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Efficacy of a locally prepared live clone vaccine against Newcastle disease virus genotype IV and genotype VII d in Egypt

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ABSTRACT

Vaccines against the virulent Newcastle disease virus (NDV) are broadly existing and can provide protection; nevertheless, better immunization practices are required to avoid clinical disease and limit virus circulation. This study evaluating the immunogenicity and protective efficiency of locally prepared clone 30 live-attenuated vaccine against the challenge impact of virulent NDV genotype IV and genotype VIId prevalent in Egypt in comparison with the commercially prepared live Lasota vaccine as a positive control group. The efficacy of the vaccine was evaluated based on the antibody titer, protection rate, oropharyngeal, and cloacal shedding. Therefore, 150 one day old specific pathogen free chicks (SPF) were divided in to three groups 50 bird per group (G), G1and G2 received 100 µl containing 6 $\log_{10}$ EID50 via the oculo-nasal route of clone 30 vaccine and lasota vaccine in order, while G3 (unvaccinated) received sterile saline at the same route and dose. On day 21 post vaccination (pv) 40 bird from each group were challenged with a dose of 6.5 $\log_{10}$/ml EID50 intramuscular per bird for both genotype IV and genotype VIId (20 bird /genotype virus), the other 10 birds left from each group were kept separate for antibody level monitoring for the 6th week pv. Results revealed that, during vaccine preparation, the clone 30 virus showed a high virus titer when propagated in SPF embryonating chicken eggs (SPF-ECEs), which reached 10^{12}/EID50/ml. The protection rate due to the clone 30 vaccine and the lasota vaccine was alike and showed 75% and 70% against challenge with genotype IV and genotype VIId, respectively, there were no significant differences ($p > 0.05$) between the antibody titer produced by the clone 30 vaccinated group and lasota group. Both the clone 30 and lasota vaccines showed nearly similar levels of oropharyngeal and cloacal shedding. The results clarify that, although there were no detected differences between the immune response and the protective efficacy of clone 30 vaccine and lasota vaccine but, the use of clone 30 vaccine is still advantageous for its superior immunogenicity and low post-vaccinal reaction, which will make the clone 30 vaccine suggestive for primary immunization, especially in immunologically naive birds. In conclusion, the prepared Clone 30 vaccine in the current study is safe for chicks and can be used as an effective vaccine against the circulating NDV.

Introduction

Since its discovery in Java, Indonesia, in 1926, the Newcastle disease virus (NDV) has been one of the most important viruses affecting the poultry industry. NDV is a variant of avian ortho-avulavirus 1 (AOAV-1) (Dimitrov *et al*., 2019; Walker *et al*., 2020), single-stranded negative-sense RNA virus that belongs to the subfamily Avulavirinae and infects large poultry populations (Rima *et al*., 2019). Newcastle disease virus is a list A disease (OIE, 2021), and since its discovery in Egypt in 1948 (Daubney and Mansy, 1948), it has continued to cause severe economic losses. Its control in Egypt or all over the world depends mainly on vaccination. Many vaccines and vaccination tactics are available in the Egyptian field; yet, despite all these intense vaccination programs and strategies, NDV remains a bothersome problem for the poultry industry. Vaccination is primarily based on the use of live attenuated strain vaccines (Lasota, HB1) and inactivated Lasota-dependent vaccinations. During using live attenuated vaccine, it is important to consider the level of vaccine reaction, and that is why HB is widely used during initial vaccination of poultry due to its low vaccinal reaction (Bell *et al*., 1990), on the other hand lasota vaccine is not suggested for primary vaccination because it yield a moderate post vaccinal reactions, particularly in immunologically naive birds. Clone 30 is a lentogenic vaccine strain that is cloned from lasota strain and characterized by superior immunogenicity compared to a HB1- virus and less vaccinal reaction than lasota strain virus (Alexander *et al*.,2004).

In Egyptian records, virulent genotype VIId and genotype VI were identified as NDV strains that produce outbreaks and losses in egg production, as well as a high mortality rate and substantial losses in the poultry industry (Miller and Koch, 2013). Many studies have attempted to undertake various sorts of vaccine formulations in an attempt to counteract or reduce the losses caused by the virus. (Hossain *et al*., 2023; Megahed *et al*., 2023). As a result, this study is considered a further attempt to limit the virus's morbidity and to reduce the virus's environmental load by investigating the effect of the manufactured vaccine on virus shedding through the production and evaluation of a locally produced clone 30 vaccine in Egypt.

