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Short Communication

Recombinant duck enteritis virus works as a single-dose vaccine in broilers providing rapid protection against H5N1 influenza infection

Jinxiong Liu [1], Pucheng Chen [1], Yongping Jiang, Guohua Deng, Jianzhong Shi, Li Wu, Yuan Lin, Zhigao Bu, Hualan Chen *

Animal Influenza Laboratory, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, CAAS, 427 Maduan Street, Harbin 150001, PR China

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ABSTRACT

Although vaccination is an important strategy for controlling H5N1 avian influenza virus infections, broilers (short-lived meat chickens) remain the major victims in disease-endemic countries. Inactivated vaccine usually requires 2–3 weeks to establish solid protection, and recombinant vaccines, including the recombinant fowlpox virus vaccine and the recombinant Newcastle disease virus vaccine are affected by maternal antibodies against the vectors. These disadvantages compromise the protective efficacy of these vaccines in broilers. Here, we evaluated the safety and efficacy of a new recombinant duck enteritis virus that expresses the HA gene of an H5N1 virus (rDEV-re6) in specific-pathogen-free chickens and broilers. We found this new rDEV-re6 virus to be safe in chickens and to induce rapid and solid protection after a single dose. This virus may therefore serve as an ideal single-dose vaccine for broilers.

The H5N1 avian influenza viruses (AIVs) continue to pose challenges for the poultry industry and public health system. The H5N1 influenza virus is endemic in China, Vietnam, Indonesia, India, Bangladesh, and Egypt (Food and Agriculture Organization of the United Nations (FAO); http://www.fao.org/index_en.htm), and the populations of these countries combined represent 45% of the world's total population. For economic and cultural reasons, broilers (i.e., fast-growing chickens that are usually slaughtered at 5–6 weeks of age) are the most important source of animal protein for the people in these countries. However, the broiler industry is severely affected by H5N1 influenza infections in these areas. Vaccination is an important strategy to control H5N1 AIVs among poultry in endemic countries (Chen and Bu, 2009; Swayne, 2012). High levels of maternal antibodies derived from the H5N1-vaccinated breeders usually provide 1–2 weeks of protection to the newly hatched broilers against H5N1 viruses in their environment. However, the high level of maternal antibody and the immature immune system of these very young broilers adversely affect their active response to the vaccines (Chu and Rizk, 1975; Grossi et al., 1977; Kim et al., 2010; Lydyard et al., 1976). Therefore, in practice, vaccines are usually administered to broilers when they are more than one-week old. Inactivated vaccine is expensive, and usually requires 2–3 weeks to establish solid protection (Tian et al., 2005). Recombinant vaccines, including the recombinant fowlpox virus (FPV) vaccine (Beard et al., 1991; Qiao et al., 2003; Swayne et al., 2000), the recombinant Newcastle disease virus (NDV) vaccine (Ge et al., 2007; Park et al., 2006; Veits et al., 2006) and the recombinant herpesvirus of turkey (HVT) (Gimeno et al., 2011; Morgan et al., 1993) are affected by maternal antibodies against the vectors, and must usually be given multiple doses to induce sufficient protection in broilers. With these vaccines, therefore, broilers have a 1–2 weeks "unprotected" period. Accordingly, a fast-acting, low-cost, single-dose vaccine for broilers is desperately needed. We recently developed a recombinant duck enteritis virus (rDEV)-vectored vaccine and demonstrated that it provides rapid protection against both DEV and H5N1 virus infection in ducks (Liu et al., 2011). DEVs mainly circulate among *Anseriformes* (ducks, geese, and swans) (Sandhu and Shawky, 2003). Since chickens lack immunity to DEV, we explored whether the rDEV vaccine could be safe and effective in broilers.

