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The Chemotaxonomic Relationship of *Vibrio cholerae* non-O1 by Fatty Acid Compositions

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지방산 조성에 의한 *Vibrio cholerae* non-O1의 화학분류학적 관계

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ABSTRACT — The authors attempted utilization of fatty acid composition of vibrios as a tool for identification of the strains. Fatty acid of 49 strains of *Vibrio cholerae* non-O1, *V. cholerae* O1, *V. mimicus, V. vulinificus* and *V. parahaemolyticus* was analyzed by gas-liquid chromatography column. According to the statistical analysis of the fatty acid data, the relationship between the *Vibrio* species and serotypes of the strains was discussed. Forty one kinds of fatty acid were detected from the tested strains and 35 kinds of fatty acids among the detected fatty acids were significant factors to identify the vibrios. The predominant fatty acids were 16:0, 16:1 cis 9, 18:1 trans 9/6/cis 11 and 15:0 iso 2OH/16:1 cis 9 as above about 20% in total. Fatty acid compositions of the *Vibrio* species were an important factor in identifying their subspecies either predominant fatty acids or minor ones. According to the analysed results by a conventional statistical processing method (UPGMA) and prepared dendrogram, *V. cholerae* non-O1 had more closer relationship with *V. mimicus* compared with *V. cholerae* O1. Moreover, the distribution of hydroxy acid was a significant factor for identifying *V. cholerae* subspecies. Comprising all the 10 serotypes detected from *V. cholerae* non-O1 examined such as O2, O5, O8, O10, O14, O27, O37, O39, O45 and O69, we could group them into seven subspecies by cluster analysis with the similarity value of fatty acid composition as above 92%. It means that there is a significant relationship between serotypes and fatty acid composition of *V. cholerae*. These results indicated that numerical analysis of fatty acid composition data of *V. cholerae* non-O1 could classify them into subspecies, and also which may provide a useful epidemiologic information or a basis for further analysis such as PCR and DNA probe analysis.

Key words □ fatty acid, *Vibrio cholerae* non-O1, serogroup, chemotaxonomic relationship

The identification of *Vibrio cholerae* (*V. cholerae*) is commonly based on a wide range of biochemical and physiological tests.[1-5] The major problems are the need for the high cost and time required for the preparation of cultures. Recently, the availability of miniaturized multitest of commercial kit (API 20E, ATB ID 32E etc) that allow the simultaneous determination of numerous phenotypic characters has overcome the previous difficulties.[6-8] However, the identification for epidemiological utilities remains problematic because there is often a lack of agreement between the biochemical tests for the type strain and the results of fresh isolates. The reason items from the similar physiological and biochemical characters shared from the differences in the biochemical features of fresh and stored isolates.[9,10] In fact, not only plasmid DNA may be lost during storage, but also fresh isolates may have greater enzymatic activity than their counterparts which have been stored in laboratory.

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More sensitive and reliable molecular biological techniques ara cleavage of DNA by restriction endonucleases (REA, Ribotyping, PFGE, etc) and PCR but they are generally difficult to perform, require many expensive reagents, and have the problem of long preparation times.[11-14] Also, *V. cholerae* has been subdivided on the basis of its O antigens, but undoubtedly there are many more serovars as about 60% of strains are typable at present. Moreover, serotyping for epidemiological study require high cost in using commercial antisera.[16] Therefore, many of those method are not easy to perform and have demonstrated limitations in terms of sensitivity, specificity, and general applicability.[15]

In this study, the use of fatty acid analysis by gas chromatography for the identification of bacteria since its initial introduction of Abel *et al.*[17] has given results in agreement with DNA-DNA hybridization data.[18-20] This technique has practical advantages, such as the simplicity of the analytical method, the speed of analysis and the low cost of reagents.[20-22] Moreover, the whole cell fatty acid profile is a phenotypic character that is not affected by mutation caused by environmental conditions[10,23] and is a direct and stable expression of the cellular genome.

We describe a method for identification at the species and subspecies level of *Vibrio cholerae* non-O1 by using gas liquid chromatography profile of fatty acid methyl esters (FAME). The column of gas liquid chromatography (GLC) used a fused silica capillary for high separated rate. And it was to determine if chemotaxonomical analysis of the quantitative fatty acid data obtained by GLC could be used to differentiate strains of *V. cholerae* according to its serogroups.

