Characterization of a G1P[8] rotavirus causing an outbreak of gastroenteritis in the Northern Territory, Australia, in the vaccine era

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In 2010, a large outbreak of rotavirus gastroenteritis occurred in the Alice Springs region of the Northern Territory, Australia. The outbreak occurred 43 months after the introduction of the G1P[8] rotavirus vaccine Rotarix®. Forty-three infants were hospitalized during the outbreak and analysis of fecal samples from each infant revealed a G1P[8] rotavirus strain. The outbreak strain was adapted to cell culture and neutralization assays were performed using VP7 and VP4 neutralizing monoclonal antibodies. The outbreak strain exhibited a distinct neutralization pattern compared to the Rotarix® vaccine strain. Whole genome sequencing of the 2010 outbreak virus strain demonstrated numerous amino acid differences compared to the Rotarix® vaccine strain in the characterized neutralization epitopes of the VP7 and VP4 proteins. Phylogenetic analysis of the outbreak strain revealed a close genetic relationship to global strains, in particular RVA/Human-wt/BEL/BE0098/2009/G1P[8] and RVA/Human-wt/BEL/BE00038/2008/G1P[8] for numerous genes. The 2010 outbreak strain was likely introduced from a globally circulating population of strains rather than evolving from an endemic Australian strain. The outbreak strain possessed antigenic differences in the VP7 and VP4 proteins compared to the Rotarix® vaccine strain. The outbreak was associated with moderate vaccine coverage and possibly low vaccine take in the population.

Keywords: Australia; diarrheal outbreak; G1P[8]; full-genome analysis; rotavirus, Rotarix®

INTRODUCTION

Rotavirus is the predominant cause of acute gastroenteritis in young children worldwide.1 A high burden of rotavirus disease is present in both developing and developed countries resulting in an estimated 453,000 annual deaths, principally in Asia and sub-Saharan Africa.1,2 Rotavirus belongs to the Reoviridae virus family and is a non-enveloped, icosahedral virus and the 11 segment double-stranded RNA (dsRNA) genome encodes six structural viral proteins (VP1–4, VP6, VP7) and six non-structural proteins (NSP1–5/6).3 Rotavirus strains can be classified into eight groups (groups A–H) based on the genetic characteristics of the inner capsid protein (VP6); group A strains are the most common cause of rotavirus disease in humans.5

Rotavirus strains can be further classified based on the two outer capsid proteins into G (glycoprotein, VP7) and P (protease-sensitive, VP4) genotypes respectively; these proteins also elicit type-specific and cross-reactive neutralizing antibody responses.7 A genotyping classification system based on the open reading frame of each gene has been adopted; Gx–P[x]–Ix–Rx–Cx–Ax–Nx–Tx–Ex–Hx.5 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.5–9

Two live-oral rotavirus vaccines; Rotarix® (GlaxoSmithKline Vaccines, Rixensart, Belgium) and RotaTeq® (Merck and Co. Inc., Whitehouse Station, NJ, USA) have been demonstrated to be efficacious in large clinical trials and are included in vaccination programs of numerous countries worldwide.10–12 Rotarix® and RotaTeq® have been highly efficacious in decreasing the burden of rotavirus gastroenteritis in several countries worldwide including Brazil, Belgium, the United States, Nicaragua, Austria and Mexico.13–17 Rotarix® is a live-attenuated monovalent vaccine comprised of a single G1P[8] strain and is administered in a two-dose schedule at 2 and 4 months of age.18 RotaTeq® is a live-attenuated, pentavalent, human-bovine reassortant vaccine administered in a three-dose schedule at 2, 4 and 6 months of age.19

Rotavirus vaccines were introduced into the Australian National Immunization Program in July 2007. Rotarix® was introduced in the Northern Territory in October 2006 due to the high burden of rotavirus disease experienced in the region, particularly in Indigenous infants.20 Vaccine introduction has decreased the burden of rotavirus disease in several locations around Australia.20 The sole exception is Central Australia a large region of the Northern Territory encompassing Alice Springs and surrounding communities with a high Indigenous Australian population.21 In Central Australia there has been no clear decline in rotavirus notification rates despite the introduction of routine infant vaccination with Rotarix®.21,22 Outbreaks of rotavirus gastroenteritis have continued to occur in Central Australia in the vaccine era, particularly affecting the town of Alice Springs and