We constructed a new recombinant DEV virus, rDEV-re6, by inserting the HA gene of a clade 2.3.2.1 virus, A/duck/Guangdong/S1322/2010 (H5N1) (GD/322), between the unique short (US) 7 and 8 genes of the DEV vaccine strain as described previously (Liu et al., 2011). To evaluate the safety of rDEV-re6 in chickens, 15 one-week-old specific-pathogen-free (SPF) chickens were intramuscularly (i.m.) injected with 10^6 plaque forming units (PFUs) of rDEV-re6. Three chickens were euthanized at 3 days, 1, 2, 3, and 4 weeks post-inoculation, and brain, duodenum, liver, bursa of Fabricius, blood, and oropharyngeal and cloacal swabs were collected for re-isolation of the rDEV-re6 virus in chicken embryo fibroblasts (CEFs). Chickens showed no signs of disease after receiving the rDEV-re6 virus, and the virus was not recovered from any tissues or swabs that were collected from the chickens at any

* Corresponding author.
E-mail address: hlchen1@yahoo.com (H. Chen).
[1] These authors contributed equally to this work.

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of the time-points tested. These results indicate that the rDEV-re6 virus does not replicate efficiently in chickens and is avirulent in this species.

To investigate the immunogenicity of the rDEV-re6 virus vaccine in chickens, three groups of 15 one-week-old SPF chickens, housed in an enhanced animal biosafety laboratory level 3 facility, were injected i.m. with 10^6 PFUs of rDEV-re6, DEV parent virus, and PBS, respectively. Twenty-four hours later, five naïve chickens were placed in the same cage with the rDEV-re6-immunized chickens so that we could monitor the transmissibility of rDEV-re6 by checking for seroconversion against H5N1 influenza and DEV virus. Sera were collected weekly from the chickens for hemagglutination inhibition (HI) antibody detection by using 0.5% chicken red blood cells (cRBCs). As shown in Fig. 1A, in the rDEV-re6-inoculated group, HI antibody was detected from 12 of the 15 chickens as early as 1 week post-vaccination (p.v.), and from all of the birds at the other time-points tested, with the mean titers ranging from 3.3 to 6.6 log2. HI antibody was not detected in the DEV virus- or the PBS-inoculated chickens (data not shown). Moreover, seroconversion was not detected in the chickens that were exposed to the rDEV-re6-inoculated chickens, indicating that rDEV-re6 is not transmissible among chickens.

At 6 weeks p.v., chickens were challenged intranasally with a 100 50% chicken lethal dose (CLD_{50}) of GD/322 virus. Three chickens in each group were euthanized at day 3 post-challenge (p.c.) and their brains and lungs were collected for virus titration in eggs. As shown in Fig. 1B, the challenge virus was not recovered from the lungs or brains of any of the birds in the rDEV-re6-vaccinated group, but high titer virus (5.5–7.5 $\log_{10}EID_{50}$) was detected in the lungs and brains of the birds in the DEV- and PBS-inoculated groups (Fig. 1B). In the rDEV-re6-vaccinated birds, no virus shedding was detected from any bird on days 3, 5, and 7 p.c., and all chickens survived the two-week observation period. However, in the DEV- and PBS-inoculated groups, challenge virus shedding was detected in all birds from the oropharyngeal and cloacal swabs on day 3 p.c. (Fig. 1C), and all of the chickens died within 5 days of the challenge (Fig. 1D). These results demonstrate that rDEV-re6 is immunogenic and provides sound protection against lethal H5N1 virus challenge in SPF chickens.

We further tested the immunogenicity and protective efficacy of rDEV-re6 in commercial broilers. Four hundred 1-week-old commercial broilers housed in a small farm were randomly divided into four groups and injected i.m. with 10^6 PFUs of rDEV-re6, DEV parent virus, 0.3 ml (containing 2.8 μg of HA protein) of H5N1 inactivated vaccine (Re-6 strain, whose HA and NA genes are derived from GD/322HA), or PBS, respectively. The broilers were also inoculated with vaccines that are routinely used against Newcastle disease, infectious bursal disease, and infectious bronchitis. Sera were randomly collected from 15 birds from each group weekly for H5 HI antibody detection by using 0.5% CRBCs. As shown in Fig. 2A, maternal HI antibodies to H5 AIV were detected from all birds in all four groups on the day of vaccination (week 0) with mean titers of 3.7–3.9 log2. At 1 week p.v., HI antibody was detected from 6–9 birds in each of the four groups, with mean titers of 0.8–1 log2. HI antibody titers were not detectable in the chickens that received the DEV and PBS inoculation by 2 weeks p.v.; however, at 2 weeks p.v., HI antibodies were detected in 14 and 15 birds, with mean titers of 3.4 and 4.8 log2, respectively, and at 3, 4, and 5 weeks p.v., HI antibodies were detected in all 15 birds, with mean titers ranging from 4.8 to 6.0 log2 in the rDEV-re-6-inoculated group and 6.6 to 8.2 log2 in the inactivated vaccine-inoculated group (Fig. 2A). These results indicate that maternal antibody is still detectable in 1-week-old broilers but declines to a very low level in 2-week-old broilers, and both the recombinant DEV vaccine and the inactivated vaccine induce comparable antibody responses when delivered to one-week-old broilers.

