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Word counts

Abstract: 82

Text: 1607

Running head: Host-range shift of P[8] rotaviruses

Short Abstract: P[8]-3 and P[4] rotavirus strains attach to saliva glycans of Secretor individuals, preferentially infecting the same individuals. By contrast, co-circulating and recently emerged P[8]-4 strains attach to saliva regardless of the secretor status and infect preferentially nonsecretor children.

Footnotes

Conflict of interest statement: None of the authors has a commercial or other association that might pose a conflict of interest.

This work was supported by a joint grant from the Agence Nationale de La Recherche and the Direction Générale de l'Offre de Soin (DGOS), France: GASTROVIM 15-0007-01to JLP and BMI, respectively. AK was supported by a PhD studentship and travel grant from the Tunisian government.

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Abstract

In Tunisia, we observed that rotavirus P[8]-3 and P[4] in young children with gastroenteritis associate with the secretor histo-blood-group phenotype. Inversely, the emerging P[8]-4, representing 10% of cases, was exclusively found among nonsecretor patients. Unlike VP8* from P[8]-3 and P[4] strains, the P[8]-4 VP8* protein attached to glycans from saliva samples regardless of the donors Secretor status. Interestingly, a high frequency of FUT2 enzyme deficiency (nonsecretor phenotype) was observed in the population. This may allow co-circulation of P[8]-3 and P[8]-4 strains in secretor and nonsecretor children, respectively.

Keywords: rotavirus, histo-blood group antigen, secretor/nonsecretor phenotypes, fucosyltransferase, FUT2, genetic susceptibility, host-range

Background

Rotavirus remains the leading cause of severe diarrheal disease in children under 5 globally despite a remarkable decrease in the yearly number of associated children deaths since vaccine introduction [1]. Unlike in high-income countries, vaccine efficacy appears limited in many low-income areas, reasons for this likely being multifactorial although still ill-defined [2]. At the initiation of infection, human rotavirus strains bind to glycans of the histo-blood group antigens type (HBGAs) through the VP4 capsid spike protein that define strains P types [3]. HBGAs are expressed in the gut mucosa, mainly as O-glycans from soluble or cell surface bound mucins and as membrane glycolipids. P[8] strains that account for 74% of the global prevalence of VP4 [4] attach to fucosylated glycans dependent on the FUT2 fucosyltransferase for synthesis. As a result, children of the said nonsecretor phenotype who lack a functional *FUT2* allele appear to be much more resistant to the disease than the so-called secretor children who possess at least one functional allele, thereby expressing α1,2-linked fucose residues in their small intestinal mucosa [5]. Although the strong protective effect of the nonsecretor phenotype was observed in various countries from Europe, North America and Africa, it could not be detected in either Tunisia or Bangladesh [6, 7]. Here we conducted a new study in Tunisia to understand those between-studies or geographical discrepancies.

Methods

Patients and control samples:

Fecal, serum and saliva samples were collected between November 2015 and October 2018 from 189 young children in Tunisia. Only 42 saliva samples were obtained and used to control for the match with the genotype performed on serum samples. The specimens were collected from infants and young children between 20 days and 4.5 years of age presenting with acute gastroenteritis to the hospital Fattouma Bourguiba (Monastir). Saliva samples from a control population composed of 103 adults coming from different regions were also collected in order to obtain HBGA phenotypes and genotypes frequencies in Tunisia. The study was approved by the Ethics Committee of the Fattouma Bourguiba University Hospital in Monastir.

Virus detection and P type genotyping:

Nucleic acids were extracted from approximately 100 mg stool samples carried by an automatic extractor type IpreP, using the manufacturer's iPrep™ PureLink™ Virus Kit (Invitrogen). Rotavirus was diagnosed by real-time multiplex PCR FTD Viral gastroenteritis (Fast-track diagnostics) allowing the detection of enteric viruses. An 850 bp fragment of VP8* sequences was obtained from a subset of 63 cases using consensus primers [8]. Sequences accession numbers are given in the Supplementary data. These sequences allowed classification of circulating strains at the P-type level. When Ct values were <18-20 a one-step RT-PCR was performed using the Takara's one step PrimeScript RT-qPCR with 5µL nucleic acids extract and ROTA CON3 M13F / ROTA CON2 M13R primers. When Ct values were >18-20, a first RT-PCR was performed using ROTA CON3F / ROTA CON2R and between 5-10 µL nucleic acids extract, followed by a nested PCR with ROTA VP4 M13F / ROTA VP4 M13R primers and the Takara's Premix Ex Taq kit. In cases no amplification was obtained, a first RT-PCR

was performed with ROTA VP4F / ROTA VP4R primers followed by the nested PCR as above. Primers sequences are given in the Supplementary data.