MATERIALS AND METHODS

Strains and culture conditions

The test strains studied were *V. cholerae* non-O1 of 47 environment condition (sea water) and 18 clinical source were isolated, and *V. cholerae* non-O1 ATCC 25872, *V. vulnificus*, *V. cholerae* O1, *V. mimicus* ATCC 33653 and *V. parahemolyticus* Kanagawa No. 4750. For the cellular fatty acid analysis, all of the test strains were subcultured on trypticase soy agar (TSA, BBL Cockeysville, MD) and incubated at 28°C for 24 hrs.

Fatty acid analysis

Total fatty acid was analyzed using the Microbial Identification System (MIDI; newark, DL) by the standardized procedure described by Miller and Berger (1985). From tertiary quadrant of TSA plate, approximately 40 mg of colonies was got into a clear dried screw cap tube (13×100 mm) with 4 mm loop and boiled for 30 min at 100°C after addition of 1 m*l* of 15% (wt/vol) NaOH to 50% methanol for saponification.

Then the samples were acidified to 2nd of methanolic HCl for methylation, and the methylated fatty acids were further extracted with 1.25 m*l* of a 1:1 (vol:vol) solution of methyl tert-buthyl ether-hexane. The organic extract was washed with of 1.2% (wt/vol) NaOH, and centrifugated at about 900×g for 3 mins.

FAMEs were analysed by gas-liquid chromatography on an HP5890A gas chromatography (Hewlett-Packard Co, USA) equipped with a flame ionization detector. A fused-silica capillary column (0.2 mm×25 m); cross-linked (methyl phenyl silicone[Hewlett-Packard Co. USA]) with ultra high-purity hydrogen as carrier gas was used. The gas-liquid chromatography conditions were as follows; injector temperature, 250°C; detector temperature 300°C; initial column temperature 170°C; increasing by 5 °C/min to 270°C in 20 min; Carrier gas flow rate 50 m*l*/min; sample volume, 1 m*l*, and the detection was FID. The peak retention time and peak area values were recorded with an HP3392A integrator (Hewllett-Packard Co. USA). FAMEs were calibrated against a standard mixture of known fatty acids provided by MIDI. Detected sample peaks were named by interpolation of retention time using the equivalent chain length. The result was expressed as area % relative to the total peak area for reproducibility under the standardized growth conditions.

RESULTS AND DISCUSSION

The results of the fatty acid composition analysis of the *V. cholerae* non-O1, *V. mimicus*, *V. vulnificus* and *V. parahaemolyticus* are listed in Table 1.

Carbon numbers of the detected fatty acids from all the tested strains were ranged from 10 to 20, and the major fatty acids were 16:1 cis 9 and 16:0 and 18:1 trans 9/6/cis11, while all of them contained a small hydroxy fatty acid. Forty one kinds of fatty acids were detected from all strains tested and 35 kinds of fatty acids among them were useful factors to identify the Vibrio species.