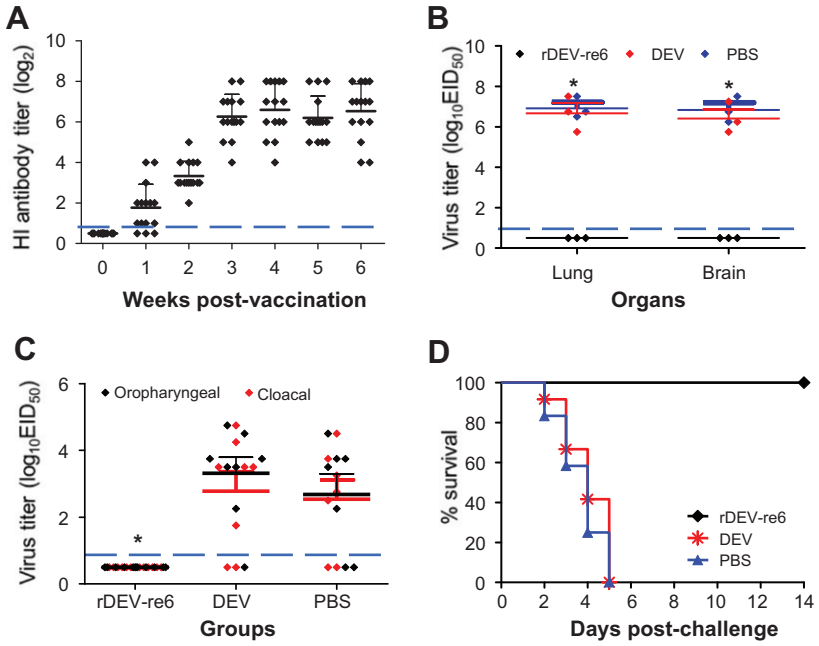

Fig. 1. Protective efficacy of the rDEV-re6 vaccine in SPF chickens against lethal H5N1 influenza virus challenge. Three groups of 15 one-week-old SPF chickens were injected i.m. with 10^6 PFUs of rDEV-re6, DEV parent virus, and PBS, respectively, and then challenged with 100-fold CLD_{50} of H5N1 virus GD/322. (A) HI antibody against H5N1 virus in chickens inoculated with the rDEV-re6 vaccine. The HI antibody was negative in the DEV- and PBS-inoculated groups (data not shown). (B) Challenge virus replication in the lungs and brains of chickens euthanized on day 3 p.c. (C) Challenge virus shedding detected on day 3 p.c. Virus shedding was also negative at day 5 and 7 in the chickens that were vaccinated with the rDEV-re6 vaccine (data not shown); no samples were collected from the chickens in the other two groups because all of the birds died within 5 days. (D) Percentage of surviving birds after challenge. The blue dashed line in panels A, B, and C indicates the limit of detection. The challenge virus replication among the different groups was compared by use of the two-sided *t*-test.*$p < 0.01$ when the titers in the chickens inoculated with rDEV-re6 were compared with those in the chickens inoculated with DEV or PBS.

