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First Report of Putative *Streptococcus pneumoniae* Serotype 6D among Nasopharyngeal Isolates from Fijian Children

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Background. A putative *Streptococcus pneumoniae* serotype, 6D, resulting from the introduction of $wciN_{\beta}$ into serotype 6B has been proposed.

Methods. We studied 98 unique serogroup 6 isolates from Fijian children, two-thirds of whom had received at least 1 dose of 7-valent pneumococcal conjugate vaccine, and 51 invasive isolates from Australian children. We used a polymerase chain reaction (PCR) system that targets both $wciN_{\beta}$ and the single-nucleotide polymorphism that differentiates serotypes 6A and 6B—*wciP584g* (6A) and *wciP584a* (6B).

Results. Two (9%) of 22 Australian isolates and 24 (38%) of 64 Fijian isolates previously identified as 6A by the Quellung reaction and *wciP584g* PCR contained $wciN_{\beta}$ and were designated as 6C; 14 (41%) of 34 Fijian isolates previously identified as 6B by the Quellung reaction and *wciP584a* PCR contained $wciN_{\beta}$ and were designated as the new putative serotype 6D. A significantly smaller proportion of children from whom serotype 6D was isolated (2/14 [14%]) had not received PCV-7, compared with the proportion of those from whom serotype 6B was isolated (11/20 [55%]) ($P < .05$).

Conclusion. This is the first report of naturally occurring *S. pneumoniae* serotype 6D.

Streptococcus pneumoniae is an important human pathogen, especially in developing countries; it accounts for >1.2 million deaths among children annually [1]. There are 48 serogroups comprising 91 serotypes, including the recently identified 6C [2, 3]. Serotypes belonging to serogroup 6 are among the most common that cause invasive disease in children [4].

Classically, serogroup 6 comprised 2 serotypes—6A and 6B [5]—that produce biochemically similar capsules, differing only in their rhamnose-ribitol linkages. The only difference in their capsular gene loci is a single-nucleotide polymorphism (SNP) in *wciP* (584a [195N] in 6B and 584g [195S] in 6A), which encodes rhamnosyl transferase [6, 7]. This SNP is the basis of polymerase chain reaction (PCR) methods developed to differentiate serotypes 6A and 6B [8, 9]. The new serotype 6C was identified in 2007 among isolates initially identified as 6A by the Quellung reaction, on the basis of differential reactions with 2 monoclonal antibodies [2].

The only difference between 6A and 6C polysaccharides is the replacement of galactose in 6A with glucose in 6C [2], which is apparently the result of homologous recombination resulting in the substitution of *wciN* of 6A ($wciN_{6A}$) with a different gene ($wciN_{\beta}$, also referred to as $wciN_{6C}$) of an unknown source [3].

It has been postulated that $wciN_{\beta}$ could be also introduced into the serotype 6B operon to form another new serotype, tentatively designated 6D [10]. This possibility was recently confirmed experimentally [11], but

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the putative serotype 6D has not been identified among pneumococcal clinical isolates to date [11–13].

We have developed a new serotype-specific PCR assay to differentiate serotypes 6A, 6B, and 6C [9], and we used this assay in the present study to test serogroup 6 isolates from Fijian and Australian children.

METHODS

S. pneumoniae *strains.* A total of 179 *S. pneumoniae* serogroup 6 isolates, which had been typed by the Quellung reaction and multiplex PCR–based reverse line blot hybridization assay [14, 15] at the Centre for Infectious Diseases and Microbiology (CIDM), were used for this study. They included 121 nasopharyngeal and 7 invasive isolates collected between 2004 and 2007 from Fijian children aged 6–18 months (85 initially identified as serotype 6A and 43 as 6B) and 51 invasive isolates collected from children aged <5 years in New South Wales in 2005 (22 initially identified as 6A and 29 as 6B).

Children enrolled in the Fijian vaccine study were randomized to receive either 0, 1, 2, or 3 doses of 7-valent pneumococcal conjugate vaccine (PCV-7) at 14 weeks, at 6 and 14 weeks, or at 6, 10, and 14 weeks of age. Half of each group received 23-valent pneumococcal polysaccharide vaccine (PPV-23) at 12 months of age. Nasopharyngeal swab samples were collected at 6, 9, 12, and 17 months to identify carriage of *S. pneumoniae*. Isolates were sent to CIDM for serotyping and were tested blind, without knowledge of the children's ages or immunization history. This study was jointly approved by the Fiji National Research Ethics Review Committee and the University of Melbourne Human Research Ethics Committee.

