Emergence of a novel equine-like G3P[8] inter-genogroup reassortant rotavirus strain associated with gastroenteritis in Australian children

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During 2013, a novel equine-like G3P[8] rotavirus emerged as the dominant strain in Australian children with severe rotavirus gastroenteritis. Full genome analysis demonstrated that the strain was an inter-genogroup reassortant, containing an equine-like G3 VP7, a P[8] VP4 and a genogroup 2 backbone I2-R2-C2-M2-A2-N2-T2-E2-H2. The genome constellation of the equine-like G3P[8] was distinct to Australian and global G3P[8] strains. Phylogenetic analysis demonstrated a genetic relationship to multiple gene segments of Japanese strains RVA/JPN/S13-30/2013/G3P[4] and RVA/Human-wt/JPN/HC12016/2012/G1P[8]. The Australian equine-like G3P[8] strain displayed a distinct VP7 antigenic profile when compared with the previously circulating Australian G3P[8] strains. Identification of similar genes in strains from several geographical regions suggested the equine-like G3P[8] strain was derived by multiple reassortment events between globally co-circulating strains from both human and animal sources. This study reinforces the dynamic nature of rotavirus strains and illustrates the potential for novel human/animal reassortant strains to emerge within the human population.

Received 30 September 2015
Accepted 19 November 2015

INTRODUCTION

Rotavirus infection causes an estimated 114 million episodes of diarrhoea annually, resulting in 24 million clinic visits and 2.4 million hospitalizations (Glass et al., 2006). The majority of an estimated 453 000 annual deaths occur in developing countries of Asia and sub-Saharan Africa (Tate et al., 2012).

The rotavirus genome comprises 11 segments of dsRNA, encoding six structural viral proteins (VP1-4, VP6 and VP7) and six non-structural proteins (NSP1-5/6) (Estes & Kapikian, 2007). A binary genotype classification system based on the two outer capsid proteins VP7 and VP4 is used in molecular epidemiology (Estes & Kapikian, 2007). In addition, whole-genome classification is used to assign genotypes to each gene. The nomenclature Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Hx represents the genotypes of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4/NSP5/6, respectively. Currently there are 27 G, 37 P, 17 I, nine R, nine C, eight M, 18 A, 10 N, 12 T, 15 E and 11 H types (Guo et al., 2012; Papp et al., 2012; Trojnar et al., 2013; Jere et al., 2014). There are two major genotype constellations of human rotaviruses, also termed genogroup 1 (G1-P[8]-I1-R1-C1-M1-A1-N1-T1-H1) and genogroup 2 (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2) (Matthijnssens et al., 2008).

The genotype constellations of genogroup 1 and 2 human rotavirus strains have origins in distinct animal species, highlighting evolutionary links between rotavirus in humans and animals (Matthijnssens et al., 2008). The segmented rotavirus genome facilitates reassortment between strains, allowing both intra- and inter-genogroup reassortment (Estes & Kapikian, 2007). Zoonotic transmission increases the genetic diversity within circulating rotavirus strains causing human infection. Strains bearing mixed genome constellations derived from both human and animal viruses are detected more commonly than strains possessing a genome only derived from animal viruses, suggesting the presence of specific human proteins may provide better adaptation to the human host (Martella et al., 2010; Matthijnssens et al., 2011).
Rotavirus vaccines are likely to alter the immune pressure on the circulating WT strains. A more dynamic and diverse WT strain population has been observed in the post-vaccine era (Kirkwood et al., 2011, 2014). However, rotavirus vaccines continue to be highly effective across the globe (Tate & Parashar, 2014). The Australian Rotavirus Surveillance Program (ARSP) monitors the diversity and distribution of rotavirus genotypes in children hospitalized with severe gastroenteritis (Kirkwood et al., 2011, 2014). This study describes the genetic and antigenic characterization of a novel human/equine reassortant G3P[8] rotavirus strain that emerged in 2013 associated with acute gastroenteritis in children in multiple locations across Australia (Kirkwood et al., 2014).

