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THEORETICAL PAPERS AND REVIEWS

Genetic Diversity and Evolution of the Influenza C Virus

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Abstract—The influenza C virus is spread worldwide and causes diseases of the upper and (less frequently) lower respiratory tract in human. The virus is not pandemic, but it circulates together with pandemic influenza A and B viruses during winter months and has quite similar clinical manifestations. The influenza C virus is also encountered in animals (pigs and dogs) and is known to override the interspecific barriers of transmssion. The immune system of mammals often fails to recognize new antigenic variants of influenza C virus, which invariably arise in nature, resulting in outbreaks of diseases, although the structure of antigens in influenza C virus in general is much more stable than those of influenza viruses A and B. Variability of genetic information in natural isolates of viruses is determined by mutations, reassortment, and recombination. However, recombination events very rarely occur in genomes of negative-strand RNA viruses, including those of influenza, and virtually have no effect on their evolution. Unambiguous explanations for this phenomenon have thus far not been proposed. There is no proof of recombination processes in the influenza C virus genome. On the contrary, reassortant viruses derived from different strains of influenza C virus frequently appear in vitro and are likely to be common in nature. The genome of influenza C virus comprises seven segments. Based on the comparison of sequences in one of its genes (*HEF*), six genetic or antigenic lineages of this virus can be distinguished (Yamagata/26/81, Aichi/1/81, Mississippi/80, Taylor/1233/47, Sao Paulo/378/82, and Kanagawa/1/76). However, the available genetic data show that all the seven segments of the influenza C virus genome evolve independently.

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INTRODUCTION

The influenza C virus first isolated in 1947 belongs to the genus *Influenzavirus C* of the family Orthomyxoviridae and leads to the development of upper or lower respiratory tract inflammations, may cause bronchities, bronchiectasie, and broncho-pneumonia, especially in children, including infants under two years of age [1–5]. This virus is most frequently encountered in infants up to six years of age [1]. The virus is able to cause reinfections [2]. The symptoms are expressed in humans after the time period of nearly 11 days, which is comparable to the duration of this disease in dogs, which is about 10 days [5, 6]. Influenza C virus does not cause the appearance of pandemics and is not introduced into vaccines against seasonal influenza. In winter it circulates together with influenza A and B viruses causing epidemics of influenza and virtually does not differ from them in clinical features [2, 7]. It has been shown that influenza C virus is able to cause reinfection with A and B influenza viruses [1].

Although influenza C was extensively studied, information about its epidemiology is more scarce in general, compared to data on A and B influenza [8–11].

The majority of identified sequences of influenza C virus recorded in GenBank database NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/) are from Japan [5, 12], but there are also some data obtained by researchers from other countries. The main information on isolates from Japan has been gained during the last ten years, whereas most of data from other strains were ushered in during the 1980s. Information on nucleotide sequences from Russian isolates of influenza C virus is currently lacking in this database.

Serological and epidemiological studies show that influenza C is distributed worldwide, since many young people carry antibodies against this virus, which indicates that this disease is widely distributed among children. However, cases of diseases induced by influenza C virus were rarely reported [1, 13]. Up to very recently, the virus has been identified by serological methods, and also by isolating virus cultures. Yet despite the fact that these methods are successfully employed for research purposes, they are not close enough to effective methods used in clinical diagnostics and probably produced false negative results to a great extent because of low viral load in tissues of respiratory tract. Therefore, procedures of diagnostics of this disease are being developed at present with the use

of more sensitive methods, such as real-PCR assay, which allow identification of the lowest amounts of viral genetic material. There are also some works that show detection of influenza C virus using PCR with multiplex reverse transcription [2, 14].

STRUCTURE OF INFLUENZA C VIRUS

Influenza C virus generates cord-like structures of about 500 microns on the surface of infected cells [15, 16]. The viral genome comprises seven segments of negative-strand RNA (*PB2, PB1, P3, HE, NP, M*, and *NS*), unlike influenza A and B viruses that contain eight segments [14, 17]. The genome encodes polymerases (PB2, PB1, and P3), haemagglutinin-esterase (HEF, which is also designated as HE), nucleoprotein (NP), membrane protein (M1), the CM2 protein and two nonstructural proteins NS1 and NS2 [13, 17]. The testing of nucleotide sequences in segments of the influenza C virus genome somewhat differs: information on 154 sequences encoding haemagglutinin-esterase is recorded in GenBank database Influenza Virus Resource, other segments are represented by nine tens.