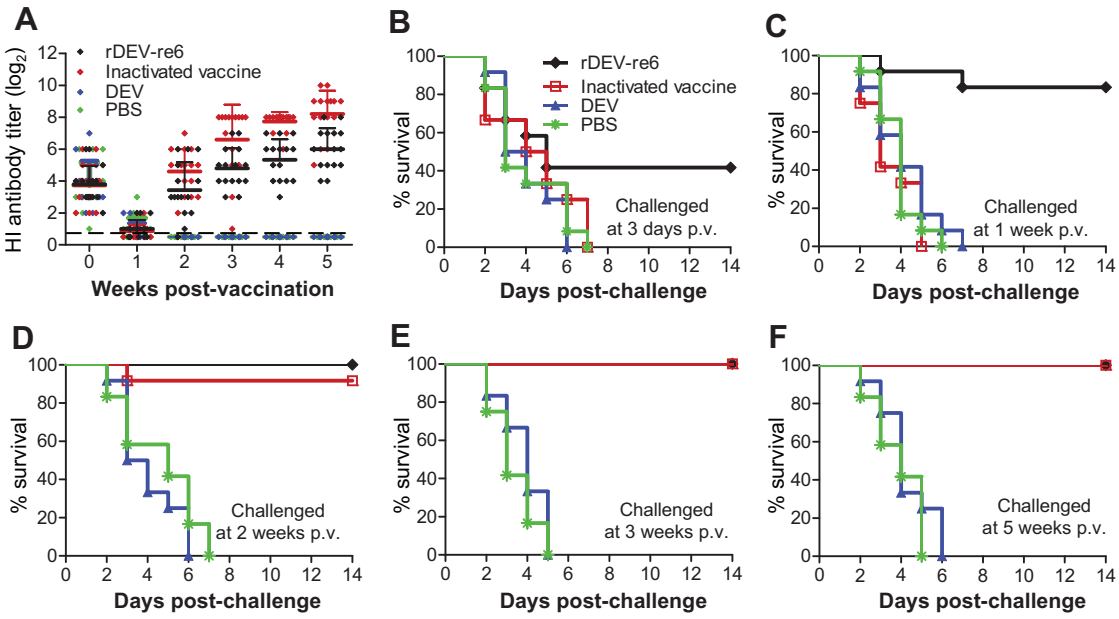

Fig. 2. Antibody response and protective efficacy of different vaccines in broilers challenged with H5N1 virus. A. HI antibody response against H5N1 virus. GD/322 was used as the antigen. The dashed line indicates the limit of detection. The percentage of surviving broilers challenged at 3 days, 1, 2, 3, and 5 weeks p.v. are shown in (B–F), respectively.

Fifteen birds from each group were transferred at 3 days, 1, 2, 3, and 5 weeks p.v., respectively, to the enhanced animal biosafety laboratory level 3 facility in Harbin Veterinary Research Institute, China and challenged with 100 CLD_{50} of GD/322 virus. Three birds from each group were euthanized on day 3 p.c. to test in eggs for challenge virus replication in the lungs and brain, while the remaining chickens were monitored for virus shedding and signs of disease or death for 2 weeks.

The challenge virus replication in the lungs and brains of birds was calculated by using the method of Reed and Muench (Reed and Muench, 1938) and compared by use of the two-sided *t*-test. The results are shown in Fig. 3. Virus was detected in the lungs, with mean titers that ranged from 7.5 to 8.5 $\log_{10}EID_{50}$, and in the brains, with mean titers that ranged from 6.0 to 7.6 $\log_{10}EID_{50}$, of all chickens inoculated with DEV and PBS and challenged at all five time-points p.v. (Fig. 3). In the rDEV-re6-vaccinated groups, challenge virus was detected in the lungs, with a mean titer of 2.8 $\log_{10}EID_{50}$, and the brains, with a mean titer of 2.5 $\log_{10}EID_{50}$, of all three birds that were challenged on day 3 p.v. and two of the three birds that were challenged at 1 week p.v, the mean titers in the lungs and brains of which were 1.1 and 1.2 $\log_{10}EID_{50}$, respectively. The titers were significantly lower than those in the birds of the other groups ($p < 0.01$); however, challenge virus was not detected in any birds that were challenged at 2, 3, or 5 weeks p.v. (Fig. 3). In the inactivated vaccine-inoculated groups, challenge virus replication was detected in all three birds challenged at 3 days p.v. and 1 week p.v. The mean titers were 7.8 and 5.8 $\log_{10}EID_{50}$, respectively, in the lungs and brains of the birds that were challenged on day 3 p.v., and were 8.2 and 6.1 $\log_{10}EID_{50}$, respectively, in the lungs and brains of the birds that were challenged at 1 week p.v.; these titers were comparable to those in the DEV- and PBS-inoculated chickens. Challenge virus was also detected from the lungs of two birds, with a mean titer of 1.4 $\log_{10}EID_{50}$, and the brain of one bird, with a titer of 1.8 $\log_{10}EID_{50}$, that was challenged at 2 weeks p.v.; titers were significantly lower than those in the chickens of the DEV- and PBS-inoculated groups