RESULTS

Emergence of equine-like G3P[8] rotavirus strain in Australia

In 2013, a total of 518 rotavirus-positive faecal samples from children aged <5 years were referred to the ARSP for genotype analysis. We identified 193 samples which were GntP[8] (G-non-typable) using the standard genotyping protocol (Kirkwood et al., 2014). Sequence analysis of VP7 genes from representative GntP[8] samples demonstrated highest nucleotide identity to human/equine reassortant virus strains RVA/JPN/S13-30/2013/G3P[4] (99.7%), RVA/JPN/S13-45/2013/G3P[4] (99.6%) and the equine strain RVA/Horse-wt/IND/Env105/2004-05/G3P[X] (90.9%). Specific equine-like G3 VP7 primers were designed [EQG3FWD 5'-CTGACATACGCTAATTTCTAACAAGG-3' (nt 242–266) and EQG3REV 5'-GATCG-TACAGTGCTAAGG-3' (nt 786–763)] which amplified a 544 bp fragment of the equine-like G3 VP7 gene (Kirkwood et al., 2014). All 193 rotavirus-positive samples were genotyped as G3P[8] using these primers. PAGE demonstrated all equine-like G3P[8] strains with a visible electropherotype (n=112) had an identical pattern (data not shown).

The frequency and distribution of G3P[8] strains detected in children between January 2009 and December 2014 (n=463) was determined. G3P[8] strains were differentiated as either equine-like or WT G3P[8], based on their ability to be genotyped using the routine G3 genotyping primer (5’-ACGAACTCAACACGAGAGG-3’) or the specific equine-like G3 primer set. WT G3P[8] strains were detected sporadically between April 2009 and January 2013, with peaks in August–September 2009, April 2011 and September 2011 (Fig. 1). The equine-like G3P[8] strain was first identified in April 2013 and circulated until December 2014. During 2013, the equine-like G3P[8] was the dominant strain across Australia, detected in 37.6% of rotavirus-positive faecal samples. The equine-like G3P[8] was the major strain in Northern Territory (94.7%) and Western Australia (50.7%), and a minor strain in New South Wales (21.0%), Queensland (14.6%), Victoria (8.2%) and South Australia (1.5%) (Kirkwood et al., 2014). The equine-like G3P[8] continued to circulate in all states during 2014, representing the third most common strain, detected in 14.4% of rotavirus-positive faecal samples collected across Australia.

Antigenic reactivity of equine-like and WT G3P[8] strains to VP7 neutralizing mAbs


Comparison of the VP7 gene of equine-like G3P[8] strain with WT G3P[8]

The amino acid sequences of the VP7 genes from equine-like G3P[8] strains were compared with WT G3P[8] strains (Fig. 2). Multiple amino acid differences were observed between the equine-like and WT G3P[8] VP7 genes, including several in antigenic regions C (T212A and N213T) and F (N238D and N242A). The N238D substitution in region F is associated with the loss of a potential glycosylation site. This correlated with the binding of mAb F45:2 to the equine-like G3P[8] strains, which binds to G3 and G9 strains lacking this suspected glycosylation site (Kirkwood et al., 1993). The neutralizing mAb RV3:1 selects escape mutants with amino acid changes in antigenic regions A and C (Dyall-Smith et al., 1986). Binding differences of RV3:1 between the equine-like and WT G3P[8] correlate with sequence changes in antigenic region C. The neutralizing mAb RV3:4 binds to antigenic region B (Lazdins et al., 1995); this region is identical in both equine-like and WT G3P[8] strains, which correlated with the neutralizing mAb reactivity observed in all samples from both virus groups.

Whole-genome analysis of representative equine-like G3P[8] strains

Whole-genome analysis was performed on one equine-like G3P[8] strain from the Northern Territory (RVA/Human-wt/AUS/D388/2013/G3P[8]) and two from Western Australia (RVA/Human-wt/AUS/WAPC1740/2013/G3P[8] and RVA/Human-wt/AUS/WAPC2016/2014/G3P[8]). These strains demonstrated high nucleotide identity for all gene segments

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The VP7 genes of D388, WAPC1740 and WAPC2016 clustered in a lineage divergent to the majority of human G3 strains (Fig. 3a). These strains clustered with RVA/Human-wt/JPN/S13-30/2013/G3P[4], RVA/Human-wt/JPN/S13-45/2013/G3P[4] and the equine strain RVA/Horse-wt/IND/Erv105/2004-05/G3P[X]. These strains were within a lineage

(99.6–100%), with the genome constellation G3-P[8]-I2-R2-C2-M2-A2-N2-T2-E2-H2.

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that contained two additional discrete sublineages that were supported by strong bootstrap values – one composed of equine strains, and one composed of canine, feline, human and lapine strains. The VP4 genes of D388, WAPC1740 and WAPC2016 clustered with RVA/Human-wt/JPN/HC12016/2012/G1P[8] (Fig. 3b), within a sublineage predominantly comprising strains from India and Thailand.