Polymerases PB2, PB1, P3 and nucleoprotein NP encoded by corresponding segments 1, 2, 3 and 5 are united with viral RNA, composing ribonucleoprotein complex of virus.

Haemagglutinin-esterase encoded by the fourth segment forms thorns on the surface of virus and have three biological functions: binds cell receptors, destroys receptors (acetylesterase activity), and possesses the ability for fusion to cell membrane (penetration activity) [17–21]. The three-dimensional structure of HEF consisting of three domains responsible for the indicated biological functions of the protein has been establised [19]. It has been shown that mature HEF contains two subunits (HEF1 and HEF2) connected with disulfide link [21]. In HEF, there are eight sites of glycosylation with four of them located in the domain responsible for fusion to membrane, three being located in the acetylesterase domain, and one in the domain that binds the receptor [22–24]. Glycosylation was shown to be essential for the formation of epitopes recognized by antibodies [25]. Analysis of the interaction of mutant protein with monoclonal antibodies against HEF revealed that glycosylation in position 144 is responsible for the formation of the epitope [24].

The sixth and seventh segments of viral RNA are represented by bicistronic genes. The nonsplicing mRNA of the sixth segment encodes the P42 protein 374 amino acid residues in length. This protein is hydrolized with signal peptidase to give two proteins: p31 (M1) and CM2, the splicing mRNA encodes the matrix CM1 protein (M1) that corresponds to 242 first amino acid residues of the P42 protein. The p31 protein, which is the N-terminal fragment of the P42 protein degrades immediately after its formation [26].

The M1 protein is present in the envelope in high amount and ensures the formation of the virus particle. It has been shown that the virus containing the M1 protein carrying amino acid substitution Ala24 $\longrightarrow$ Thr24 is unable to generate cordlike structures [15, 27, 28].

The CM2 protein is introduced into the envelope and forms dimers and tetramers bound with disulfide links, with the N-terminal end oriented outside the membrane [26]. This protein was shown to be pH modulating tok-activated ion channel with penetration activity of chlorine ions [29, 30].

The nonsplicing mRNA of the seventh segment potentially encodes the NS1 protein 246 amino acid residues in length, the splicing mRNA, encodes the NS2 protein 182 amino acid residues in length [31, 32]. The NS1 protein (but not NS2) is involved in the splicing of mRNA [33].

The NS2 protein is located inside the virus particle and seems to be associated in this particle with the ribonucleoprotein complex [34]. NS2 is functionally active in virus replication and was shown to play the role of protein essential for nuclear export (mediating transport of the ribonucleoprotein complex from nucleus into cytoplasm) [35].

THE FORMATION OF INFLUENZA C VIRUS VARIABILITY

Recombination

Study of variability of virus genomes is an important basic problem. Principally, it is connected with that the immune system of the organism often fails to recognize influenza viruses because of constant formation of new antigenic variants. It is known that nearly half of volunteers developed diseases after vaccination despite the high level of population seropositivity [5, 36]. In addition, we note the actuality of studying genetic alterations, which may be markers of high virulence and are connected with the most severe disease process [37].

RNA viruses evolve rapidly due to the high mutation rate, short period of replication, and large population size [38]. The source of genetic variability of RNA viruses is, apart from mutations, the processes of reassortment and recombination. The first process is encountered only in viruses whose genome is divided into several portions and represents the exchange of one or a number of discrete parts of RNA molecules, which are segments of the viral genome. The second process, recombination, is encountered in viruses with both segmented and nonsegmented genome [39]. Still, the frequency of recombination strongly varies among identified viruses, and this process is relatively rarely encountered among negative-strand RNA viruses. Homologous recombination proceeds in these viruses at a significantly lower rate than the rate of mutations [40]. In particular, it was shown that

homologous recombination is very rare in influenza A and B viruses and has virtually no effect on their evolution [40, 41]. Information about recombination events leading to the generation of definite influenza A strains [42] was not confirmed in later works [40].