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surrounding communities which place a high demand on health-care facilities.21

During May and June 2010, a large G1P[8] rotavirus outbreak occurred in Alice Springs that resulted in a considerable number of children hospitalized with severe gastroenteritis. The aim of this study was to perform a genetic and antigenic characterization of the G1P[8] rotavirus strain responsible for the outbreak. A comparison of the outbreak strain to other Australian and international G1P[8] strains was performed to establish its context within the global rotavirus population.

MATERIALS AND METHODS

Stool specimens

A total of 43 fecal samples were collected from infants (≤38 months of age) presenting to hospital with severe gastroenteritis during a rotavirus outbreak in the Alice Springs region of the Northern Territory between 10 May and 15 June 2010. Patient information including date of birth, date of sample collection and gender was routinely collected. The length of hospitalization, immunization status with regard to the Rotarix® rotavirus vaccine was obtained where possible. Fecal samples were frozen, stored at −70°C and forwarded to the Australian Rotavirus Reference Centre in Melbourne, Victoria. G1P[8] samples collected in 2010 were analyzed from Darwin (n=20), Gove (n=12), Katherine (n=26), Tennant Creek (n=16) and remote regions of the Northern Territory and Western Australia (n=28). Additional G1P[8] samples circulating in neighbouring states South Australia (n=7), Queensland (n=2) and Western Australia (n=5) in 2010 were also analyzed.

Nucleic acid extraction

Rotavirus dsRNA was extracted from clarified 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.

Polyacrylamide gel electrophoresis

The 11 segments of 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. The 11 segments of dsRNA were separated on 10% (w/v) polyacrylamide gel and 3% (w/v) stacking gel. The genome migration patterns (electropherotypes) were visualized.

Viruses and adaptation of strains to MA104 culture

The 2010 G1P[8] outbreak strain was adapted to culture in MA104 cells. Filtered 20% (w/v) fecal extracts were activated with 10 µg/mL porcine trypsin (Sigma, St Louis, MO, USA) at 37°C for 30 min and inoculated onto 2.5×10⁶ MA104 cells in DMEM supplemented with 1 µg/mL porcine trypsin (Sigma). Cultures were incubated at 37°C with 5% CO₂ and maintained in suspension culture using a rotary mixer. At 24 h post inoculation, an additional 1×10⁶ MA104 cells were added to the suspension culture. At 96 h post inoculation, the virus was released by three cycles of freeze–thaw at −80°C and clarified by centrifugation at 4000g for 10 min. The viruses underwent serial passage in suspension for six passages and were adapted to stationary phase by three passages in MA104 cells. The electropherotypes of the adapted strains were compared to the pattern derived from the original stool sample. The VP7 and VP4 genes of the adapted virus was sequenced as previously described to ensure conservation of protein sequence following adaptation.25 Virus titer was monitored during passage using indirect immunofluorescence in MA104 cells.26 The standard human rotavirus strains RV4 (G1P[8]), F45 (G9P[8]) and D (G1P[8]), as well as Rotarix®, were propagated in MA104 cells in the presence of trypsin.

Neutralization of strains with monoclonal antibody and polyclonal antisera

Adapted 2010 G1P[8] outbreak virus and control viruses were studied in a fluorescent focus reduction neutralization assay with neutralizing monoclonal antibodies (N-MAbs) and polyclonal sera, as described previously.26 Four rotavirus N-MAbs RV4:1, RV4:2, RV4:3 and RV4:5, reactive with the VP7 antigen of human G1P[8] rotavirus RV4, were used.27 The VP4-specific N-MAb F45:4 reactive to P[8] antigen of F45 was also used.28,29 Rabbit hyperimmune antisera raised to RV4 was also used.27 The criteria for resistance to neutralization was defined as a reduction in neutralization titer of at least 1 log, when compared to the homologous virus titer.