Materials and methods

Ethical approval

All Veterinary Serum and Vaccine Research Institute (VSVRI) institutional guidelines for animal care and use were followed.

Vaccine strain and challenge viruses

Lentogenic Genotype II NDV clone 30 virus was used for the preparation of the clone 30 vaccine and the vaccination of birds, supplied kindly by Pirbright, for the vaccination of chicks. Lasota vaccine (used as control positive vaccine) is a commercial live attenuated vaccine with a known titer (Batch #22037), supplied by the poultry viral vaccine department (VSVRI).

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Two virulent Egyptian local isolates of Newcastle disease virus (NDV) strains were utilized as challenge viruses, genotype VIId, kindly provided by the National Laboratory for Veterinary Control of Poultry Production (accession # KM288609). Genotype IV, virulent NDV strain (SR/76) isolated by Sheble and Reda (1976), graciously donated by the poultry vaccine production unit (VSVRI).

All used viruses were propagated on nine day old SPF embryonating chicken eggs (SPF-ECEs), and the allantoic fluid was harvested (after 5 days for clone 30 and after 48hrs for both virulent NDV genotypes) aseptically and preserved at 80°C till use.

According to Anon (1971), a tenfold serial dilution of each virus strains were inoculated in 9 days old -SPF embryonating chicken eggs (ECE) (5 eggs for each dilution) to determine the titer of all the used viruses. The egg infective dose fifty (EID50) was then estimated using Reed and Munch's formula (1938).

According to OIE (2021), the intracerebral pathogenicity index (ICPI) was tested for both virulent NDV genotypes by intracerebral inoculation of one-day-old SPF chicks with 0.05 ml of 1/10 diluted fresh infective allantoic fluid free from any extraneous contaminant. Birds were observed daily for 8 days and scored: 0 if normal, 1 if sick, and 2 if dead. (ICPI) represents the mean score per bird per observation during an 8-day period.

Preparation of the vaccine, sterility, and safety testing

The vaccine was prepared following the standard operating procedures (SOPs) and the legislation of the Veterinary Serum and Vaccine Research Institute (VSVRI) for the preparation of live attenuated lyophilized vaccine. The collected clone 30 allantoic fluids were titrated in 9-day-old SPF-ECEs following Reed and Muench (1938). Mixing of the allantoic fluid with sterile skimmed milk (10%) as a stabilizer, dispensing in to vials then lyophilized. The desired clone 30 vaccine titer per vial must be not less than 10^8 EID50/ml. Following the standard procedures of OIE (2021) and the Egyptian Legislations of the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB); Safety and sterility were tested according to the standard procedures of culture in an aerobic and aerobic bacterial and fungal growth medium for a week and was inspected daily for any microbial growth. Five birds (one day old) were inoculated with a 10-times vaccination dose to test the safety of the produced vaccine.

Evaluation of the immunogenicity and protective efficacy of the prepared vaccine

The vaccine's potency was evaluated in two ways: first testing was performed by quantifying the virus titer per dosage for each bird using titration of the vaccine vial by EID50 according to OIE (2021). Second, the efficacy of the produced vaccine on SPF chicks against the challenge with two locally circulating virulent strains, (genotype IV and genotype VIId).