Serotype-specific PCR methods. A 25-μL PCR volume contained 2 μL of template DNA, 0.125 μL of each forward and reverse primer (50 pmol/μL), 1 μL of deoxynucleoside triphosphates (2.5 mmol/L each dNTP), 2.5 μL of 10× PCR buffer (Qiagen), and 0.1 μL of Qiagen HotStar *Taq* polymerase (5 U/μL); molecular biology–grade H_2O (Eppendorf) was added to 25 μL. The PCR program was performed according to the Qiagen HotStar *Taq* polymerase kit instructions, as follows: 95°C for 15 min for 1 cycle; 94°C for 30 s, 62°C for 60 s, and 72°C for 60 s for 35 cycles; 72°C for 10 min for 1 cycle; and 22°C hold. Primers wciN_βS1 and wciN_βA2 were used to amplify *wci*N_β (359 bp), primers wciP584gS and wciP-r were used to amplify *wciP* of serotype 6A (149 bp), and primers wciP584aS and wciP-r were used to amplify *wciP* of serotype 6B (155 bp). PCR products were detected by electrophoresis in a 1%–2% agarose gel.

Isolates were also tested by 2 previously published PCR protocols, using primers 6C-fwd and 6C-rev, which amplify a por-

Table 1. Oligonucleotide Primers Used in the Present Study

Primer	Specificity		GenBank accession no.	Sequence (5′→3′)[a]
	Serotypes	Target		
wciN_βS1[b]	6C (6D)	*wci*N_β	EF538714	6960 ATCTCTAAATCTGAATATGAAGCGGCTCAATC 6991
wciN_βA2[b]	6C (6D)	*wci*N_β	EF538714	7319 GAACTGAGCTAAATAATCCTCTGGATTATCCACC 7286
wciP584gS[b]	6A (6C)	*wciP*	CR931638	8855 ATTTATATATAGAAAAACTGGCTCATGATAG 8885
wciP584aS[b]	6B (6D)	*wciP*	CR931639	8747 AAGATTATTTATATATAGAAAAACTGTCTCATGATAA 8783
wciP-r[c]	6A (6C)	*wciP*	CR931639	8902 GCGGAGATAATTTAAAATGATGACTAGTTG 8873
	6B (6D)		CR931638	9004 GCGGAGATAATTTAAAATGATGACTAGTTG 8975
5106[d]	6A, 6B, 6C (6D)	*wchA*	CR931639/	5795 TACCATGCAGGGTGGAATGT 5814/
			CR931638/	5897 TACCATGCAGGGTGGAATGT 5916/
			EF538714	6143 TACCATGCAGGGTGGAATGT 6162
3101[d]	6A, 6B, 6C (6D)	*wciO*	CR931639/	7803 CCATCCTTCGAGTATTGC 7786/
			CR931638/	7905 CCATCCTTCGAGTATTGC 7888/
			EF538714	7958 CCATCCTTCGAGTATTGC 7941
6C-fwd[e]	6C (6D)	*wci*N_β	EF538714	6540 CATTTTAGTGAAGTTGGCGGTGGAGTT 6566
6C-rev[e]	6C (6D)	*wci*N_β	EF538714	7266 AGCTTCGAAGCCCATACTCTTCAATTA 7240
wciPS1[f]	6A, 6B, 6C (6D)	*wciP*	AF316640	8231 CCTATAATGGTGAGCGATATTTGT 8254
wciPS2[f]	6A, 6B, 6C (6D)	*wciP*	AF316640	8203 GGAAAGTCAGTTGCAATTTTAATG 8226
wciPA1[f]	6A, 6B, 6C (6D)	*wciP*	AF316640	9094 TTCGATTCTGATAGAAATATCTCACAC 9068
wciPA2[f]	6A, 6B, 6C (6D)	*wciP*	AF316640	9041 TCCTGAATTTCAAGTAGTCTATCACTTA 9014

NOTE. A, antisense; fwd, forward; r and rev, reverse; S, sense.