Amplification and nucleotide sequencing of the rotavirus genome segments

The extracted dsRNAs of five representative G1P[8] 2010 outbreak samples were sent to the J Craig Venter Institute (Rockville, MD, USA) for high-throughput reverse transcription-polymerase chain reaction and Sanger sequencing as previously described.26 Briefly, reverse transcription-polymerase chain reaction primers were designed at 600 bp intervals along the sense and antisense RNA strand of each gene to ensure high coverage by reverse transcription-polymerase chain reaction.

Phylogenetic analysis

Nucleotide similarity searches were conducted using the Basic Local Alignment Search Tool server on the GenBank database at the National Center for Biotechnology Information, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). The nucleotide (nt) and deduced amino acid (aa) sequences of each gene were compared with sequences available in the GenBank database that possessed the entire open reading frame and multiple alignments constructed using the MUSCLE algorithm in the MEGA5.20 program.31,32 The optimal evolutionary model was selected for each gene based upon the Akaike information criterion (corrected) ranking implemented in jModelTest.33,34 Maximum likelihood phylogenetic trees using the models of nucleotide substitution GTR+GG4 (VP1, VP2, VP3, NSP1 and NSP3), GTR+GG4 (VP4 and VP6), TrN+GG4 (VP7, NSP2 and NSP4) and HKY+GG4 (NSP5) were generated using MEGA5.20.31 The robustness of branches was assessed by bootstrap analysis with 1000 pseudoreplicate runs. Nucleotide and amino acid distance matrices were calculated using the P-distance algorithm in MEGA5.20.31 Structural analysis of the VP7 protein (PDB ID: 3FMG) was performed using PyMOL.35

Assignment of genotypes

The genotypes of each of the 11 genome segments of the 2010 G1P[8] outbreak strains were determined using the online rotavirus genotyping tool RotaC v2.0 (http://rotac.regatools.be).36

Accession numbers

The nucleotide sequences for genes described in this study have been deposited in GenBank under the accession numbers RVA/Human-wt/AUS/CK000096/2010/G1P[8] (JX027934–JX027944), RVA/Human-wt/AUS/CK000097/2010/G1P[8] (JX027945–JX027955), RVA/Human-wt/AUS/CK000099/2010/G1P[8] (JX027956–JX027966) and RVA/Human-wt/AUS/CK00100/2010/G1P[8] (JX027967–JX027977). For simplicity,
the strains will be referred to by their common name CK00096, CK00097, CK00098, CK00099 and CK00100.

RESULTS
Sample characterization
GIP[8] (n=43) samples were collected from patients hospitalized with severe gastroenteritis during the 2010 Alice Springs rotavirus outbreak. The average age of infants was 5.9 months (1–38 months), and 53.3% of patients were male. Based on age, 42/43 infants were eligible to be vaccinated with at least the primary dose of Rotarix®. A single patient was too young to be vaccinated. Vaccination status was available for 29 patients, seven patients had received the first dose of Rotarix® and ten patients had received both doses. Five patients did not receive the second dose despite being eligible. Seven patients had not been vaccinated despite being of an eligible age. The remaining 13 patients had no vaccination history recorded and were assumed to not have been vaccinated.

Polyacrylamide gel electrophoresis
All Alice Springs samples analyzed during the outbreak had an identical electropherotype and strains possessing the outbreak electropherotype circulated in the region until mid-August, 2010. Strains analyzed from other regions of the Northern Territory (excluding Gove) and remote Western Australia exclusively possessed the outbreak electrophotype detected between May and July 2010. The outbreak electropherotype was also observed in a single South Australian, a single Queensland and three Western Australian samples circulating in June/July 2010.

