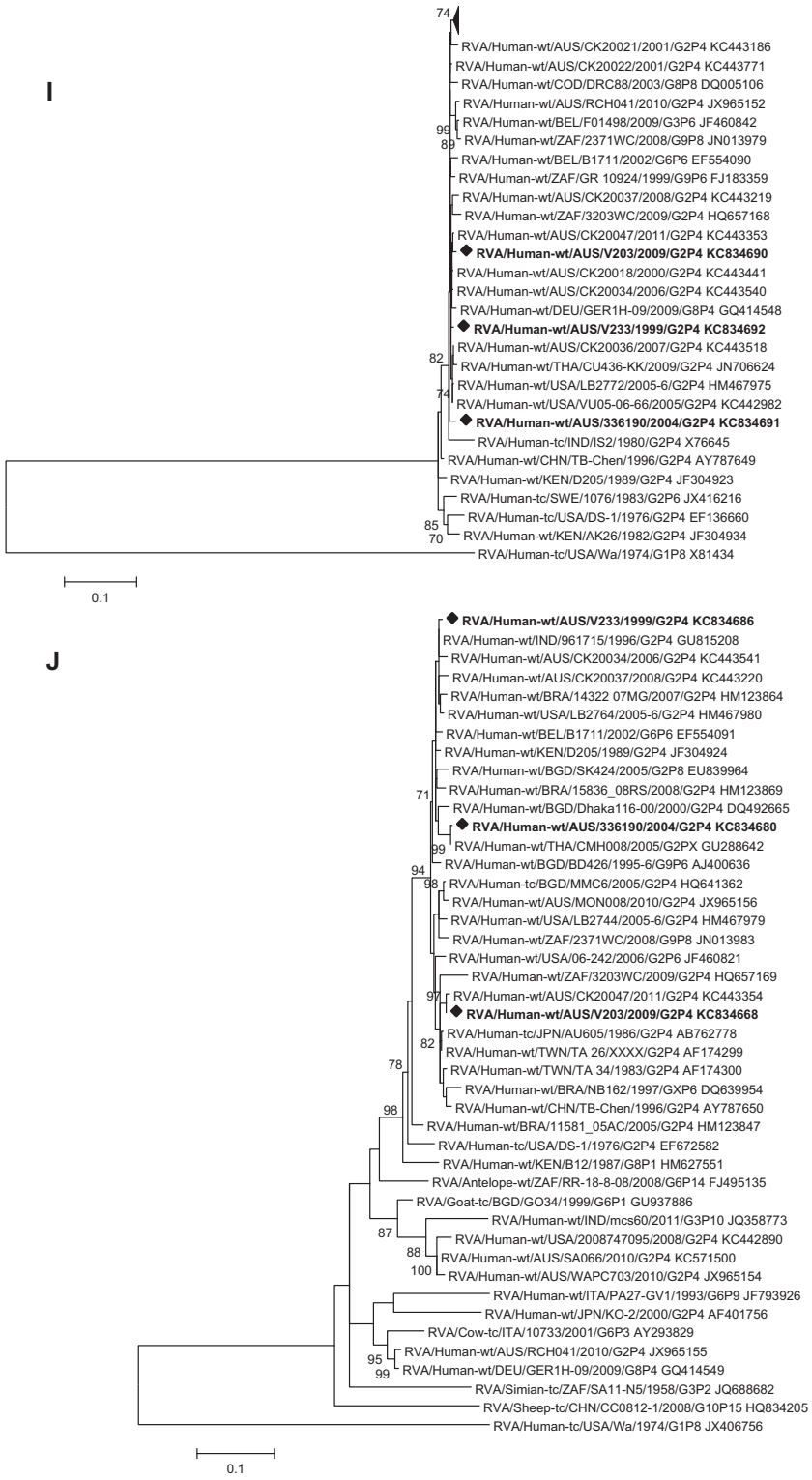

Fig. 2 (*continued*)

Indigenous population contribute to reduced protective responses following vaccination.

