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1 **HOST-RANGE SHIFT BETWEEN EMERGING P[8]-4 ROTAVIRUS AND**
2 **COMMON P[8] and P[4] STRAINS**

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

25

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34

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41

42 **Abstract**

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

50

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

53

54

55 **Background**

56 Rotavirus remains the leading cause of severe diarrheal disease in children under 5 globally
57 despite a remarkable decrease in the yearly number of associated children deaths since
58 vaccine introduction [1]. Unlike in high-income countries, vaccine efficacy appears limited in
59 many low-income areas, reasons for this likely being multifactorial although still ill-defined
60 [2]. At the initiation of infection, human rotavirus strains bind to glycans of the histo-blood
61 group antigens type (HBGAs) through the VP4 capsid spike protein that define strains P types
62 [3]. HBGAs are expressed in the gut mucosa, mainly as O-glycans from soluble or cell
63 surface bound mucins and as membrane glycolipids. P[8] strains that account for 74% of the
64 global prevalence of VP4 [4] attach to fucosylated glycans dependent on the FUT2
65 fucosyltransferase for synthesis. As a result, children of the said nonsecretor phenotype who
66 lack a functional *FUT2* allele appear to be much more resistant to the disease than the so-
67 called secretor children who possess at least one functional allele, thereby expressing α 1,2-
68 linked fucose residues in their small intestinal mucosa [5]. Although the strong protective
69 effect of the nonsecretor phenotype was observed in various countries from Europe, North
70 America and Africa, it could not be detected in either Tunisia or Bangladesh [6, 7]. Here we
71 conducted a new study in Tunisia to understand those between-studies or geographical
72 discrepancies.

73

74 **Methods**

75 **Patients and control samples:**

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

85 **Virus detection and P type genotyping:**

86 Nucleic acids were extracted from approximately 100 mg stool samples carried by an
87 automatic extractor type IpreP, using the manufacturer's iPrep™ PureLink™ Virus Kit
88 (Invitrogen). Rotavirus was diagnosed by real-time multiplex PCR FTD Viral gastroenteritis
89 (Fast-track diagnostics) allowing the detection of enteric viruses. An 850 bp fragment of
90 VP8* sequences was obtained from a subset of 63 cases using consensus primers [8].
91 Sequences accession numbers are given in the Supplementary data. These sequences allowed
92 classification of circulating strains at the P-type level. When Ct values were <18-20 a one-
93 step RT-PCR was performed using the Takara's one step PrimeScript RT-qPCR with 5µL
94 nucleic acids extract and ROTA CON3 M13F / ROTA CON2 M13R primers. When Ct values
95 were >18-20, a first RT-PCR was performed using ROTA CON3F / ROTA CON2R and between
96 5-10 µL nucleic acids extract, followed by a nested PCR with ROTA VP4 M13F / ROTA VP4 M13R
97 primers and the Takara's Premix Ex Taq kit. In cases no amplification was obtained, a first RT-PCR

98 was performed with ROTA VP4F / ROTA VP4R primers followed by the nested PCR as above.

99 Primers sequences are given in the Supplementary data.

100 **Determination of the Secretor status and saliva OSGE treatment:**

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

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

114

115

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 α 1,2fucosylated 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

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

147 **Discussion**

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

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

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

167 Zeller et al indicated in 2015 that P[8]-4 originated in Asia and can be considered endemic in
168 Bangladesh, unlike in Sub-Saharan Africa, Europe and North America [13]. This was
169 confirmed by a recent study where a large fraction of cases in Bangladesh were reported to be
170 due to P[8]-4 rotavirus [7]. An earlier study showed that all P[8] rotavirus circulating in
171 Tunisia between 2006 and 2011 were of the P[8]-3 subtype [14]. Similar to our present
172 observation in Tunisia, a recent report detected 10% P[8]-4 in Ghana, suggesting a recent
173 emergence of P[8]-4 in both Northern and Sub-Saharan Africa. Alternatively, in earlier
174 studies P[8]-4 strains may have been undetected due to primers mismatches [15].

175 Interestingly, the nonsecretor phenotype appears at high frequency both in Bangladesh [7] and
176 Tunisia. This may allow P[8]-4 viruses to occupy a host niche of sufficiently large size to
177 maintain transmission in the context of competition with P[8]-3 strains.

178 In accordance with the fact that the commonly used vaccines Rotarix and RotaTeq are
179 attenuated live vaccines based on P[8] strains that require an α 1,2-linked fucose residue for
180 attachment, recent studies reported reduced vaccine efficacy and seroconversion in
181 nonsecretor children in comparison with secretors [5, 7]. Considering that P[8]-4 strains
182 represent antigenic variants that readily infect nonsecretor children, the presence of such
183 variants may have a significant impact on vaccine efficacy in populations where HBGA
184 polymorphisms allows their circulation at high frequency.

