

Higher Immunological Protection of Pandemic 2009 H1N1 Influenza Live Virus Infection than Split Vaccine Against the Homologous Virus for Long Term Immunization in Ferret

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Abstract The study was to evaluate the long term immunological efficacy of pandemic 2009 H1N1 influenza live virus infection and split vaccine against the homologous virus challenge in ferrets. Antibodies in ferrets were monitored by haemagglutination inhibition (HI) assay for 200 days, the HI titers of both infected-only and vaccinated plus infected ferrets could maintain a high level for at least 182 days, without significant difference between the two infected groups. While one-dose and two-dose vaccinated ferrets could last a moderate antibody titers for 81 days, with its peak value at day 7 post immunization. After the virus challenge at day 207, the two groups of vaccinated ferrets shed virus for longer time than the two infected groups, while the latter two groups basically did not shed any virus particles. Furthermore, the vaccinated and infected ferrets which were sacrificed at day 211 exerted moderate immune protection against the challenge by alleviating clinical signs and lung lesion without obvious difference between groups. These data supported that both one-dose and two-dose vaccination of 2009 influenza A (H1N1) split vaccine conferred a moderate protection against challenge after 207 days, and there was no significant difference between the two groups. Either the infected only or vaccinated plus infected ones exerted more effective protective immune than one-dose and two-dose vaccination against the challenge, especially in

preventing virus shedding, and vaccination primed before infection had no additional efficacy.

Keywords Pandemic 2009 (H1N1) split vaccine · One-dose · Two-dose · Infected vaccinated plus infected

Introduction

Rapidly spread worldwide since April 2009, pandemic influenza A (H1N1) virus was endangering public health. Although the post-pandemic period of H1N1 influenza virus was controlled [13], the recurrence of H1N1 influenza as seasonal influenza cannot be excluded as now ([13], <http://www.who.int/csr/disease/swineflu>).

Several types of vaccine were developed to guard against 2009 pandemic influenza A (H1N1) virus. Split virus vaccine had been broadly used as an economical and effective weapon for the prophylaxis [7].

To evaluate the efficacy and safety of the vaccination, one mass vaccination program associated with different strategy and age group was conducted in several countries. The study revealed that one single dose was sufficient to elicit a potentially protective antibody response in the majority of vaccinees more than 10 years of age in short term, while a second dose of vaccine after 21 days was needed to boost immune responses in infants and toddlers 6 months to 3 years of age and, with some vaccines, in children aged 3 to 9 years. But for the long term, whether a second dose was needed or not, it was unknown. [5, 6, 14].

Furthermore, for the one-dose vaccinated or infected-only people, a certain 180-days study was carried out, and HI antibody analysis and adverse effect were monitored, but no further study was reported with the actual evidence in infection/re-infection, especially for the vaccinated plus

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infected ones, which even had no attention be paid on [11, 12]. So the risk of infection/re-infection were not assessed for long term, and the immune regimes were not clear either the infected or the vaccinated plus infected people.

In addition, it was believed that the mucosal immune elicited by live attenuated virus was more promising than system immune mounted by inactive influenza virus vaccine. However, the immune efficacy of live virus was hindered for the reason that the broad application both in experimental animals and people was not identified [6].

Considered as an excellent model for influenza vaccine efficacy assessments, ferrets were used to evaluate the inactive split or whole virus vaccine against A/Mexico/InDRE4487/2009 H1N1 virus challenge 5 weeks post immunization [1, 2, 9, 10]. But there were no reports about the long term study for the vaccinated (one-dose and two-dose vaccinated) and infected (the infected-only and vaccinated plus infected) ferrets against any H1N1 virus strain challenge.

This study is to investigate the antibody dynamics of one-dose and two-dose 2009 H1N1 split vaccine or live virus infection in a 200-day-period, evaluate the immunologic protection against 2009 pandemic influenza for vaccinated, infected-only and vaccinated-infected individuals after 207 days, then speculate the possible risk of transmission among individuals. On the other hand, this study may provide clues for the mucosal immune induced by intranasal infection and the system immune induce by intramuscular vaccination in long term. Besides, evidence-based guidelines were provided for 2009 pandemic influenza vaccination.

Materials and Methods

Vaccination and Challenge

Mustela Pulourius Furo (ferret), 4–5 months of age (Marshall Farms, USA), were serologically negative (HI titer <1:40) for currently circulating influenza viruses including A/California/7/2009 (H1N1), seasonal influenza virus H1N1, H3N2 and H5N1, detected by hemagglutination inhibition (HI) assay.