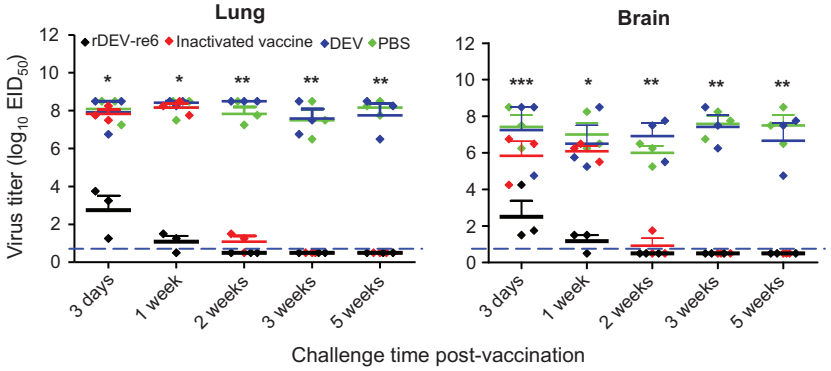

Fig. 3. H5N1 challenge virus replication in the lungs and brains of vaccinated broilers. Three chickens in each group were euthanized at day 3 p.c. at different time-points p.v., and their lungs and brains were harvested for virus titration in eggs. The blue dashed line indicates the limit of detection. The challenge virus replication among the different groups was compared by use of the two-sided t-test. $^{*}p < 0.01$ when the titers in the chickens inoculated with rDEV-re6 were compared with those in chickens inoculated with the inactivated vaccine, DEV, or PBS. $^{**}p < 0.01$ when the titers in the chickens inoculated with rDEV-re6 or the inactivated vaccine were compared with those in chickens inoculated with DEV or PBS. $^{***}p < 0.01$ when the titers in the chickens inoculated with rDEV-re6 were compared with those in chickens inoculated with DEV or PBS.

Table 1
Protective efficacy of rDEV-re6 and inactivated vaccine in broilers against H5N1 lethal virus challenge.[a]

Challenge time (post-vaccination)	Vaccine	No. of swabs shedding virus/total No. on day p.c.[b]						No. survival/total
		3		5		7		
		Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
3 Days	rDEV-re6	3/8	5/8	1/5	0/5	0/5	0/5	5/12
	Inactivated vaccine	6/8	7/8	3/5	0/5	NA[c]	NA	0/12
	DEV	5/6	6/6	3/3	3/3	NA	NA	0/12
	PBS	4/5	5/5	4/4	4/4	NA	NA	0/12
1 Week	rDEV-re6	1/11	2/11	1/11	0/11	0/10	0/10	10/12
	Inactivated vaccine	5/5	4/5	NA	NA	NA	NA	0/12
	DEV	4/7	5/7	2/2	2/2	NA	NA	0/12
	PBS	7/8	6/8	1/1	1/1	NA	NA	0/12
2 Weeks	rDEV-re6	0/12	0/12	0/12	0/12	0/12	0/12	12/12
	Inactivated vaccine	2/11	2/11	2/11	3/11	1/11	0/11	11/12
	DEV	3/6	4/6	3/3	3/3	NA	NA	0/12
	PBS	7/7	6/7	4/5	4/5	NA	NA	0/12
3 Weeks	rDEV-re6	0/12	0/12	0/12	0/12	0/12	0/12	12/12
	Inactivated vaccine	0/12	0/12	0/12	0/12	0/12	0/12	12/12
	DEV	6/8	7/8	NA	NA	NA	NA	0/12
	PBS	3/5	5/5	NA	NA	NA	NA	0/12
5 Weeks	rDEV-re6	0/12	0/12	0/12	0/12	0/12	0/12	12/12
	Inactivated vaccines	0/12	0/12	0/12	0/12	0/12	0/12	12/12
	DEV	8/9	9/9	3/3	3/3	NA	NA	0/12
	PBS	6/7	6/7	NA	NA	NA	NA	0/12