Determination of the Secretor status and saliva OSGE treatment:

Patients serum samples were used to extract genomic DNA for *FUT2* genotyping as previously described and the secretor/nonsecretor phenotype was deduced from the genotype [9]. Saliva samples obtained from a control Tunisian population composed of 103 adults were used for HBGA phenotyping and genotyping as previously described [10] in order to obtain HBGA phenotypes frequencies. Genotyping was used for validation of the phenotypes.

To ascertain attachment to O-glycans, salivary mucins were diluted 1 in 250 in PBS and incubated or not with 12 µl of O-Sialoglycoprotein Endopeptidase (1,2 mg/ml; CEDARLANE, Burlington, Canada) in a water bath at 37°C overnight. After a 1 in 4 dilution in coating buffer pH 9,5 (Pierce, Thermo Fisher Scientific), 100 µl of the treated mucins were dispatched in each well of a Maxisorp 96-well plate (Nunc) which was then incubated overnight at 4°C. The plate was washed 3 times with PBS-0,05% Tween20, blocked with PBS-5% BSA for several hours at 37°C and ELISA was performed with VP8* proteins as previoulsy described [11].

116 **Results**

117 All rotavirus cases (n=115) were infected by P[8] or P[4] strains. Rotavirus was the single
118 viral agent detected in 45 (39%) cases, whilst co-infection with either astrovirus, adenovirus,
119 norovirus and/or sapovirus was found in the remaining 70 (61%) cases. Construction of a
120 phylogenetic tree from the 63 available sequences revealed that 48 sequences (76%) were of
121 the P[8]-3 subtype, 9 (14%) were of the P[4] genotype and 6 (10%) were of the P[8]-4
122 subtype (Fig. 1S). The latter strains, initially termed OP-354-like, correspond to an antigenic
123 variant also called P[8]b contrasting with P[8]a strains [12, 13].

124 The Secretor phenotype (or FUT2 genotype) could be unambiguously obtained for 114 RVA+
125 patients. In comparison with the adult control group where the proportion of nonsecretors was
126 high (36% as compared with <20% among people of European origin), a lower frequency of
127 nonsecretors was found among rotavirus-infected patients (20% vs 36%, $p<0.01$). Analysis of
128 the patients group without co-infection showed no significant effect of the FUT2 status, likely
129 owing to the small number of cases. Splitting cases between those infected with P[4], P[8]-3
130 versus those infected by P[8]-4 revealed a clear-cut demarcation since all P[8]-4 were found
131 among nonsecretors, whilst all P[4] were found among secretors and P[8]-3 were also
132 preferentially found among secretors (Table 1).

133 Previous studies documented binding of the VP8* domain from P[8] and P[4] strains to
134 α1,2fucosylated glycans [3]. Our group showed attachment of the VP8* from recent P[8]-3
135 clinical as well as the P[8]-1 and P[8]-2 vaccine strains to salivary mucins from secretor
136 individuals only [11]. In order to relate the above described epidemiological profile to HBGA
137 binding, VP8*-GST fusion proteins from a P[4] (TU35) and a P[8]-4 strain (TU7) were
138 produced as previously described in order to assay by ELISA their binding to saliva samples
139 from previously HBGA-phenotyped individuals [11]. As shown on Fig. 1a, binding of the

P[4] VP8* was limited to saliva from secretor donors. By contrast, P[8]-4 VP8* attached to saliva regardless of the Secretor character (Fig. 1b). To ascertain that binding occurred to O-linked glycans, a saliva sample was pretreated by the enzyme O-sialoglycoprotein endopeptidase (OSGE) that cleaves O-sialoglycoproteins but not unglycosylated or N-glycosylated proteins. We observed that binding to saliva of both the P[4] and the P[8]-4 VP8* was strongly diminished following OSGE treatment, indicating similar O-glycan dependence in the saliva binding assay (Fig. 1c).

Discussion

Here we confirm that commonly circulating P[8]-3 strains cause gastroenteritis in children of the secretor phenotype preferentially, albeit not exclusively since a fraction of nonsecretors also appeared among patients. We also showed that P[4] strains infected secretors only. At variance, the emergent P[8]-4 strain was found among nonsecretors but not among secretor children. As the number of patients with P[4] and P[8]-4 was limited, it can be stated that P[8]-4 preferentially infects nonsecretors, whilst other P[8] strains, as well as P[4] preferentially infect secretors. This confirms that, by mediating virus attachment, HBGAs are important host factors determining susceptibility to rotavirus gastroenteritis, but that depending on the circulating strains distinct subgroups in the population may be at higher risk.