V. cholerae non-O1, *V. cholerae* O1 and *V. mimicus* were quite different from *V. vulnificus* and *V. parahae-*

Table 1. Comparison of fatty acid compositions of genus *Vibrio* by species

Kinds of Fatty acid	*V. cholerae* non-O1	*V. cholerae* O1	*V. mimicus*	*V. vulnificus*	*V. parahaemolyticus*
$C_{10:0\ 3OH}$	0~3	·	0~3	·	·
$C_{11:0\ iso\ 3OH}$	·	0~3	·	·	·
unknown 12.486	0~1	1	0~1	2	·
$C_{12:0\ iso\ 3OH}$	·	3	·	·	·
$C_{12:0}$	·	·	·	·	0~4
$C_{12:1\ 3OH}$		·	0~3	·	·
$C_{12:0\ 3OH}$	3~7	3~7	4~6	·	1~2
$C_{12:0\ 2OH}$	·	·	0~3	·	·
$C_{14:0\ iso}$	0~3	3	·	·	·
$C_{14:0}$	4~7	4~7	·	3~16	·
$C_{15:0}$	0~3	0~3	0~3	·	0~3
$C_{14:0\ iso\ 3OH}$	0~3	0~3	0~3	·	·
$C_{16:0\ iso}$	0~3	0~5	0~3	·	·
$C_{16:0\ cis\ 9OH}$	0~3	·	0~3	·	·
$C_{16:1\ cis\ 7}$	·	·	0~3	·	·
$C_{16:1B}$	1	0~2	0~3	0~3	0~1
$C_{16:1\ cis\ 9}$	35~43	23~43	24~46	34~42	30~43
$C_{16:1\ cis\ 11}$	0~3	·	0~3	·	·
$C_{16:0}$	19~24	16~25	20~27	22~34	20~37
$C_{15:0\ iso\ 3OH}$	·	·	·	0~3	·
$C_{17:1B}$	·	0~3	·	·	0~3
$C_{17:1\ cis\ 9}$	0~3	·	0~3	·	·
$C_{17:0\ iso}$	·	·	·	·	0~3
$C_{17:0}$	0~3	0~3	0~3	·	0~3
$C_{18:0\ iso}$	0~3	0~3	0~3	·	·
$C_{18:1\ cis\ 9}$	0~1	0~1	0~3	·	·
$C_{18:1\ trans\ 11}$	·	·	0~3	·	·
$C_{18:1\ cis\ 13}$	0~3	0~3	0~3	·	·
$C_{18:0}$	1~3	1~3	1~3	·	·
$C_{19:0\ methyl}$	0~1	0~1	·	·	·
$C_{20:1\ trans\ 11}$	0~3	0~3	·	·	·
$C_{14:0\ 3OH/16:1\ iso\ I}$	3~4	3~4	3~4	4~6	4
$C_{16:1\ trans\ 9/15:0\ 2OH}$	·	·	0~16	·	·
$C_{18:1\ trans\ 9/6/cis\ 11}$	20~27	20~27	20~25	6~24	4~26
unknown $C_{18:0\text{-}19:0\ cyclo}$	0~3	0~3	0~3	·	·

Each strain was analyzed at least 5 times for reproducibility and values are percentages of total fatty acid expressed as range of detection

molyticus. First of all, much more kinds of fatty acids were isolated from the former 3 species compared with those of latter 2 species. Some fatty acids were not detected from the examined Vibrio species except one kind of Vibrio species.

For example, 12:0 iso 3OH detected only from the strain V. cholerae O1, while 12:0 2OH, 12:1 3OH, 16:1 cis 7 and 16:1 trans 9/15:0 2OH were detected only from the strain *V. mimicus*. Some patterns were appeared 15:0 iso 3OH in *V. vulnificus* and 17:0 iso in *V. parahaemolyticus*.

These results of fatty acid composition were in agreement with other published data.[19-21] To eveluate the similarity between the species and subspecies in taxonomic relationship, these data were analyzed using unweighted pair group methods using arithmetic averages (UPGMA) by Sneath and Sokal.[27] Results given in Fig. 1 showed that the similarity of *V. cholerae* non-O1 was higher in *V. mimicus* (68.9%) than *V. cholerae* O1 (64.3%) and were other *Vibrio* spp. (less than 40%). These results were quite different from other studies[12,25,26] but in agreement with 16S rRNA sequences analyzed by Tzukamoto *et al.*.[27] The most distinguishing feature of *V. vulnificus* were the higher amounts of 14:0, the small amounts of 15:0 iso 3OH and the simple fatty acid composition. Finally it was possible to differentiate the other serogroups as difference of fatty acid profiles and amounts. Especially, moderately high fatty acid of *V. cholerae* non-O1 contained 12:1 3OH, 14:0 iso 3OH, 16:0, 10:0 3OH and 14:0 3OH/16:1 iso. 12:1 3OH for *V. cholerae* was higher than in the other *Vibrio* spp. On the other hand, *V. cholerae* O1 were the absence of 10:0 3OH and 16:0 cis 9 OH, and the presence of 11:0 3OH and 12:0 iso 3OH. Therefore, in the identification of *V. cholerae*, we indicated a significant factor which demonstrated the distribution of hydroxy acid.