No decisive evidence was provided for the existence of recombination processes in the influenza C virus genome. Analysis of variability types among gene sequences reveals that these processes most probably exist indeed [40]. However, 722 sequences referring to all seven segments of the influenza C virus genome, which are recorded in GenBank database NCBI Influenza Virus Resource, underwent recombination analysis in April 2010 by means of special algorithms, and in not one of the segments statistically significant results were obtained that might indicate the effect of recombination [4]. In any case, with consideration of the data obtained in study of influenza A and B viruses, one may conclude that homologous recombination exerts a minimal effect on evolution of influenza viruses.

There are some assumptions about the reasons for the rare incidence of homologous recombination among negative-strand viruses. First, supporting this view is the finding that these viruses often cause the acute stage of infection lasting for a short time, which diminishes the probability of infection with several strains simultaneously [40], but this view was disproved by the fact that negative-strand viruses, including influenza viruses, successfully generate reassortant viruses. Secondly, some data suggest that RNA of these viruses never exists in a free state; the RNA was only found in the ribonucleoprotein complex, and this can promote the ability of RNA polymerase to bring about template changes during replication. However, numerous data also suggest that RNA polymerase is capable of rapid template-changing; thus, the existence of ribonucleoprotein complex does not account well for impeding recombination processes. Thus, the reasons for this decrease in the level of recombination in negative-sense RNA viruses remain unknown [4, 40].

Reassortment

There is the justified notion that several strains of influenza C virus cocirculate in nature, which is the basis of the appearance of reassortants [31, 43]. For example, three different types of viruses were detected in Japan during a short period of time (from March 1981 to February 1982) [44]. Analysis of 15 strains isolated during two years, which was conducted with monoclonal antibodies against HEF and total RNA mapping, revealed the simultaneous presence of two distinct groups of viruses in one of cities [45]. The cocirculation of strains assigned to different lines was also detected in one of Japan cities [13].

Reassortants of RNA viruses appear as a consequence of exchange among genome segments of various strains, and this leads to the exchange of genetic information and the generation of new strains better adapted to the environment.

Reassortants between different strains of influenza C virus arise at high frequency in vitro [46]. Analysis of genomic RNA allows one to ascertain that frequent reassortment among different types of influenza C virus occurs in nature [4, 13, 46–49]. In particular, the strain C/Yamagata/9/88 is the reassortant with two segments inherited from C/Nara/2/85-like virus, and with five segments from C/Aichi/1/81-like virus [46]. The strain C/Yamagata/5/92 is the reassortant that inherited genes *HEF*, *P3*, *NP*, and *M* from C/Mississippi/80-like virus, whereas *PB2, PB1*, and *NS* came from C/pig/Beijing/115/81-like virus. A comparison of the complete sequence of gene *HEF* and partial sequences of other genes in strains C/Nara/1/85, C/Nara/2/85, and C/Nara/82 dhowed that C/Nara/1/85 inherited segments *HEf* and *NP* from C/Nara/2/85-like virus [46]. Phylogenetic analysis of sequences *M* and *HEF* from strains C/Yamagata/64, C/Kanagawa/1/76 C/Miyagi/77, and *HF* or *NS* from 34 different strains indicated that some of the examined strains are reassortants [31, 48]. Strains isolated in 1996–1998 in one of the cities in Japan are also reassortants with genes *PB2*, *PB1, HEF, M* and *NS* derived from pig/Beijing/115/81-like virus, and *P3* together with *NP*, from Mississippi/80-like gene [49]. Phylogenetic comparison of partial or complete sequences of all seven genomic segments showed that 44 of 45 examined strains isolated in 1990–1999 from various cities of Japan are reassortants [13].

Although influenza A, B, and C viruses have common ancestors, no reasortants between these viruses were observed, despite the fact that in artificially obtained influenza A viruses glycoprotein HEF was expressed instead of their own HA and NA [50].

Phylogeny of Influenza C Virus Strains

Phylogenetic analysis of genes in influenza C virus was conducted. For example, sequences of *HEF* genes from 18 strains isolated in Japan during 1964-1988 were examined. The results demonstrated that these strains are four discrete lines (Yamagata/26/81, Aichi/1/81, Aomori/74, and Mississippi/80), one of which (Aomori/74) ceased to exist after 1970 [51]. Thus, the stochastic hypothesis on so-called birth and death of lines in the process of virus evolution was put forward [5].