[a] Nos. indicate sequence positions in corresponding GenBank sequences.

[b] Primers used in our previous study [9].

[c] Previously published primers [8].

[d] Previously published primers [3].

[e] Previously published primers [13].

[f] New primers designed for the present study.

Table 2. Sequence Polymorphism Sites of Serotypes 6C and 6D in Partial *wchA-wciN$_\beta$-wciO* (wcANO)

Sequence types (no. of isolates)	Gene locations and positions of polymorphisms, relative to position 1 of EF538714[a]															
	wchA							*wciN$_\beta$*							*wciO*	GenBank accession no.
	6201	6245	6248	6250	6269	6275	6284	6586	6733	7062	7198	7311	7326	7624	7699	
wcANO-6C1 (15)	C[b]	C[b]	T[b]	T	C	A	G	A	C	A	T	G	G	A[b]	G	FJ899597
wcANO-6C2 (1)	C[b]	C[b]	T[b]	T	C	A	G	A	T[c]	A	T	G	G	A[b]	G	FJ899598
wcANO-EF538714[a]	T[d]	T[d]	T	T	T[d]	T[d]	T[d]	T[d]	C	C[d]	G[d]	G	G	A	C[d]	EF538714
wcANO-6D1 (7)	T[b]	T[b]	A[b]	G	T	T	G	A	C	A	T	G	G	G[b]	G	FJ899599
wcANO-6D2 (7)	T[b]	T[b]	A[b]	G	T	T	G	A	C	A	T	A[e]	G	G[b]	G	FJ899600
wcANO-EU714777[a]	T	T	T	T	T	T	G	A	C	A	T	G	A[f]	A	G	EU714777

NOTE. Amplification was done using primer pair 5106 and 3101, and sequencing was done using primers 5106, 3101, wciN$_\beta$S1, and wciN$_\beta$A2 (see Figure 2 for details).

[a] Polymorphisms in Fijian serotype 6C and 6D isolates were compared with each other and with GenBank sequences EF538714 (serotype 6C *cps* gene cluster) and EU714777 (artificial serotype 6D/6X1 *cps* gene cluster). Position 1 of EF538714 was used as the alignment position.
[b] Four base differences, causing 4 corresponding amino acid differences between Fijian serotype 6C and 6D isolates.
[c] One wcANO-6C2–specific site.
[d] Nine base differences between EF538714 and Fijian serotype 6C; 5 were unique or specific for EF538714.
[e] One wcANO-6D2–specific site.
[f] One EU714777–specific site.

tion of *wciN$_\beta$* [13], and primers 5106 and 3101, which amplify portions of *wchA-wciN-wciO* for 6A and 6B and *wchA-wciN$_\beta$-wciO* for 6C and the putative 6D [3]. All primers used in the present study are shown in Table 1.

Sequencing and sequence analysis. PCR products (*wciP* and partial *wchA-wciN$_\beta$-wciO*) of all 6D isolates and of selected 6C isolates were amplified and sequenced. Products were purified using the PCR Product Pre-Sequencing kit (USB) and were directly sequenced in both directions using the BigDye Terminator cycle sequencing kit (version 3.1) in an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Primers wciPS1 and wciPA2 were used for amplification (810 bp) of the *wciP* region, and the inner primer pair wciPS2 and wciPA1 were used for sequencing. For the partial *wchA-wciN$_\beta$-wciO* region, primers 5106 and 3101 were used for amplification (1.8 kb), and primers 5106 and wciN$_\beta$A2 as well as primers 3101 and wciN$_\beta$S1 were used for sequencing. Sequences were compared with known sequences of serotype 6C and the artificial 6D *cps* loci in GenBank (accession numbers EF538714 and EU714777, respectively) [6, 11] and were analyzed with the BLASTn tool in BioManager (Sydney Bioinformatics; available at: https://www.angis.org.au/).

Six new Fijian 6C and 6D sequences were submitted to GenBank: FJ899597 (6C1), FJ899598 (6C2), FJ899599 (6D1), and FJ899600 (6D2) for partial *wchA-wciN$_\beta$-wciO*, and FJ899601 (6C) and FJ899602 (6D) for near-full-length *wciP* (Tables 2 and 3).