The VP8* fragment of the P[8]-4 VP4 protein attached equally well to saliva from secretors and nonsecretors, indicating a change in carbohydrate specificity in comparison with all other P[8] strains (P[8]-1, P[8]-2, P[8]-3) that bind exclusively to saliva from secretors [11]. The precise carbohydrate motif recognized by the VP8* of P[8]-4 strains remains to be characterized but it is unlikely to involve an α1,2-linked fucose as we failed to observe binding to various synthetic oligosaccharides containing this motif (data not shown). The

reason why P[8]-4 was detected in nonsecretors only is unclear but may be due to a competition with the more frequently encountered P[8]-3 and P[4] that largely spare nonsecretors.

Zeller et al indicated in 2015 that P[8]-4 originated in Asia and can be considered endemic in Bangladesh, unlike in Sub-Saharan Africa, Europe and North America [13]. This was confirmed by a recent study where a large fraction of cases in Bangladesh were reported to be due to P[8]-4 rotavirus [7]. An earlier study showed that all P[8] rotavirus circulating in Tunisia between 2006 and 2011 were of the P[8]-3 subtype [14]. Similar to our present observation in Tunisia, a recent report detected 10% P[8]-4 in Ghana, suggesting a recent emergence of P[8]-4 in both Northern and Sub-Saharan Africa. Alternatively, in earlier studies P[8]-4 strains may have been undetected due to primers mismatches [15]. Interestingly, the nonsecretor phenotype appears at high frequency both in Bangladesh [7] and Tunisia. This may allow P[8]-4 viruses to occupy a host niche of sufficiently large size to maintain transmission in the context of competition with P[8]-3 strains.

In accordance with the fact that the commonly used vaccines Rotarix and RotaTeq are attenuated live vaccines based on P[8] strains that require an α1,2-linked fucose residue for attachment, recent studies reported reduced vaccine efficacy and seroconvertion in nonsecretor children in comparison with secretors [5, 7]. Considering that P[8]-4 strains represent antigenic variants that readily infect nonsecretor children, the presence of such variants may have a significant impact on vaccine efficacy in populations where HBGA polymorphisms allows their circulation at high frequency.

Acknowledgements

186 We thank Laure Barbé (Inserm, Nantes University) and the recombinant protein core facility
187 P2R of the Nantes University for expert assistance with the production of VP8* proteins, and
188 Mickaël Jacquet (CHU Nantes) for his help with the virus detection.

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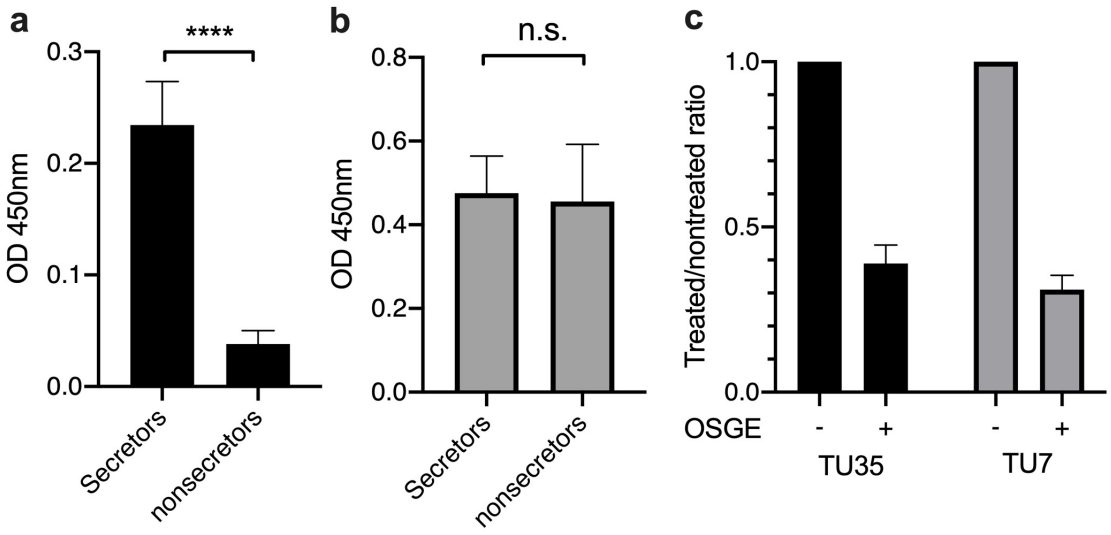
Table 1