In conclusion, a large G2P[4] rotavirus outbreak occurred 28 months after the introduction of the Rotarix® vaccine in the Alice Springs region of the Northern Territory. Full genome analysis suggested that the 2009 G2P[4] outbreak strain was an intra-genogroup reassortant. The 2009 outbreak strain was not directly derived from the 2004 G2P[4] outbreak strain, but shared an evolutionary history with the 1999 G2P[4] outbreak strain. Levels of immunity to G2P[4] strains generated by both wild-type infection and vaccination was likely to be low in this population, providing an opportunity for this genotype to cause disease. The global increase in the circulation of G2P[4] strains has been observed in both vaccinated and vaccine naive populations suggesting a

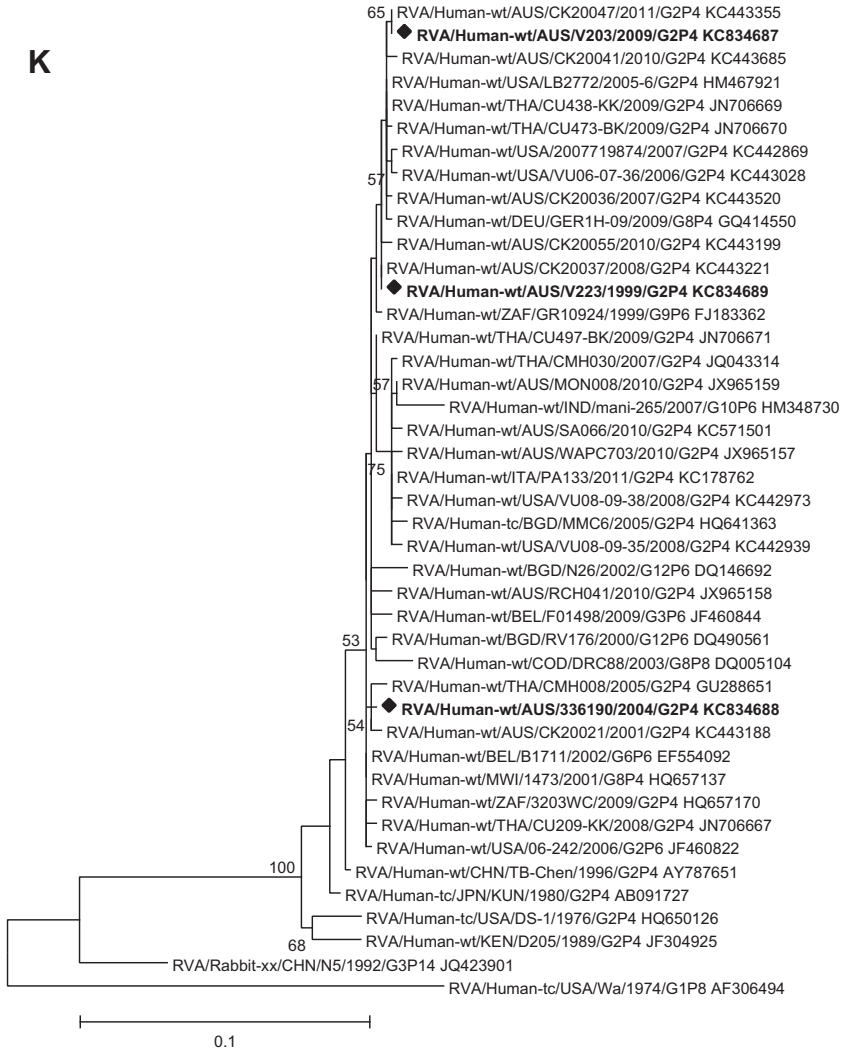

Fig. 2 (*continued*)

range of factors are associated with the emergence of this genotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.08.009.

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