RESULTS AND DISCUSSION

Fijian isolates. By means of primers wciN$_\beta$S1 and wciN$_\beta$A2, 52 (41%) of 128 serogroup 6 isolates from Fijian children were shown to contain *wciN$_\beta$*. Results of serotype 6A– and serotype 6B–specific PCRs with primers wciP584gS and wciP-r and

Table 3. Sequence Polymorphisms Sites of Serotypes 6C and 6D in Partial *wciP*

Sequence types (no. of isolates)	Position of polymorphisms in *wciP*, relative to position 1 of EF538714[a]									GenBank accession no.
	8456	8540	8730	8828	8928	8938	8957	8990	9114	
wciP-6C (16)	A[b]	A	A[c]	A[c]	G[c]	G[c,d]	G	A	A[c]	FJ899601
wciP-EF538714[a]	G	A	C	A	G	G[c,d]	G	A	A	EF538714
wciP-6D (14)	G	A	C[c]	T[c]	T[c]	A[c,d]	A	G	G[c]	FJ899602
wciP-EU714777[a]	G	C[e]	A	T	T	A[c,d]	A	G	G	EU714777

NOTE. Amplification was done using primers wciPS1 and wciPA2, and sequencing was done using primers wciPS2 and wciPA1 (see Figure 3 for details).

[a] EF538714, serotype 6C *cps* gene cluster sequences, and EU714777 artificial serotype 6D/6X1 *cps* gene cluster sequences. Position 1 of EF538714 was used as the alignment position.
[b] One wciP-6C–specific site, causing a corresponding amino acid difference.
[c] Five base difference, causing 5 corresponding amino acid differences between Fijian serotype 6C and 6D isolates.
[d] *wciP*584g (6A/C) and *wciP*584a (6B/D) single-nucleotide polymorphism in position 8938 in EF538714.
[e] One EU714777-specific site, causing a corresponding amino acid difference.

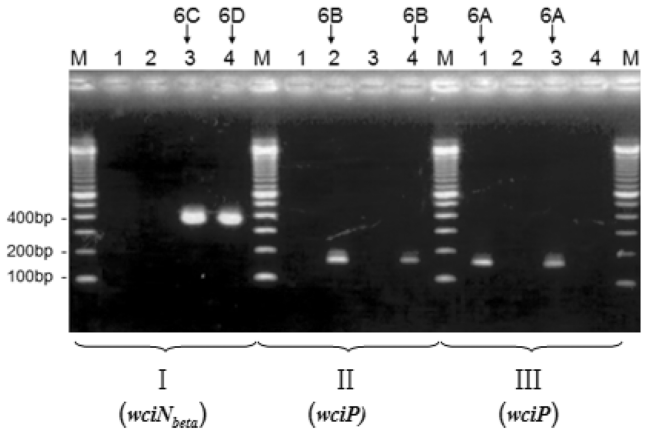

Figure 1. Electrophoretic pattern of the polymerase chain reaction products of serotypes 6A, 6B, 6C, and 6D. Lanes 1, 2, 3, and 4 represent 4 *Streptococcus pneumoniae* strains that had been identified as serotypes 6A, 6B, 6A, and 6B, respectively, by Quellung methods. Lane M indicates a standard marker (100-bp DNA Ladder, catalogue number 15628-019; Invitrogen). Group I isolates were amplified using the primer pair wciN$_\beta$S1 and wciN$_\beta$A2, which target *wciN*$_\beta$. Lanes 1 and 2 did not amplify. Lanes 3 and 4 amplified with wciN$_\beta$S1 and wciN$_\beta$A2, consistent with serotype 6C. Groups II and III isolates were amplified using the primer pair wciP584aS and wciP-r (6B) and the primer pair wciP584gS and wciP-r (6A), respectively, which target *wciP*. Lane 1 amplified by wciP584gS and wciP-r only and was identified as serotype 6A. Lane 2 amplified by wciP584aS and wciP-r only and was identified as serotype 6B. Lane 3 (*wciN*$_\beta$ positive in I) amplified by wciP584gS and wciP-r (as for 6A) and was designated as serotype 6C. Lane 4 (*wciN*$_\beta$ positive in I) amplified by wciP584aS and wciP-r (as for 6B) and was designated as serotype 6D.