Experimental design

SPF chicks were achieved from the national project Nile-SPF-Eggs Farm, Koom Oshiem, Fayoum, Egypt. All chicks were maintained in isolators under positive pressure, received water and food ad libitum. One-day-old SPF chicks (n = 150) (serologically evaluated to ensure absence of maternal immunity) were divided into three groups: two vaccinated groups, the clone 30 vaccine group (G1), the Lasota vaccine group (G2) (n = 50 per group), and the unvaccinated group (G3) (n = 50). The vaccination was done on day 7 of age. Each chick received 100 µl containing 6 $\log_{10}$ EID50 via the oculo-nasal route, while the unvaccinated group received 100 µl of sterile distilled water via the same route. All chicks were kept in a bio isolator and received sterile food and water. On day 21 post-vaccination (PV), 40 chicks from each vaccinated group (20 chicks challenged with genotype IV and another 20 chicks challenged with genotype VIId) and 40 chicks from the unvaccinated group were challenged with 0.5 ml of genotype IV and genotype VIId (20 birds per virus) at a dose of 6.5 $\log_{10}$/ml EID50 intramuscular per bird. All the challenged chicks were kept under observation and monitoring for mortalities and clinical signs. Post-challenge (pc) oropharyngeal and cloacal swabs were taken from 5 birds and pooled in sterile saline at days (3, 5, and 7) and (5, 7, and 10) (pc), respectively, from all challenged birds (vaccinated and unvaccinated) to measure the virus shedding. The unchallenged G1 and G2 vaccinated groups (n = 10 /group) and unchallenged unvaccinated G3 (n = 10) were kept separated from the challenged ones for the 6th week post-vaccination for measuring the antibody titer, utilizing five birds per group each week to measure the antibody titer in their sera.

Measuring antibody titer following vaccination by Haemagglutination inhibition test (HI)

The antibody titers were measured in G1 and G2 (5 birds / group), utilizing 50 µl of a twofold serially diluted tested serum sample against the HAU Lasota virus (as commonly used). The antibody titer is calculated by the highest serum dilution that can inhibit the Haemagglutination activity of the virus. (OIE 2021).

RNA extraction and measuring viral shedding after challenge by quantitative Real-time Reverse Transcription PCR (RT-qPCR)

RNA was isolated from cloacal and oropharyngeal swabs following the manufacture instructions of the Pure Link® (Invitrogen, USA) RNA Mini Kit, and RNA was stored at -20 until use. One-step quantitative (RT-qPCR) using TOPrealTM One-Step SYBR Green with Low ROX RT-qPCR Kit (Enzynomics, Korea) following the manufacturer's instructions and using the CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, USA). Specific primers target the matrix protein (M) gene of APMV-1 virus were used. (MF: 5'-AGTGATGTGCTCGGACCTTC-3'; MR: 5'-CCTGAGGAGAGGCATTTGCTA-3') according to (Wise *et al*., 2004). The cycling conditions were as follows: firstly, reverse transcription step at 50 °C for 30 min, followed by 10 min at 95°C for inactivation of reverse transcriptase enzyme and initial denaturation. Followed by 35 cycles of 95°C denaturation for 5 seconds, then 52°C annealing for 10 seconds, and 60°C extension for 30 seconds. To determine the specificity of amplification, a melting curve was performed. A standard curve was created by qRT-PCR utilizing RNA extracted from known EID50 titers of a commercially licensed lasota virus vaccine (Batch #22037) to convert the resulted cycle threshold (Ct) value into viral titers.

Statistical analysis

The statistical analysis of antibody titers was estimated by one way ANOVA analysis using Tukeys the analysis was done at confidence interval of 95%.

Results

Vaccine and viruses

Using Read and Munch equation the titer of clone 30 virus was 10^{12}/EID50/ml while the titer of genotype IV and genotype VIId viruses was 10^9 and 10^{10}/ml respectively.

The ICPI values of both genotype IV and genotype VIId ranged from 1.64 and 1.72 respectively.

The produced clone 30 vaccine fulfill the needed criteria for the physically inspected vaccine, as presence of vacuum and uniformed desk appearance. The sterility test showed that the produced vaccines is sterile and free from any contaminant, which was consistent with the safety test, in which all chicks were alive and exhibited excellent vitality.

Safety, sterility and quantifying the virus titer of the prepared vaccine

The clone 30 vaccine was safe for the chicks where no clinical signs were observed on them. The sterility testing of the vaccine showed no growth of any contaminant along the inspection period. The vaccine potency was measured by testing the titration of the clone 30 vaccine titer was 10^{11}/ml.

Antibody titer following vaccination by Haemagglutination inhibition test (HI)

Experimental birds showed negative antibody titer when tested with HI test for the existence of maternal antibodies against NDV.