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189

190

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231

232

233 **Table 1**

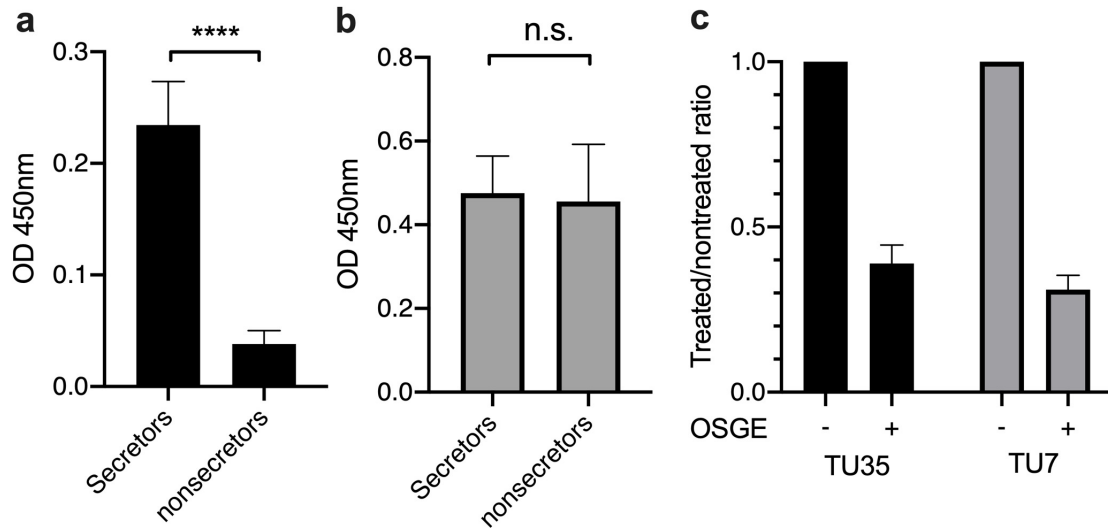
234 Associations between the Secretor type and rotavirus positivity

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

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

239

240



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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255 **Supplementary data**

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

258	Case identification	GenBank number	Patient's phenotype ^a
259	TU187	MN836721	Sec
260	TU219	MN836722	Sec
261	TU55	MN836723	Sec
262	TU138	MN836724	Sec
263	TU173	MN836725	nonsec
264	TU93	MN836726	Sec
265	TU132	MN836727	Sec
266	TU123	MN836728	Sec
267	TU83	MN836729	Sec
268	TU200	MN836730	Sec
269	TU91	MN836731	Sec
270	TU63	MN836732	Sec
271	TU33	MN836733	Sec
272	TU232	MN836734	nonsec
273	TU008	MN836735	nonsec
274	TU131	MN836736	Sec
275	TU84	MN836737	Sec
276	TU37	MN836738	Sec
277	TU29	MN836739	Sec
278	TU52	MN836740	Sec
279	TU76	MN836741	Sec
280	TU118	MN836742	Sec
281	TU81	MN836743	?
282	TU44	MN836744	Sec
283	TU98	MN836745	nonsec
284	TU263	MN836746	Sec
285	TU176	MN836747	Sec
286	TU249	MN836748	Sec
287	TU42	MN836749	Sec
288	TU108	MN836750	Sec
289	TU90	MN836751	Sec
290	TU26	MN836752	Sec
291	TU286	MN836753	nonsec
292	TU9	MN836754	Sec
293	TU18	MN836755	Sec
294	TU48	MN836756	Sec
295	TU69	MN836757	nonsec
296	TU58	MN836758	Sec
297	TU170	MN836759	Sec
298	TU49	MN836760	Sec
299	TU283	MN836761	Sec
300	TU281	MN836762	Sec
301	TU15	MN836763	Sec
302	TU147	MN836764	Sec
303	TU369	MN836765	?
304	TU341	MN836766	Sec
305	TU31	MN836767	Sec
306	TU50	MN836768	nonsec
307	TU7	MN836769	nonsec
308	TU41	MN836770	nonsec

309	TU115	MN836771	nonsec
310	TU24	MN836772	nonsec
311	TU124	MN836773	nonsec
312	TU102	MN836774	nonsec
313	TU19	MN836775	?
314	TU35	MN836776	Sec
315	TU95	MN836777	Sec
316	TU40	MN836778	Sec
317	TU85	MN836779	Sec
318	TU92	MN836780	Sec
319	TU334	MN836781	Sec
320	TU342	MN836782	Sec
321	TU340	MN836783	?