The VP1, VP2, VP6, NS1, NS2, NS3 and NS5 genes of D388, WAPC1740 and WAPC2016 clustered with a similar group of strains, including RVA/Human-wt/JPN/HC12016/2012/G1P[8], RVA/Human-wt/JPN/S13-30/2013/G3P[4], RVA/Human-wt/JPN/S13-45/2013/G3P[4], RVA/Human-wt/JPN/NEU/ERN5523/2012/G3P[4] (not for NS2), RVA/Human-wt/AUS/C2004/2011/G2P[4] and RVA/Human-wt/AUS/C2005/2010/G2P[4] (not for NS3) (Fig. S1A, B, D, E–G, I, available in the online Supplementary Material). The VP6 genes of D388, WAPC1740 and WAPC2016 also clustered with RVA/Human-wt/JPN/OH3385/2012/G1P[8], RVA/Human-wt/JPN/OH3493/2012/G1P[8], RVA/Human-wt/JPN/OH3625/2012/G1P[8] and RVA/Human-wt/JPN/OH3506/2012/G1P[8].


The concatenated genomes of D388, WAPC1740 and WAPC2016 were compared with G3 reassortant strains identified in Japan and Hungary, and Australian G2P[4] strains (Fig. 4). Four distinct patterns were observed when the concatenated genomes were compared. With the exception of VP4 gene, the genome of equine-like Japanese G3P[4] strains RVA/Human-wt/JPN/S13-30/2013/G3P[4] and RVA/Human-wt/JPN/S13-45/2013/G3P[4] exhibited highest overall genetic similarity to D388, WAPC1740 and WAPC2016. With the exception of VP7 and NSP4 genes, the Japanese G1P[8] strains RVA/Human-wt/JPN/HC12016/2012/G1P[8] and RVA/Human-wt/JPN/KN039/2012/G1P[8] shared a highly conserved genome with D388, WAPC1740 and WAPC2016. Similarly, with the exception of three genes (VP4, VP7

**DISCUSSION**

G3 rotavirus strains have a broad host range, including humans, cats, cows, dogs, horses, pigs, rabbits, sheep, monkeys and bats, in association with numerous P genotypes (Matthijnssens et al., 2011). The VP7 gene of the equine-like G3P[8] strain was divergent to the majority of human G3 strains and shared highest genetic similarity to the VP7 gene of G3P[4] strains from Japan (RVA/Human-wt/JPN/S13-30/2013/G3P[4] and RVA/Human-wt/JPN/S13-45/2013/G3P[4]). These strains are suggested to be human/equine reassortants due to genetic similarity of the VP7 gene to the equine G3 strain (RVA/Horse-wt/IND/Erv105/2004-05/G3P[X]) (Malasao et al., 2015). The majority of equine rotavirus strains are genotypes G3P[12] and G14P[12] (Papp et al., 2013). However, VP7 genes of equine G3 strains exhibited moderate similarity to the Australian equine-like G3P[8]. This demonstrated that the VP7 gene of the Australian equine-like G3P[8] strain is genetically distinct from currently circulating equine G3P[12] strains.

The genome constellation of the Australian equine-like G3P[8] strain was distinct compared to global strains. However, several genes demonstrate high genetic similarity with reassortant strains causing outbreaks of gastroenteritis in Japan. The strains with the highest genetic identity were...

The NS4 genes of the Australian equine-like G3P[8] strains do not share a close relationship with the Australian G2P[4] strains that demonstrated genetic similarity in other gene segments. The NS4 gene may have been derived from an additional reassortment event, potentially a bovine-like strain given that the gene clusters closely with the human zoonotic strain RVA/Human-wt/AUS/RCH272/2012/G3P[14] and other bovine and bovine-like human reassortant strains. Thus the VP7, VP4 and NS4 genetic relationships within the genogroup 2 backbone suggest that the equine-like G3P[8] strain likely evolved by multiple reassortment events, possibly including an interspecies transmission as well as inter-genogroup reassortment within the global rotavirus strain population.

The rotavirus vaccines RotaTeq and Rotarix were introduced into the Australian National Immunization Program in July 2007. In 2013, 83.6% of Australian children were immunized with a rotavirus vaccine (Hull et al., 2013). High vaccine coverage in Australia is associated with a significant reduction in disease (Buttery et al., 2011). The mechanisms of protection following natural infection and vaccination remain unclear, but partially involve neutralizing antibody responses to both outer capsid proteins (Estes & Kapikian, 2007; Desselberger & Huppertz, 2011). The equine-like G3P[8] VP7 protein is antigenically distinct when compared with WT G3P[8] strains previously circulating in Australia, and amino acid differences in known antigenic regions were also identified between the equine-like G3P[8] VP7 and the G3 component of RotaTeq (W178-8) (data not shown). However, it remains unclear whether the unique antigenic profile of the equine-like VP7 was implicated in the emergence of the equine-like G3P[8] strain.