primers wciP584aS and wciP-r, respectively, confirmed those of conventional serotyping. Of the 85 isolates initially serotyped as 6A, 33 (39%) contained *wciN*$_\beta$ and were redesignated as 6C; of the 43 isolates initially serotyped as 6B, 19 (44%) contained *wciN*$_\beta$ and were redesignated as putative serotype 6D (Figure 1); retesting of these 19 isolates by 2 previously published PCR protocols and the Quellung reaction confirmed the original results (6B).

Review of collection dates and study participant data showed that 18 isolates were duplicates (ie, were from the same specimen) and were excluded. There were 14 pairs of isolates from swab samples collected from the same participants at different times. Serotypes were the same for both members of 12 pairs and the immunization status of the children from whom they were isolated had not changed between collection of swab samples, so 1 of each pair was excluded. Two pairs of isolates from swab samples collected at different times were of different serotypes—6C and 6B were isolated at 6 and 12 months of age, respectively, from an unvaccinated child, and 6B and 6A were isolated at 12 and 17 months, respectively, from a child who had received a single dose of PCV-7 at 14 weeks of age. Both isolates of these pairs were included in the analysis. After these 30 exclusions, 98 unique serogroup 6 isolates from 96 Fijian children remained for analysis.

Of these 98 isolates, 32 were recovered from children who had not received PCV-7 (3 had received PPV-23 only at 12 months of age), and 26, 26, and 14 had received 1, 2, or 3 doses of PCV-7, respectively. The distribution of serotypes 6A, 6B, 6C, and 6D according to the immunization status of the participants is shown in Table 4. A significantly smaller proportion of the serotype 6D than 6B isolates were from children who had not received PCV-7 ($P < .05$). Differences in immunization status between children from whom other serotypes were isolated were not significant. All 7 invasive isolates (for 6A, 1; for 6B, 4; and for 6C, 2) were from unimmunized children.

Australian isolates. Serogroup 6 isolates from Australia were tested as for the Fijian isolates. With primers wciN$_\beta$S1 and wciN$_\beta$A2, two (4%) of 51 isolates were shown to contain *wciN*$_\beta$. By means of primers wciP584gS and wciP-r and primers wciP584aS and wciP-r, Quellung results were confirmed (22 serotype 6A and 29 serotype 6B). The 2 containing *wciN*$_\beta$ were among those previously identified as 6A and were therefore redesignated as 6C. No serotype 6D isolates were identified.

Is the formation of serotype 6D from 6B plausible? Pneumococcal serotypes 6A and 6B are closely related but are distinguishable using polyclonal antisera. Apart from the one well-defined SNP in *wciP*, any other genetic differences have not been well defined. We have previously found several sequence polymorphisms in *cpsA-cpsB* [16], some of which are apparently unique to either 6A or 6B. Others have found evidence for relatively frequent switching between serotype 6A and 6B [6] and sharing of sequence types, suggesting that capsular switching occurs among serotypes 6A, 6B, and 6C [13, 17].

Serotype 6C was apparently derived from 6A by the replacement of *wciN* with *wciN*$_\beta$, which replaces galactosyltransferase

Table 4. Distribution of *Streptococcus pneumoniae* Serogroup 6 Serotypes, According to the Immunization Histories of the Fijian Children from Whom They Were Isolated

Serotype, no. of isolates	No. of PCV-7 doses			
	0	1	2	3
6A, 40	12 (30)[a]	12 (30)	8 (20)	8 (20)
6C, 24	7 (29)	6 (25)[b]	10 (42)[a]	1 (4)
6B, 20	11 (55)[b,c]	4 (20)	4 (20)	1 (5)
6D, 14	2 (14)[c]	4 (29)[b]	4 (29)	4 (29)
Total, 98	32	26	26	14

NOTE. Data are no. (%) of isolates by PCV-7 dose group. PCV-7, 7-valent pneumococcal conjugate vaccine; PCV-23, 23-valent pneumococcal vaccine.

[a] Two of these children received a dose of PCV-23 at 12 months.