322 ^aSec= secretor ; nonsec= nonsecretor patient

323

324

Rotavirus primers

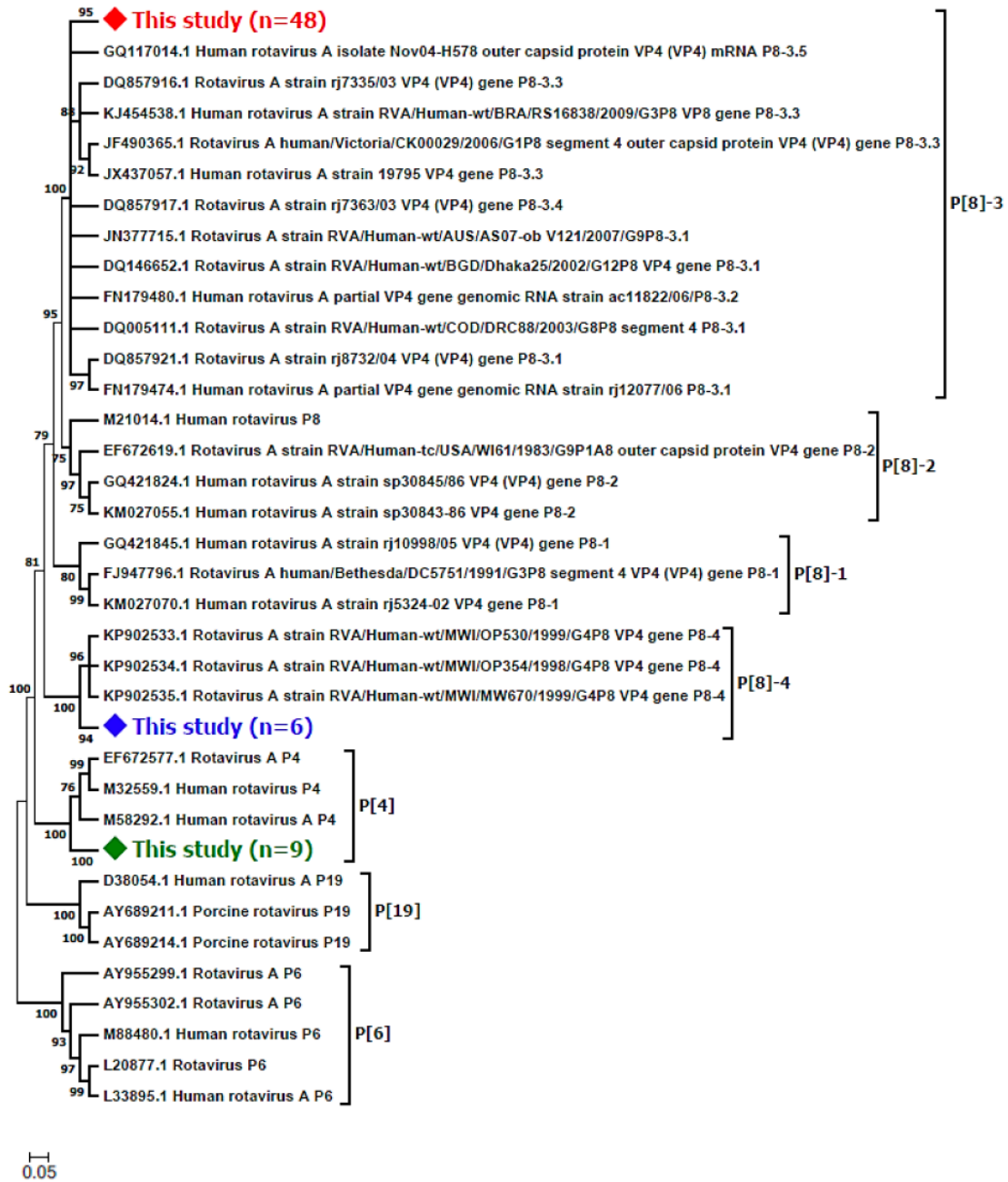
ROTA CON3 M13F	TGTAAAACGACGGCCAGTTGGCTTCGCTCATTATAGACA
ROTA CON2 M13R	CAGGAAACAGCTATGACCATTTCCGACCATTATAACC
ROTA VP4 M13F	TGTAAAACGACGGCCAGTTATGCTCCAGTNAATTGG
ROTA VP4 M13R	CAGGAAACAGCTATGACCATTGCATTTCTTTCCATAATG
ROTA CON3F	TGGCTTCGCTCATTATAGACA
ROTA CON2R	ATTTCCGACCATTATAACC
ROTA VP4F	TATGCTCCAGTNAATTGG
ROTA VP4R	ATTGCATTTCTTTCCATAATG

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

336