Three viral strains of 14 tested strains (Yamagata/1/92, Yamagata/1/93, and Miyagi/5/93) isolated in 1992-1993 in Japan were identical, but they differ from 60 strains isolated in the same country prior to 1992. A comparison of these three strains with eight isolates obtained outside Japan (from France, Republic of South Africa, United States, Greece, and Brazil) revealed that they constitute a single group with Brazilian isolate Sao Paulo/78/82. They were assigned to a separate line (the remaining 11 strains were attrib-

uted to earlier identified lines Yamagata/26/81, Aichi/1/81, and Mississippi/80). In the same work, another line (Taylor/1233/47) was detected [52].

To date, a total of six genetic or antigenic groups (lines) of influenza C virus (Yamagata/26/81, Aichi/1/81, Mississippi/80 Taylor/1233/47, Sao Paulo/378/82 and Kanagawa/1/76) have been isolated on the basis of comparing sequences of *HEF* genes [13, 51, 52]. Moreover, one of Yamagata/26/81 lines was additionally divided into two subgroups (Yamagata/26/81 and Pig/Beijing/115/81) [13].

From these data and with consideration of data on sequencing followed by phylogenetic analysis of sequences of *M* genes from 24 viral isolates obtained in 1964–1991, we also showed the evolution of three independent lines. One of M lines contains strains assigned to line Yamagata/26/81 isolated after analysis of *HEF* genes in this line, the other contains strains belonging to lines Aichi/1/81 and Mississippi/80, whereas the third line contains strains belonging to line Aomori/74 [48].

For genes *PB2*, *P3*, and *NP*, the trees were constructed with consideration of the data on partial nucleotide sequences, and three-four to five-six random lines were isolated [13, 31, 49].

Phylogenetic analysis of *NS* genes from 34 strains isolated in various strains (Japan, China, United States, Republic of South Africa, Great Britain) between 1947 and 1992 revealed that these genes are divided into two rather close groups A and B. Intragroup differences in gene sequences are equal to 0.1-2.5% for group A, while for group B, these differences vary from 1.5 to 4% [31].

Interestingly, the group A of *NS* genes contains 16 strains isolated prior to 1983, 12 of which were isolated prior to 1980. The group B comprise strains (a total of 18 strains) isolated after 1981; and 15 of these strains were isolated after 1985. The presented data testify in favor of the assumption that viruses of group B, which, for the most part, are reassortants, gradually replace more conserved viruses of group A (these viruses lack reassortants). Meanwhile, lines isolated after analysis of phylogenetic trees constructed for other genes of influenza C virus showed the simultaneous presence of strains isolated both over a long period of time and also in recent years [31].

The Rate of Influenza C Virus Evolution

Some publications have been devoted to the evolution of influenza C virus and analysis of rates of substitution accumulation in different segments of this virus. The rate of evolution among seven strains of the first line of *HEF* genes was estimated according to [51] as 4.9×10^{-4} nucleotide substitutions per site during one year (the same estimate of evolution was 4.87×10^{-4} nucleotide substitutions per site during one year [5]). This rate is about one-ninth times slower than the evolution rate of haemagglutinin gene of influenza A virus (the evolution rate of influenza A virus *H1* gene is equal to 4.3×10^{-3} nucleotide substitutions per site during one year, this rate being 4.44×10^{-3} for the *H3* gene [51]. Meanwhile, the HEF protein evolved 26 times slower than haemagglutinin H3 of influenza A virus [51]. The rate of substitution accumulation in influenza B virus is of the same order as in influenza C virus [5].

The second segment of influenza C virus genome (6.77×10^{-4} nucleotide substitutions per site during one year) evolves most rapidly, whereas the seventh segment (3.72×10^{-4} nucleotide substitutions per site during one year) evolves most slowly. The rate of evolution amounts to 4.32×10^{-4}, 5.49×10^{-4}, 5.74×10^{-4}, and 4.12×10^{-4} nucleotide substitutions per site for the first, second, third, fifth, and sixth segments, respectively. Thus, the rate of accumulation of nucleotide substitutions differs approximately twofold among various segments of the virus genome.