Associations between the Secretor type and rotavirus positivity

	Controls (%)	**All RVA+** (%)		**RVA+ only** (%)		
secretors	66 (64)	89 (80)**		32 (74)$^{n.s.}$		
nonsecretors	37 (36)	22 (20)		11 (26)		
		P[4]	**P[8]-3**	**P[8]-4**	**P[4]**	**P[8]-3**	**P[8]-4**
secretors		7 (100)***	39 (85)****	0 (0)	2 (100)$^{n.s.}$	20 (90)**	0 (0)
nonsecretors		0 (0)	7 (15)	6 (100)	0 (0)	2 (9)	4 (0)

Upper part: Comparisons between the group of controls and groups of RVA-infected patients: all RVA+ cases or RVA+ without co-infection (RVA+ only). Lower part: Comparisons between either the P[4] or the P[8]-3 infected groups versus the P[8]-4 infected group were performed by 2-tailed Fisher's exact test. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, n.s. $p>0.05$.



241

242 **Figure Legend**

243 VP8* saliva binding. Comparison of binding of P[4]VP8* (TU35) (**a**) and P[8]-4 VP8* (TU7)
244 (**b**) to saliva from secretors (n=43) and nonsecretors (n=14) tested by ELISA on immobilized
245 saliva samples. These saliva samples were from adults and previously described [11] Values
246 +/- SEM are shown and significance levels based on Welch's t-test. ****$p<0.0001$, n.s.
247 $p>0.05$. Effect of the sialoglycoprotein endopeptidase treatment on binding of P[4] (TU35)
248 and P[8]-4 (TU7) VP8* to the saliva sample from a secretor and Lewis positive individual.
249 Saliva diluted 1/250 was treated with 12 µL of enzyme (+) or enzyme buffer only (-)
250 overnight at 37°C prior coating at a further ¼ dilution onto ELISA plates. Results represent
251 the mean +/- SEM from three independent experiments performed in duplicates (**c**).

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Supplementary data

GenBank accession numbers of VP8* coding sequences from Tunisian cases included in the phylogenetic tree (Fig. 1S).

Case identification	GenBank number	Patient's phenotype[a]
TU187	MN836721	Sec
TU219	MN836722	Sec
TU55	MN836723	Sec
TU138	MN836724	Sec
TU173	MN836725	nonsec
TU93	MN836726	Sec
TU132	MN836727	Sec
TU123	MN836728	Sec
TU83	MN836729	Sec
TU200	MN836730	Sec
TU91	MN836731	Sec
TU63	MN836732	Sec
TU33	MN836733	Sec
TU232	MN836734	nonsec
TU008	MN836735	nonsec
TU131	MN836736	Sec
TU84	MN836737	Sec
TU37	MN836738	Sec
TU29	MN836739	Sec
TU52	MN836740	Sec
TU76	MN836741	Sec
TU118	MN836742	Sec
TU81	MN836743	?
TU44	MN836744	Sec
TU98	MN836745	nonsec
TU263	MN836746	Sec
TU176	MN836747	Sec
TU249	MN836748	Sec
TU42	MN836749	Sec
TU108	MN836750	Sec
TU90	MN836751	Sec
TU26	MN836752	Sec
TU286	MN836753	nonsec
TU9	MN836754	Sec
TU18	MN836755	Sec
TU48	MN836756	Sec
TU69	MN836757	nonsec
TU58	MN836758	Sec
TU170	MN836759	Sec
TU49	MN836760	Sec
TU283	MN836761	Sec
TU281	MN836762	Sec
TU15	MN836763	Sec
TU147	MN836764	Sec
TU369	MN836765	?
TU341	MN836766	Sec
TU31	MN836767	Sec
TU50	MN836768	nonsec
TU7	MN836769	nonsec
TU41	MN836770	nonsec

TU115	MN836771	nonsec
TU24	MN836772	nonsec
TU124	MN836773	nonsec
TU102	MN836774	nonsec
TU19	MN836775	?
TU35	MN836776	Sec
TU95	MN836777	Sec
TU40	MN836778	Sec
TU85	MN836779	Sec
TU92	MN836780	Sec
TU334	MN836781	Sec
TU342	MN836782	Sec
TU340	MN836783	?