The fatty acid data for the *V. cholerae* non-O1 according to serogroups are shown in Table 2. All of the *V. cholerae* non-O1 serogroups possessed a fatty acid composition of 16:1 cis 9, 16:0, 18:1 trans 9/6/ cis 11 to be major, and branched short chain acids were 12:0 to 18:0. Among the *V. cholerae* non-O1 tested, 16:1 cis 9 ranged from 29.6 to 36.1%, 16:0 from 15.2 to 26.4% and 18:1 trans 9/6/cis 11 from 12.9 to 25.0%. O2 contained 23 fatty acids and most fatty acids were similar to the other serogroups, but these were distinguished by the absence

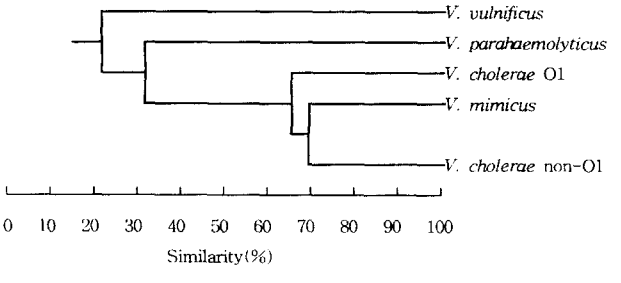

Fig. 1. Similarity dendrogram of *Vibrio* spp. by fatty acid composition.

Table 2. Difference of fatty acid composition of *V. cholerae* non-O1 by their serotypes

Serotypes Fatty acid	O2 (4)	O5 (1)	O8 (5)	O10 (3)	O14 (28)	O27 (3)	O37 (1)	O39 (2)	O45 (1)	O69 (1)
$C_{10:0\ 3OH}$	0.2	0.2	0.1	·	0.2	0.1	0.3	0.2	0.8	0.2
unknown 12.486	0.6	0.7	0.5	0.5	0.6	0.9	1.1	0.6	0.8	0.5
$C_{12:0\ 2OH}$	0.1	0.1	0.1	·	·	0.2	·	0.1	·	0.1
$C_{12:0\ iso\ 3OH}$	·	·	·	·	·	0.1	·	·	3.7	·
$C_{12:1\ 3OH}$	0.1	0.1	0.1	·	0.2	·	0.2	0.1	·	·
$C_{12:0\ 3OH}$	3.9	4.8	4.6	4.7	4.2	5.0	8.6	3.4	·	4.4
$C_{14:0\ iso}$	0.1	0.1	·	·	0.2	0.1	·	0.2	0.5	·
$C_{13:0\ iso}$	·	·	·	·	0.3	·	·	·	·	·
$C_{14:0}$	6.6	5.6	5.6	·	4.1	8.7	7.3	5.5	2.0	7.0
$C_{15:0}$	0.2	0.2	0.1	·	0.2	0.2	0.2	0.3	0.7	0.1
$C_{14:0\ iso\ 3OH}$	0.3	0.4	0.2	·	0.3	0.2	0.6	0.4	0.6	0.1
$C_{15:0\ iso}$	·	·	·	·	0.5	·	·	·	·	·
$C_{15:1\ A}$	0.3	·	·	·	·	·	·	·	·	·
$C_{16:1\ iso\ E}$	·	0.1	0.3	·	0.3	0.3	0.8	0.2	0.3	0.3
$C_{16:1\ cis\ 9\ OH}$	·	·	·	·	0.2	·	·	·	·	·
$C_{16:1\ B}$	·	·	·	·	·	·	1.1	0.8	0.8	0.6
$C_{16:0\ iso}$	1.3	1.4	0.7	·	0.3	3.5	1.3	1.8	3.6	0.6
$C_{16:1\ cis\ 9}$	30.9	29.6	36.1	30.6	35.4	30.6	34.6	33.4	31.5	30.2
$C16:1\ C$	0.9	0.9	1.0	·	0.3	0.9	·	·	0.5	0.9
$C_{16:1\ cis\ 7}$	·	·	·	·	·	·	·	0.2	·	·
$C_{16:1\ cis\ 11}$	0.3	0.2	0.2	·	0.3	0.2	·	0.3	·	0.2
$C_{15:0\ iso\ 3OH}$	·	·	·	·	·	·	·	·	0.2	·
$C_{16:0}$	23.8	22.9	23.4	22.6	20	23.9	15.2	24.4	16.6	26.4
$C_{17:1\ cis\ 9}$	0.3	0.4	0.2	0.7	·	0.1	·	0.3	0.2	0
$C_{17:0}$	0.2	0.2	0.2	·	0.3	0.1	0.2	0.3	0.7	0.1
$C_{17:1\ C}$	·	·	·	·	·	·	·	·	0.4	·
$C_{17:1\ B}$	·	·	·	·	·	·	·	0.5	1.3	·
$C_{17:0\ iso}$	·	·	·	·	·	·	·	·	0.9	·
$C_{18:0\ iso}$	0.4	0.4	0.2	0.7	0.5	0.2	·	0.4	0.5	0.2
$C_{18:0}$	·	4.1	1.2	2.5	0.5	1	0.5	1.3	0.7	0.9
$C_{18:1\ cis\ 13}$	0.2	0.2	0.1	·	8.2	0.1	·	0.1	·	·
$C_{20:1\ trans\ 11}$	0.6	1.3	0.2	·	·	0.1	·	0.1	·	0.1
$C_{16:0\ iso/14:0\ 3OH}$	3.5	0.1	3.5	3.0	4.0	4.7	6.1	3.1	5.5	3.9
$C_{15:0\ iso\ 2OH/16:1\ cis\ 9}$	5.6	7.3	·	4.3	7.8	5.0	8.6	32	2.8	6.9
$C_{18:1\ trans\ 9/6/cis\ 11}$	18.4	18.0	18.2	22.8	17.8	16.1	12.9	19.4	25.0	16.3
Unknown $C_{18:0\text{-}19:0\ cyclo}$	0.2	0.1	0.1	·	0.2	0.1	·	0.1	0.2	0.3