Regarding the antibody titer due to the clone 30 vaccine, along the immunization period, the antibody titer showed significant differences ($p<0.05$) between the 3rd week post vaccination (pv) and both 5th and 6th week (pv).also, significant difference seen between the 4th and 6th week (pv).While no significant differences ($p>0.05$) between the antibody titers produced by the clone 30 vaccinated group when compared with the positive control lasota immunized group. (Table 1and Figure 1).

Clone 30 vaccine efficacy against NDV genotype IV and genotype VIId challenge, clinical signs and gross lesions

As shown in (Table 2) nearly 2/20 (10%) of birds in both G1 and G2 showed signs of illness, respiratory and nervous signs, nasal discharge, and diarrhea when challenged with NDV genotype IV. While vaccinated birds in both groups showed general signs of illness (3/20:4/20) (15%: 20%) in both G1 and G2 in order after challenge with genotype VIId, the unvaccinated challenge group with genotype IV and genotype VIId showed nervous signs and general signs of illness in all birds. Regarding genotype IV deaths recorded on the second day post-challenge (pc), by the 5th day (pc), all birds had died, while genotype VIId deaths were recorded on the 3rd day (pc), and by the 7th day, all birds had died.

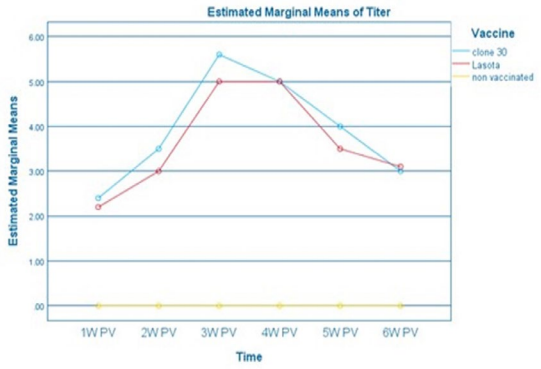

Fig.1. Mean HI Antibody titers in sera of different vaccinated chicks of different groups.

Measuring viral shedding after challenge by quantitative Real-time Reverse Transcription PCR (RT-qPCR)

Oropharyngeal and cloacal shedding were assessed for all challenged birds with both genotypes, except at some time points in the unvaccinated challenged groups that were not applicable (NA), where these chicks died. All tested samples showed positive shedding levels for NDV when estimated with the presence of the standard curve (Table 3 and Figure 2).

Table 1. Mean ND-HI antibody titers in different vaccinated chick groups.

Vaccinated groups	Mean ND-HI antibody titer expressed as log2±SE					
	1WPV*	2WPV	3WPV	4WPV	5WPV	6WPV
G1	2.4±0.18	3.5±0.15	5.6±0.18	5.0±0.15	4.0±0.31	3.0±0.35
G2.	2.2±0.2	3±0.27	5.0±0.31	4.0±0.22	3.5±0.15	3.1±0.29

*WPV: week post vaccination; G1: vaccinated with clone 30; G2: vaccinated with Lasota; G3: unvaccinated control

Table 2. Clinical observation of different chick groups 10 days post challenge

Observed clinical signs	Challenged with Genotype IV		Challenged with Genotype VIId		G3 challenged with	
	G1	G2	G1	G2	Genotype IV	Genotype VIId
Respiratory signs	2/20	2/20	2/20	2/20	8/20	18/20
Nervous signs	2/20	2/20	2/20	2/20	8/20	2/20
Nasal discharge	2/20	3/20	2/20	2/20	16/20	14/20
Diarrhea	2/20	2/20	2/20	2/20	18/20	16/20
General signs of Illness	2/20	2/20	3/20	4/20	18/20	18/20
Protection rate	75%	70%	75%	70%	0%	0%
Mortality	5 / 20	5/20	5/20	6/20	20/20	20/20

G1: vaccinated with clone 30; G2: vaccinated with Lasota; G3: unvaccinated control

Table 3. Viral shedding expressed by log 10 at different point time post challenge (pc) measured by syber green quantitative Real-time Reverse Transcription PCR (RT-qPCR)