The rate of nonsynonymous substitutions is lower than that of synonymous substitutions in all seven segments of the viral genome, which suggests the operation of stabilizing selection, although some evidence for the action of positive selection in the receptor-binding domain have been provided in the site of gene *HEF* [5].

The variability of genes in influenza C virus and the encoded proteins was estimated. It was shown that the fourth segment of the genome encoding the HEF protein is most variable among all segments. In this segment, the number of nucleotide substitutions per site is 0.042, which is twice as low as that of the least variable first segment encoding the PB2 polymerase (0.017 nucleotide substitutions per site) [5]. Analysis of 34 strains of influenza C virus revealed that proteins NS1 and NS2 are sufficiently conserved, although the sequences of amino acids in the NS2 protein are slightly more divergent. Amino acid substitutions in the NS1 protein were found in 9.8% positions, and in the NS2, substitutions were encountered in 11.3% positions. Note that the NS1 protein of strain C/Taylor/47 differed from strains isolated 33 years later than strains C/Mississippi/80, C/Aichi/1/81, C/Nara/82, C/Kyoto/41/82, C/Hyogo/1/83 in only one or two amino acid substitutions. Proteins NS1 from two strains obtained with the exception that they were isolated with the difference of eight years and ascribed to different phylogenetic groups also differed in one amino acid. Proteins NS2 from strains C/Ann Arbor/50 and C/Mississippi/80 (isolated with the difference of 30 years) differed in one amino acid, whereas these proteins from strains C/Sapporo/71 and C/California /78 (the difference of 7 years) were completely identical [31].

The time period of the appearance of the most recent general influenza virus ancestors of contemporary viral genes was established to be 1890 for gene *HEF* and the period from 1930 to 1944 was established for the appearance of many other genes (except for the

gene encoding the NS protein); the most recent general ancestor of this gene appeared approximately in the year 1916 [15].

These results demonstrate that all seven segments of the influenza C virus genome evolve independently.

Stability of Antigenic Variants of Influenza C Virus

Among isolates of influenza C virus, various antigenic variants were found by the use of antigenic analysis with HEF monoclonal antibodies. A total of nine non-overlapping or partially overlapping antigenic sites are present on the surface of the HEF protein (AI-A5 and BI-B4) [21, 25].

Influenza C viruses may exist for a long time (seven to nine or more years) without change in the specificity of HEF antigenand have a significantly higher stability in this respect than influenza A and B viruses [13, 25, 43]. Thus, in the immunodominant site of the HEF protein, virtually no divergence was observed between different strains of the single line, although the clear differences between viruses of different lines were observed. The noticeable difference among viruses of different lines was observed which, according to data of the authors, suggests that the immune selection does not play a major role in HEF evolution, at least within the range of one line [51]. Analysis by using polyclonal serum revealed cross-reactivity among all examined isolates obtained in different time periods in different countries [13, 53]. Published data of another work have been reported suggesting that 24 strains from Japan assigned to one line, which were obtained in the period of 10 years, are antigenically indistinguishable from each other along with two strains of the same line isolated 10–30 years prior to this period [13].

With the use of monoclonal antibodies, it was shown that each of proteins M and NP contained at least two non-overlapping or partially overlapping antigenic sites (M-1 and M-1) and (NP-1 and NP-1), respectively [54]. The authors of this work believe that M and NP proteins are immunologically more conserved than HEF [54].

Some observations revealed that different antigenic groups are prevailing in the same geographical area during different years. Thus, line Aichi/1/81 was identified in one of cities in Japan as dominant in 1990–1991, line Mississippi/80 was shown to become predominant from 1993 to 1996, in the same year (1996), the line Yamagata/26/81 was found to become predominant and shown to circulate up to 1999.

Obviously, the genomic composition of influenza C virus affects its ability for the spread within population, and some reassortants have epidemiologic advantages over their parental viruses and start to circulate as a prevailing strain [13, 17].