Numbers in parenthesis indicated number of tested strains.
A percentage of total cellular fatty acid was calculated as average within each strain. Each strain was analyzed at least 5 times for reproducibility.

of 16:1 iso E, 16:1 B and 18:0, and the presence of 15:1A. And the fatty acid patterns of O5 and O8 most closely resembled those of the other serogroups, but O8 was differentiated from each of the other 9 serogroups by the absence of 14:0 iso and 15:0 iso 2OH/16:1 cis 9.

For O10, the most abundant fatty acids were 16:1 cis 9, 16:0 and 18:1 trans 9/6/cis 11 followed by 12:0 3OH, 15:0 iso 2OH/16:1 cis 9, 16:0 iso/14:0 3OH and 18:0, but not 10:0 3OH, 14:0, 15:0, and 16:1 C etc, which are readily distinguishable from other groups. And amounts of 16:0 and 18:1 trans 9/6/cis 11 were similar. O14 only possessed 15:0 iso and 16:1 cis 9OH, and moderate quantities of 12:0 OH, 14:0, 18:1 cis 13, 16:0 iso/14:0 3OH and 15:0 iso 2OH/16:1 cis 9. In O14, 15:0 iso and 16:1 cis 9 OH were easily differentiated from the other groups. The overall fatty acid profiles of O27 and the other groups were the most similar, but these serogroups were distinguished by the presence of 12:0 iso 3OH and the absence of 12:1 3OH. O37 contained 16 fatty acids, and these groups were differentiated from the other groups by the absence of 12:0 2OH, 14:0 iso, 16:1 C, 16:1 cis 11, 17:1 cis 9, 18:0 iso, 18:1 cis 13, 20:1 trans

11 and 18:0-19:0 cyclo, and 16:0 and 18:1 trans 9/6/ cis 11 were lower than in the other serogroups. O39 contained 26 different fatty acids. These groups resembled that of O5 except the presence of 14:0 iso, 16:1 B and 17:1 B, and the absence of 16:1 C. O45 contained 12:0 iso 3OH, 15:0 iso 3OH, 17:1C, 17:1B and 17:0 iso but not 12:0 3OH and 18:1 cis 13, and amounts of 16:0 were lower than 18:1 trans 9/6/ cis 11. which also were easily differentiated from the other *V. cholerae* non-O1. The fatty acid profiles of O69 and the other groups were similar, but these were distinguished by the absence of 12:1 3OH, 14:0 iso, 18:1 cis 13.