Challenge virus	Groups	Oropharyngeal shedding			Cloacal shedding		
		3d pc	5d pc	7d pc	5d pc	7d pc	10d pc
Genotype IV	Clone 30	1.6	2.4	2.9	3	4.6	2.9
	Lasota	1.69	2.8	3	2.9	4.6	2.79
	unvaccinated	4.5	NA	NA	NA	NA	NA
Genotype VIId	Clone 30	2.6	3.3	2.16	1.9	3.4	3.78
	Lasota	2.7	3.35	2	1.8	3.5	3.9
	unvaccinated	3.6	4.53	NA	2.1	NA	NA

NA: (not applicable) due to death of checks at this time point; d PC: days post challenge

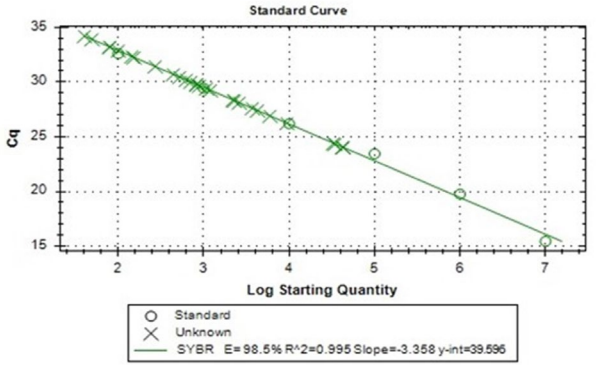

Fig. 2. Standard curve initiated to evaluate the shedding virus titer.

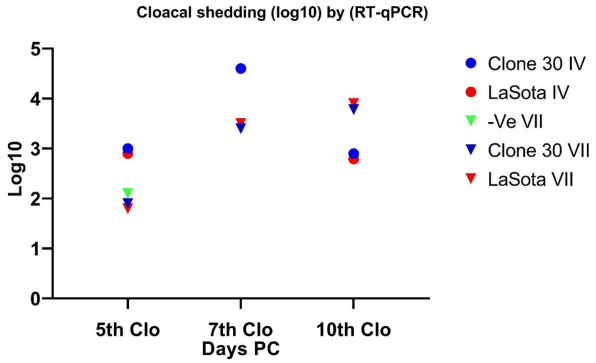

Fig. 4. Cloacal shedding of both vaccinated groups after challenge with both virulent NDV genotypes.

Regarding oropharyngeal swabs, among the vaccinated groups challenged with genotype IV NDV, the shedding titer expressed by log_{10} in both vaccinated groups showed a reduction (1.6:3) in comparison with the unvaccinated challenged group that showed a virus titer of 4.5 at the 3rd dpc (the only detected time point). On the other hand, the vaccinated groups challenged with genotype VIId showed a shedding level titer range of 2 to 3.35 versus the unvaccinated challenged group that showed a shedding titer of (3.6: 4.53) at the 3rd and 5th dpc (the only detected time points). Both clone 30 and lasota vaccines showed nearly similar levels of shedding during the oropharyngeal shedding of both challenged genotypes (1.6: 3.3) and (1.6: 3.3) for both clone 30 and lasota vaccines, respectively (Figure 3).

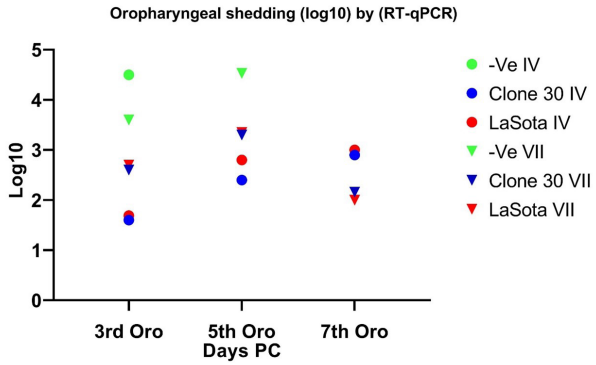

Fig. 3. Oropharyngeal shedding of both vaccinated groups after challenge with both virulent NDV genotypes.

Cloacal shedding of vaccinated groups challenged with genotype IV showed a shedding level ranging from 2.7 to 4.6, while the unvaccinated challenged group's shedding level couldn't be detected where all chicks died at this time point. Meanwhile, the vaccinated groups challenged with genotype VIId showed a shedding level of (1.8:3.9) when compared with the unvaccinated challenged group, which showed a level of 2.1 at the 3rd dpc (other days NA). Both clone 30 and lasota vaccines showed nearly similar levels of shedding during the cloacal shedding of both challenged genotypes (1.9:4.6) and (1.8:4.6) for both clone 30 and lasota vaccines, respectively (Figure 4).