DISTRIBUTION OF INFLUENZA C VIRUS IN THE WORLD, HUMAN POPULATIONS AND ANIMALS

Influenza C virus can be distributed worldwide. It is ascertained that seven isolates obtained outside Japan (in China, England, United States, and Republic of South Africa) in the period from 1966 to 1983 refer to three lines (Yamagata/26/81, Aichi/1/81, and Mississippi/80), representatives of which were also isolated during the 1980s in Japan [51]. Three strains isolated in Japan in the years 1992–1993 and one strain from Brazil also belong to the same line (Sao Paulo/378/82) [52].

Influenza C Virus in Animal Populations

The influenza C virus may cause infections not only in humans but also in pigs and dogs [17].

In the years 1981–1982, proteins and genomes of the influenza C virus were isolated from Chinese pigs. Mapping of genomic sequences revealed that they were rather similar but not identical to sequences of influenza C virus strains isolated from man [55]. Study that evaluates the presence of antibodies against influenza C virus in sera of the blood of 2000 pigs obtained in Great Britain demonstrated that 9.9% of samples were seropositive [56]. For comparison, seropositivity of pig sera pigs obtained in China and Japan was 1–8% (samples were taken each month for one year) and 19%, respectively [57, 58]. These results display that influenza C virus is widely spread in pig populations.

It was shown that the artificially introduced influenza C virus (strain Ann Arbor/1/50) causes disease symptoms (nose ailments, oedema, release of tears and slime from eyes of dogs) [6].

The influenza C virus can overcome interspecific barriers of transmssion. First, it is known that viral strains isolated from man (in particular, C/NJ/1/76) are able to reproduce in the organism of pig after experimental infection of pigs with influenza C virus [57]. Interspecific transmission was confirmed by other indirect data [59]. Therefore, in future, it is worth conducting studies for monitoring the spread of influenza C virus in economies dealing with breeding of pigs and cooperating with veterinary hospitals for the acquisition of most complete information about influenza C virus in dogs.

Seasonal Fluctuations of Influenza C Virus Distribution

Information about seasonal distribution of influenza C virus in populations of man and animals is scarce and was primarily produced due to material from Japan [2, 49, 60].

For example, analysis of this virus in infants under fifteen years of age, which had symptoms of respiratory disease, was conducted in Yamagata City (Japan) in the period from January 1996 to December 1998. More than 10000 samples were studied. Thirty three

various strains of influenza C virus were recorded; note that 20 strains were identified during a short period of time from May to August 1996 (with sixteen more strains of them registered for 24 days of June), 13 strains being found in March-June of 1998. However, not one of disease cases was registered in 1997. The frequency of virus identification in June 1996 and April 1998 reached 4.9 and 2.8%, respectively, although the average value of this virus occurrence per one year proved to be low (0.3%) [49].

Studies conducted for the period from January to July 2004 on material from 11 various prefectures of Japan demonstrated that the incidence of the virus was 1.4–2.5% (comparison with preceding works showed that it was the highest frequency for 14 years). Maximal temporal peaks of disease differed in dependence of the geographical area where material was collected and were observed in the single prefecture in March and several others, in January–February, and in one more case, were observed in February and May. In general, the researchers conclude that influenza C virus circulates in Japan over the whole studied semiperiod of 2004 [2].

In Russia, the work has been carried outwith the use of data obtained in the period from September to June in2005–2009 and in the spring of 2010 in Moscow. The detectionof virus was conducted in samples with the use of real-PCR assay.The sample consisted of 1868 children (preferentially, at the age ofone month to five years) and 31 adults (aged from 25 to 60 years) with symptoms of respiratory disease. The control group was composed of 106 samples of healthy children. The virus appeared in influenza patients with the highest frequency (8.3–18.2%) in September of 2005, January–February 2006, and in A[pril and October 2008. The average value of this virus occurrence per one year was 1–5%. In the control group, RNA of influenza C virus was found only in one sample (0.9%) in October, which coincides with the period of increased frequency of this virus appearance in children affected with influenza [37].

Experiments with animals also revealed the relation between different activities of influenza C virus and the season [57].

CONCLUSIONS

Intense migration flows in the world coupled with active evolutionary process in populations of influenza C virus create prerequisites for the occurrence of novel antigenic variants and their rapid global distribution. In this connection, they are extremely needed to quickly detect diagnostics and for typing of contemporary strains of the virus. A search for mutations, the markers of high virulence, together with constant monitoring aimed to reveal changes in the genome of this virus are of great importance.

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