Neutralization of 2010 GIP[8] outbreak samples
Fluorescent focus reduction neutralization assay was used to identify potential antigenic differences between a culture adapted isolate (V474) from the 2010 GIP[8] outbreak, Rotarix® and control viruses. A panel of VP7 N-MAbs and polyclonal sera derived against RV4 (GIP[8]) and N-MAb derived to VP4 of F45 (GIP[8]) were used to determine neutralization profiles (Table 1). The 2010 GIP[8] outbreak virus was neutralized with the N-MAbs RV4:1, RV4:2, F45:4; however, it was resistant to neutralization by the VP7 MAb RV4:3 and RV4:5, and rabbit anti-RV4 sera. In contrast, Rotarix® was neutralized by the VP7 N-MAbs RV4:1, RV4:2, RV4:3, RV4:5 and the rabbit RV4 polyclonal sera. In contrast, Rotarix® was neutralized by the VP4 N-MAb F45:4. The distinct neutralization patterns of the 2010 GIP[8] outbreak virus and Rotarix® demonstrate differences in the antigenic profile of these viruses, suggesting alterations in VP7 and VP4 proteins.

Comparison of the Alice Springs 2010 GIP[8] outbreak strain to Rotarix® vaccine VP7 and VP4 genes
The VP7 gene of the 2010 outbreak samples possessed 94.2% nt and 95.7% aa identity to the VP7 gene of Rotarix®. The amino acid differences between the outbreak strains and Rotarix® were analyzed and mapped to the VP7 trimer (Figure 1), identifying several changes in regions of biological function (Figure 2). Several changes in the VP7 protein were located within antigenic regions, T91N and N94S in antigenic region A and M217T in antigenic region C. An additional change was identified at position K291R previously identified in neutralization escape mutants. The N94S change identified in the outbreak virus correlated with the loss of neutralization by N-MAb RV4:3 and RV4 polyclonal sera. Similarly, the outbreak virus was resistant to neutralization with RV4:5 when compared to Rotarix®. The N-MAb RV4:5 requires the sequence asparagine-lysine at position 147–148 in antigenic region B for neutralization, plus additional unidentified amino acids. Resistance to RV4:5 by strain D correlates with the N147S change in antigenic region B (Figure 2). The outbreak strain is resistant to RV4:5, despite a conserved amino acid sequence in antigenic region B when compared to RV4 and Rotarix®. This suggests that additional surface exposed amino acids, potentially the S123N change, are responsible neutralization resistance.

The VP4 genes of the 2010 outbreak samples possessed 90.2%–90.3% nt and 94.5%–94.6% aa identity to the Rotarix® vaccine strain. The VP4 protein undoes proteolytic cleavage by trypsin into two subunits: VP8* (aa 1–247) and VP5* (aa 248–776) which enhances viral infectivity. The Y385D change that differentiated Rotarix® and the outbreak virus was identified within the hydrophobic apex of VP5*, a conformationally dependent antigenic region. The change at position 385 correlates with neutralization resistance of Rotarix® with N-MAb F45:4, which selects neutralization escape mutants at position 392 within this antigenic region of VP5*.

Whole genome analysis of the Alice Springs 2010 GIP[8] outbreak samples
Five representative strains collected during the outbreak were selected for whole genome sequence analysis and possessed the archetypal Wales-like genome constellation G1-P[8]–I1–R1–C1–M1–A1–N1–T1–E1–H1. A high-quality sequence read could not be achieved for CK00098 and the genome was not included in the phylogenetic analysis. CK00096, CK00097, CK00099 and CK00100 shared 100% nt and aa identity for all genes except, NSP1 (99.9% nt and 99.8% aa), NSP2 and VP4 (99.9% nt and 99.7% aa).

Phylogenetic analysis of the Alice Springs 2010 GIP[8] outbreak samples
Phylogenetic analysis of each of the 11 gene segments was conducted to investigate the genetic relationship of the outbreak strain to
Australian and global strains. In each tree, the 2010 G1P[8] outbreak strains clustered within large, diverse clades that were comprised of contemporary global isolates (Supplementary Figures S1, S2 and S3). The VP7 gene of the outbreak strain shared the highest genetic identity (99.9% nt and 99.7% aa) to numerous strains circulating in Asia between 2004 and 2010 and RVA/Human-wt/BEL/BE00098/2009/G1P[8]. The outbreak strains shared the highest genetic similarity to RVA/Human-wt/BEL/BE00098/2009/G1P[8] for the VP4 (99.7% nt and aa), VP2 (99.9% nt and aa), VP6 (99.7% nt and 100% aa), NSP1 (99.7% nt and 99.8% aa) and NSP3 (99.9% nt and 100% aa) genes.