To conclude, we characterized a novel equine-like G3P[8] strain circulating in Australia, responsible for a large number of gastroenteritis cases during 2013 and 2014. The identification of equine-like rotavirus genes in the human population emphasizes the close evolutionary links between animal and human rotavirus, and highlights the potential for emergence of novel rotavirus strains. Continued rotavirus strain surveillance is vital to understand the diversity of the WT strains circulating within the human population in order to ensure we maintain an effective vaccine programme.

**METHODS**

**Samples.** Frozen faecal specimens from gastroenteritis cases were forwarded to the ARSP in Melbourne, Australia. In 2013, 518 rotavirus-positive samples from children aged <5 years were referred to the ARSP (Kirkwood et al., 2014). In 2014, 480 rotavirus-positive faecal samples were referred. Faecal suspensions (20%, w/v) were prepared and RNA extracted using a QiAamp Viral RNA Mini kit (Qiagen) (Cowley et al., 2013). Samples were genotyped using a hemi-nested reverse transcription (RT)-PCR with G-type and P-type primers (detailed in the supplementary material) (Kirkwood et al., 2014).

**Amplification of complete rotavirus genomes.** The 11 gene segments were reverse transcribed and amplified by PCR using the PrimeScript High Fidelity RT-PCR kit (TaKaRa) as described previously (Cowley et al., 2013). Primers used in the amplification of the 11 gene segments are detailed in the supplementary material.

**Nucleotide sequencing.** PCR amplicons were purified using a Wizard SV Gel for PCR Clean-Up System (Promega) according to the manufacturer’s protocol. Purified cDNA was sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Reaction kit (Applied Biosystems) in an Applied Biosystems 3730xl DNA analyser (Applied Biosystems). Primer walking was employed to cover the complete nucleotide sequence of each gene (detailed in the supplementary material).

**Phylogenetic analysis.** Contiguous DNA sequence files were constructed utilizing Sequencher software (version 5.0.1; Gene Codes). Nucleotide similarity searches were performed using the BLAST server on the GenBank database. The nucleotide and deduced amino acid sequences of each gene were compared with sequences available in GenBank possessing the entire ORF. Multiple nucleotide and amino acid alignments were constructed using the MUSCLE algorithm in MEGA 6.0 (Tamura et al., 2013). Nucleotide and amino acid distance matrices were calculated using the p-distance algorithm in MEGA 6.0 (Tamura et al., 2013). The optimal evolutionary model was selected based upon the Akaike information criterion (corrected) (AICc) ranking implemented in jModelTest (Darriba et al., 2012). Maximum-likelihood phylogenetic trees using the selected models of nucleotide substitution GTR+G (VP2 and NSP2), GTR+G+I (VP1, VP3 and NSP1), GTR+G+I (VP4 and NSP1), TrN+I (NSP3), TrN+G+I (VP6), TrN+G+I (VP7 and NSP4), HKY+I (NSP3) and HKY+G+I (NSP5) were reconstructed using MEGA 6.0 (Guindon & Gascuel, 2003; Tamura et al., 2013). The robustness of branches was assessed by bootstrap analysis using 1000 pseudo-replicate runs. mVISTA software was used to visualize the comparative sequence similarities of concatenated whole genome of genetically related strains (Mayor et al., 2000).

**Enzyme immunoassay.** Selected G3P[8] faecal samples were tested in a binding enzyme immunoassay using a panel VP7-specific neutralizing mAbs RV3:1, RV3:4 and F45:2 as described previously (Coulson et al., 1987). A_{50} was measured using a Titertek Multiscan MCC/340MKII microplate reader. A positive mAb reactivity was defined as A_{50} > 0.2, at least double the pre-immune mouse sera A_{50}.

**Assignment of genotypes.** The genotypes of each of the 11 rotavirus genome segments were determined using the online RotaC rotavirus genotyping tool (version 2.0; http://rotac.regatools.be) (Maes et al., 2009).
ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Health and Medical Research Council of Australia (1031473). This research was supported by the Victorian Government’s Operational Infrastructure Support Program. C. D. K. is Director of Australian Rotavirus Surveillance Program, which is supported by research grants from vaccine manufacturers CSL and GSK, as well as the Australian Commonwealth Department of Health. C. D. K. is a co-developer of an investigational rotavirus vaccine.

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