On the other hand, for the identification of subspecies by numerical taxonomy we used 35 different fatty acids for UPGMA, the results of cluster analysis showed 7 subspecies among 10 serogroups, and O5 and O39 yielded a similarity value of 94%, and O8 and O27 yielded a similarity value of 92% (Fig. 2). In the further study, it was differentiated by the fatty acid compositions that O5 contained 16:1 cis 7 but O39 not possessed 16:1 B, 16:1C and 17:1B. And in similarity of O8 and O27, O8 obtained 12:1 3OH, while O27 contained 12:0 iso 3OH, 14:0, 15:0 iso 2OH and 16:1 cis 9. Therefore, for identification based on the close chemotaxonomic relationship of the *V. cholerae* serogroup examined, it should be differentiated on the basis of fatty acid composition and the absence or the presence of fatty acid contents.

In conclusion, the fatty acid profiles according to a suitable data elaboration approach of *V. cholerae* were easily differentiated from the species and subspecies (serogroups) studied and it provides a basis for further epidemiological studies such as serogroup, PCR and LCR *et al.*, and it could be a useful, rapid and reproducible tool of identification once a proper database has been constructed.

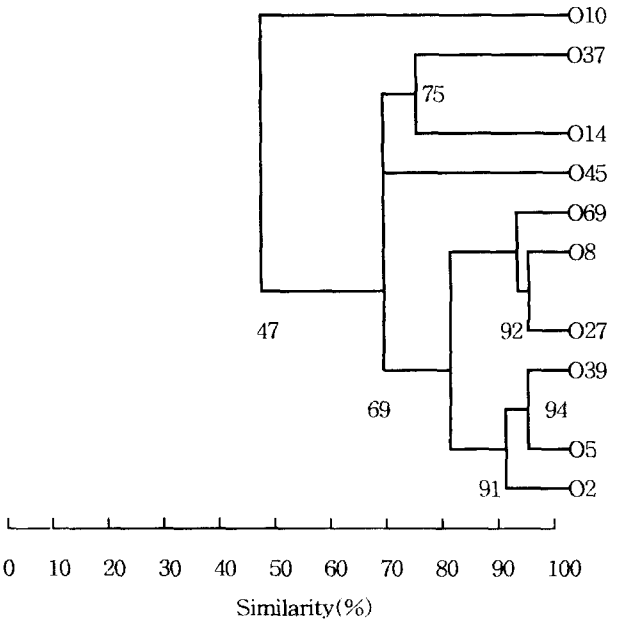

Fig. 2. Similarity dendrogram of *V. cholerae* non-O1 serotypes by fatty acid composition.

국문요약

V. cholerae non-O1 49균주와 *V. cholerae* O1, *V. mimicus, V. vulnificus*와 *V. parahaemolyticus*의 균체 지방산(fatty acid methyl ester; FAME)을 gas liquid chromatogrphy로 분석하였다. 이들 분석자료를 통계학적으로 처리하여 *Vibrio* 종과 *V. cholerae*의 혈청형별 유연성을 비교 검토하였다. 검출된 지방산은 모두 41종이었고 분포량이 많은 것은 16:0, 16:1 cis 9, 18:1 trans 9/16/cis 11과 15:0 iso 2 OH/16:1 cis 9 였다. 검출된 지방산 중에서도 35종은 *V. cholerae*를 동정하는데 주요한 인자로 작용되었다. 지방산분포를 UPGMA(비가중수리분석)으로 dendrogram을 작성한 결과 *V. cholerae* non-O1은 *V. cholerae* O1보다 *V. mimicus*가 더 높은 유사도를 나타내었다. 특히 hydoxy acid는 *V. cholerae*의 아종단위를 동정하는데 중요하였다. *V. choleare* non-O1 중에서 O2, O5, O8, O10, O14, O27, O37, O39, O45와 O69의 총 10 종류 혈청형을 대상으로 지방산 조성에 의한 유사성을 검토한 결과 유사도가 92% 이상 수준에서 7개의 아종를 형성하여 혈청형과 지방산 조성간에는 유의할 만한 상관관계가 있었다. 따라서 *V. cholerae* non-O1의 동정 및 역학적인 조사시 지방산 분석은 유용하게 활용될 수 있음을 알 수 있었다.

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