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The outbreak strain shared the highest genetic similarity to RVA/Human-wt/BEL/BE00038/2008/G1P[8] for VP1 (99.8% nt and 99.9% aa) and VP3 (99.8% nt and 99.8% aa). The NSP2 gene shared 99.7% nt and aa similarity to RVA/Human-wt/BEL/BE00098/2009/G1P[8], RVA/Human-wt/USA/VU-06-07-21/2006/G3P[8] and RVA/Human-wt/USA/2007719674/2007/G1P[8], while the NSP4 gene shared 99.6% nt and 100% aa similarity to RVA/Human-wt/USA/VU-08-09-24/2008/G3P[8], RVA/Human-wt/USA/2007719674/2007/G1P[8] and RVA/Human-wt/RUS/Nov08-3281/2008/G3P[8]. The NSP5 gene shared 99.8% nt and 99.5% aa similarity to RVA/Human-wt/THA/CU875-BK/2010/G1P[8], RVA/Human-wt/USA/2009726997/2009/G3P[8] and RVA/Human-wt/USA/VU08-09-7/2008/G3P[8].

**DISCUSSION**

The inclusion of rotavirus vaccines onto the Australian National Immunization Program has decreased rotavirus associated hospitalizations, emergency room visits and episodes of gastroenteritis in several regions. Australian and global strains. In each tree, the 2010 G1P[8] outbreak strains clustered within large, diverse clades that were comprised of contemporary global isolates (Supplementary Figures S1, S2 and S3).

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The outbreak strain shared the highest genetic similarity to RVA/Human-wt/BEL/BE00038/2008/G1P[8] for VP1 (99.8% nt and 99.9% aa) and VP3 (99.8% nt and 99.8% aa). The NSP2 gene shared 99.7% nt and aa similarity to RVA/Human-wt/BEL/BE00098/2009/G1P[8], RVA/Human-wt/USA/VU-06-07-21/2006/G3P[8] and RVA/Human-wt/USA/2007719674/2007/G1P[8], while the NSP4 gene shared 99.6% nt and 100% aa similarity to RVA/Human-wt/USA/VU-08-09-24/2008/G3P[8], RVA/Human-wt/USA/2007719674/2007/G1P[8] and RVA/Human-wt/RUS/Nov08-3281/2008/G3P[8]. The NSP5 gene shared 99.8% nt and 99.5% aa similarity to RVA/Human-wt/THA/CU875-BK/2010/G1P[8], RVA/Human-wt/USA/2009726997/2009/G3P[8] and RVA/Human-wt/USA/VU08-09-7/2008/G3P[8].

**Figure 2** Alignment of VP7 gene of the prototype G1P[8] strains RV4, D, the 2010 G1P[8] Alice Springs outbreak strain and Rotarix® vaccine strain. Amino acid differences between these strains are shaded. Residues comprising the antigenic regions are defined within brackets. The sites shown to escape neutralization with monoclonal antibodies include RV4:1 (sites 147 and 148, filled circle), RV4:2 (sites 213, filled square), RV4:3 (site 94, filled triangle) and RV4:5 (sites 147, 148 and other undefined sites, filled right pointing triangle).
In conclusion, we characterized a G1P[8] rotavirus outbreak in Central Australia in the vaccine era. The G1P[8] outbreak strain exhibited high genetic relatedness to contemporary global strains and exhibited antigenic differences to the VP4 and VP7 proteins of Rotarix®. The outbreak was more likely the result of one or more factors including suboptimal vaccine uptake, low primary immune responses or waning immunity in this population rather than the circulation of a strain associated with increased pathogenicity or vaccine escape. This study emphasizes the need for continued surveillance of rotavirus strains to help guide current and future vaccination strategies.

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Characterization of a G1P[8] rotavirus causing an outbreak of gastroenteritis in the Northern Territory, Australia, in the vaccine era

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