[a]Sec= secretor ; nonsec= nonsecretor patient

Rotavirus primers

ROTA CON3 M13F	TGTAAAACGACGGCCAGTTGGCTTCGCTCATTTATAGACA
ROTA CON2 M13R	CAGGAAACAGCTATGACCATTTCGGACCATTTATAACC
ROTA VP4 M13F	TGTAAAACGACGGCCAGTTATGCTCCAGTNAATTGG
ROTA VP4 M13R	CAGGAAACAGCTATGACCATTGCATTTCTTTCCATAATG
ROTA CON3F	TGGCTTCGCTCATTTATAGACA
ROTA CON2R	ATTTCGGACCATTTATAACC
ROTA VP4F	TATGCTCCAGTNAATTGG
ROTA VP4R	ATTGCATTTCTTTCCATAATG

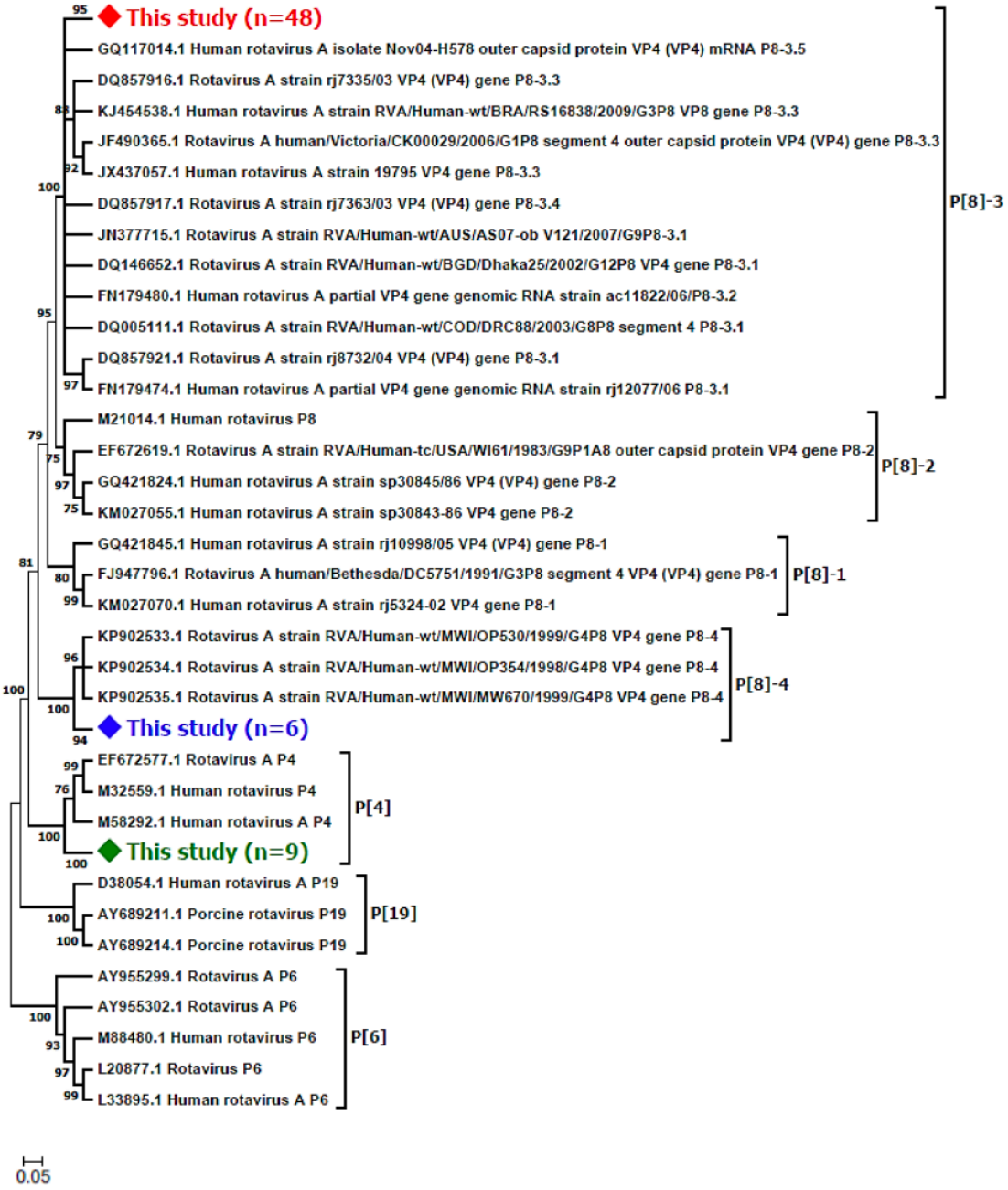

Figure 1S. Phylogenetic tree of the VP8* portion of the VP4 genes in Tunisian strains. The tree was constructed using the Neighbor Joining method (NJ) and 1000 bootstrap replicates with the MEGA 7 software. Only bootstrap support values ≥70% are shown. Strains from the present study are indicated in colored font. The scale bar indicates genetic distances expressed as nucleotide substitutions per site.