Discussion

Live-attenuated vaccines have been used since the 1950s and have made great progress in controlling and preventing ND (Dimitrov *et al.*, 2017). Due to the endemicity of the virus, many vaccine regimes were used to reduce the morbidity and motility of the disease, and the main goal of any vaccine produced is not only to produce a high antibody titer and protection rate but also to maintain the virus shedding to the lowest level in order to decrease the environmental load and decrease NDV transmissibility across surrounding poultry flocks (Palya *et al.*, 2012). Live attenuated and inactivated NDV genotype II-based vaccines are used utilizing Lasota, Hitchner B1, and inactivated virulent NDV strains during vaccine formulation (Kapczynski *et al.*, 2013; Sultan *et al.*, 2020). During our study, a clone 30 vaccine strain was prepared and evaluated together with a commercially available Lasota vaccine, locally prepared by VSVRI, for their immunogenicity and protective efficacy. During vaccine titration, clone 30 showed titer 10^{11}, and this was also very clear during egg propagation, where clone showed high replicative ability, hence the high titer produced. This is considered an important point during vaccine preparation as it helps us during large-scale preparation and decreases the costs of production. Antibodies produced against the F and HN glycoproteins are considered critical for NDV neutralization and consequently protection from vNDV (Reynolds and Maraqa, 2000). Antibodies against the F glycoprotein are responsible for fusion of the virus with the host cell membrane; on the other hand, HN antibodies block viral attachment. Interestingly, low antibody levels have the ability to protect chickens against challenge with vNDV (Gough and Allen, 1973). During our study, the results of the humoral immune response were not surprising where, no significant differences ($p>0.05$) between the antibody titers produced due to clone 30 vaccine when compared with the positive control Lasota group, where the antibody titers reached their highest levels (5 log2 and 4 log2) by the 4th week on both G1 and G2, respectively, then decreased by the 5th week to reach 4 log_2 and 3, 5 log_2 in both G1 and G2 in order. These results may be explained by the previous work of Madbouly *et al.*, (2021), who recorded that there are no differences between the F gene of both clone 30 and lasota at the amino acid levels at these positions (cleavage site, fusion peptide, glycosylation site, Heptad repeat (HR) regions). meanwhile, the analysis of the HN gene at the level of amino acids of both lasota and clone 30 revealed that both are similar at the transmembrane domain, head-stalk linker region, and neutralizing epitopes at the C-terminal globular head; but the analysis of the Heptad repeat (HR) region revealed the similarity only at the HRa (74–88) region, while the region HRb (96-110) differs in clone 30. This may be the explanation why both vaccines have such low levels of antibody titers. During the work of Cornax *et al.* (2012) they demonstrated that the live Lasota vaccination can induce protection in chickens when challenged with a virulent Newcastle disease virus of heterologous genotype. Similar results were obtained during our study, where the protection rate due to the Lasota vaccine was 70% against the challenge with genotype IV and genotype VIId, while the clone 30 vaccine induced a protection rate of 75% against the challenge with genotype IV and genotype VIId. During measuring viral shedding observed during the challenge with both genotypes, it was very clear that there are no great differences in the oropharyngeal and cloacal shedding of both vaccinated groups, G1 that was vaccinated with the clone vaccine and G2 that received the lasota group. This may be explained due to the known superior immunogenicity of clone 30 than lasota vaccine (Alexander *et al.*, 2004). Our results go with the fact that prevention of infection following virus challenge is mainly mediated by the generated neutralizing antibodies (Reynolds and Maraqa, 2000; Yu *et al.*, 2001). These results were also explained previously by Dimitrov *et al.* (2017) who concluded that using a single dose of a live attenuated vaccine can provide complete clinical protection but can't prevent challenge virus shedding.

Conclusion

The generated clone 30 vaccine is safe in chicks and can be utilized as an effective vaccine against the circulating NDV.

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Conflict of interest

The authors declare that they have no conflict of interest.

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