[a] Groups of 100 one-week-old broilers were inoculated intramuscularly with the 10^6 PFU of rDEV-re6, DEV, 0.3 ml of the H5N1 commercial inactivated vaccine, or PBS; 15 birds from each group were transferred to the laboratory at 3 days, 1, 2, 3, and 5 weeks p.v. and challenged with 100-fold CLD_{50} of H5N1 virus GD/322.
[b] Oropharyngeal and cloacal swabs were collected from all live chickens at the indicated days for virus detection in eggs.
[c] NA, no live birds were available.

($p < 0.01$). Challenge virus replication was not detected in chickens inoculated with the inactivated vaccine and challenged at 3 and 5 weeks p.v. (Fig. 3).

In the rDEV-re6-vaccinated groups, when the birds were challenged at 3 days p.v., five birds shed virus, and seven birds showed disease signs of tremor and torticollis and died within 5 days p.c.; when the birds were challenged at 1 week p.v., two of 12 birds shed virus and one died on day 3 p.c. and another died on day 7 p.c.; however, when the birds were challenged at 2, 3, and 5 weeks p.v., virus shedding was not detected from any birds, and they all stayed healthy and survived for the duration of the observation period (Table 1, Fig. 2). In the inactivated vaccine-inoculated groups, when the birds were challenged at 3 days and 1 week p.v., all of the birds shed virus, showed disease signs of tremor and torticollis and died within 7 days p.c.; when the birds were challenged at 2 weeks p.v., three birds shed virus and one of them died on day 3 p.c.; however, when the birds were challenged at 3 and 5 weeks p.c., virus shedding was not detected from any birds, and they all remained healthy and survived for the duration of the observation period (Table 1, Fig. 2). In the DEV- and PBS-inoculated chickens that were challenged at all five time-points, all birds shed virus, showed disease signs of tremor and torticollis and died within 7 days p.c. (Table 1, Fig. 2). It is noteworthy that maternal antibody was still present in some of the broilers when they were challenged at 3 days and 1 week p.v., but it did not provide protection against the lethal virus challenge, as all of the birds in the inactivated vaccine-, DEV- and PBS-inoculated groups died after challenge. These results indicated that the inoculation with rDEV-re6 could induce a rapid immune response and provide over 80% protection against lethal H5N1 virus challenge in broilers as early as 1 week p.c., which is about 1 week faster than the protective immune response induced by the inactivated vaccine.

We also investigated the potential influence of rDEV-re6 on the growth of broilers and on the efficacy of other vaccines that these birds may be inoculated with. The body weight of 20 randomly selected chickens from the rDEV-re6- and PBS-inoculated groups were recorded weekly and no differences were observed between the two groups (data not shown). HI antibody titers against Newcastle disease virus in the chickens of these two groups were also evaluated and found to be comparable (data not shown). These results indicate that inoculation with rDEV-re6 will not affect the growth of broilers or their immune response to other vaccines.

In summary, we demonstrated that the rDEV-re6 virus induces a rapid immune response and could work as a single-dose vaccine providing fast and solid protection to broilers against lethal H5N1 virus infection. rDEV does not replicate in or transmit among chickens, which means it cannot mutate into a pathogen in chickens and guarantees its safety as a live virus vaccine. The HI antibody titers at 1 week p.v. between the rDEV-re6- and the inactivated vaccine-inoculated groups are comparable, but the protective efficacy are different, suggesting that the rDEV-re6 may have induced specific cellular immune responses that play a role in the protection, though more studies are needed to fully understand the mechanism of rapid protection induced by rDEV-re6. If this vaccine is not used in breeder chickens, the broilers will never develop maternal antibodies against the DEV vector. The rapid immune response and over 80% protection in as little as 1 week p.v. induced by the rDEV vaccine will significantly shorten or cover the "unprotected" period in broilers, a problem that currently available vaccines cannot solve. Moreover, our study suggests that the DEV vaccine strain can also be used as vector to generate recombinant vaccines to protect against other lethal diseases, such as Newcastle disease and infectious bursal disease in broilers.

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