[b] One of these children received a dose of PCV-23 at 12 months.

[c] The proportion of serotype 6D isolates from children who had not received PCV-7 was significantly lower than that of serotype 6B isolates (14% vs 55%; $P < .05$; relative risk, 0.26 [95% confidence interval, 0.07–0.99]).

of 6A with glucosyltransferase in 6C. Another new serogroup 6 serotype would be generated if the *wciN*/galactosyltransferase of 6B were similarly replaced with the *wciN*$_\beta$/glucosyltransferase [11]; logically, this putative new serotype would be designated serotype 6D or genotype 6D [10]. Recently, such a strain (TIGR6X1) was produced experimentally by inserting *wciN*$_\beta$ into the 6B capsule gene locus [11].

The 14 unique isolates that we have designated as serotype 6D were consistently identified as 6B by means of polyclonal antisera and 6B-specific PCR (ie, they contained the *wciP*584a SNP). However, 3 separate PCRs using primers wciN$_\beta$S1 and wciN$_\beta$A2 and the previously published primers 5106 and 3101 [3] and 6C-fwd and 6C-rev [13] amplified *wciN*$_\beta$ from these isolates, producing amplicons of 359 bp, 1.8 kb, and 727 bp, respectively. We propose that these 14 putative serotype 6D isolates and are the first naturally occurring equivalents of experimental serotype 6X1 to be identified.

PCV-7 was licensed in the United States in 2000 and has been used widely in the United States and Europe since; it was introduced into the routine infant immunization schedule in Australia in 2005 but has not been widely used in Fiji. Most of the Fijian isolates were from children enrolled in a pneumococcal vaccine trial, two-thirds of whom had received at least 1 dose of PCV-7, which contains 6B antigen and confers cross-protection against serotype 6A but not 6C [18]. Reports that the prevalence of 6C has increased in some places after the introduction of PCV-7 [12, 13, 17, 19] suggests that it may have a selective advantage, although we found similar proportions of immunized and unimmunized children among those colonized with serotypes 6A and 6C. However, 86% of children from whom serotype 6D was isolated had received at least 1 dose of PCV-7, compared with only 45% of those colonized with 6B. This suggests that serotype 6D could have a selective advantage after immunization.

To further investigate the genetic origins of serotype 6D, we sequenced capsular gene regions containing *wciN*$_\beta$ and *wciP* from all 14 serotype 6D isolates and 16 selected serotype 6C isolates from Fiji. Two different sequence types were identified for each serotype, both of which differed slightly from GenBank sequences, with some consistent differences between 6C and 6D (summarized in Tables 2 and 3; representative sequences are shown in Figures 2 and 3). Like serotype 6C, serotype 6D has a 194-bp deletion in *wciN*$_\beta$ relative to their parental serotypes 6A and 6B [3]. Between serotypes 6C and 6D sequences, there were 6-bp and 3–amino acid differences in *wchA*, 1-bp and 1–amino acid difference in *wciN*$_\beta$, and no differences in *wciO*; in *wciP*, there were 8 consistent SNPs (representing 5–amino acid differences) in addition to the well-recognized *wciP*584g and *wciP*584a SNPs. Further biochemical and monoclonal antibody studies will be required to demonstrate the effects that these amino acid changes have on the capsular structure and antigenic specificity of serotype 6D.

In conclusion, we have identified *S. pneumoniae* isolates from Fijian children that were initially identified as serotype 6B but that contain *wciN*$_\beta$, previously found only in serotype 6C. As previously suggested [10], we have provisionally designated these isolates as serotype 6D. The influence that immunization has on the emergence of this new serotype and the evolutionary relationships between serogroup 6 members deserve further investigation.

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases.*

Figure 2. Sequence alignment of *Streptococcus pneumoniae* serotypes 6C and 6D partial *wchA-wciN*$_\beta$*-wciO*, amplified using primer pair 5106 and 3101 and sequenced using primers 5106, 3101, wciN$_\beta$S1, and wciN$_\beta$A2.

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases.*

Figure 3. Sequence alignment of *Streptococcus pneumoniae* serotypes 6C and 6D partial *wciP*, amplified using primers wciPS1 and wciPA2 and sequenced using primers wciPS2 and wciPA1.

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