The ferrets were divided into six groups: CNc, CN0 (control); T1v, T2v (vaccinated); Tv-c and Tc (infected); six ferrets for each group. Ferrets were vaccinated intramuscularly with inactivated split vaccine (SINOVAC), 0.5 ml for each, containing 30 µg/ml of antigen equivalent to A/California/7/2009 H1N1 virus (CA7 virus, SINOVAC), at day 0 (group T1v, T2v and Tv-c) and day 21 (T2v). And all ferrets in Tv-c, Tc groups were challenged intranasally with 500 µl 10^7 TCID50 CA7 virus at day 18. At day 207, all the ferrets in T1v, T2v, Tv-c, Tc and CNc

group were challenged intranasally with 10^7 TCID50 CA7 Virus. The ferrets in CN0 group were injected intramuscularly with PBS as control. Then 3 ferrets in each group were euthanized at day 211, and the rest three ferrets in each group were selected to monitor virus shedding and antibody dynamic (Fig. 1). All procedures were approved by the Institute of Animal Use and Care Committee of the Institute of Laboratory Animal Science, Peking Union Medical College (MC-09-7005).

Evaluation of Clinical Signs of Disease in Ferrets

Animals were monitored daily for weight, body temperature, and scored with a 0-1-2-3 scale for activity and such nasal symptoms as sneezing, coughing and rhinorrhea for 9 days. Symptoms were scored with the method developed by Reuman (1989) summarized in Table 1. The mean score and deviation were calculated as in Table 1.

Hemagglutination Inhibition (HI) Assay

The serum samples were collected from vaccinated ferrets at day 0, 7, 14, 18, 21, 28, 81, 116 and 200, and infected ones at day 0, 7, 14, 18, 21, 28, 37, 72, 107 and 200, while the serum samples of control ferrets (CNc, CN0) were collected at day 0, 7, 14, 18, 21, 28, 37, 72, 81, 107, 116 and 200. Antibody titers of serum samples were determined by haemagglutination inhibition (HI) assay. Before detection, the serum samples were treated with receptor destroying enzyme (RDE) from *Vibrio cholerae* (Denka Seiken, Cat lot 370013) for 18 h at 37 °C, and were heat-inactivated at 56 °C for 30 min according to WHO's standard procedure. Serum samples were diluted in serial two-fold dilutions from 1:10 to 1:640 and then mixed with 1 % suspension of Turkey red blood cells and four hemagglutinating units of virus strain A/California/7/2009 H1N1. Specific positive antiserum and negative serum controls were set for the assay. All the experiments were carried out in the Animal Biosafety Level 3 (ABSL-3) laboratory [3].

SYBR Green real time RT-PCR (RT-PCR) and Virus isolation (VI)

Intramuscularly injected with Pentobarbita sodium (30 mg/kg, ILAS-PC-2010-003), ferrets were euthanized on day four post challenge. Necropsy was carried out in a biosafety level three facility according to the standard protocol described previously [15]. After total 1 ml nasal wash was centrifuged at 4 °C; 12,000 rpm for 5 min; 100 µl of the supernatant was aliquoted for virus detection by RT-PCR and the VI respectively, which were done according to procedures described previously [4, 15]. Tissue liquids

Fig. 1 The groups and study design of the long term study. *Top*: the groups for the study; *bottom*: the schedule for the long term study from day 0 to day 200

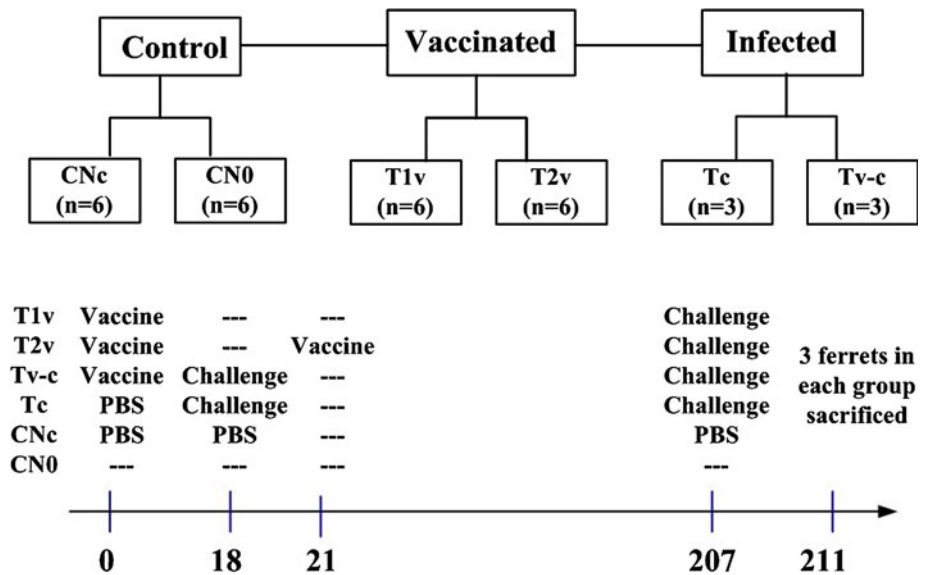


Table 1 Ferret clinical sign scoring

Score	Nasal symptoms	Activity level (playfulness)
0	No symptoms	Fully playful
1	Nasal rattling or sneezing	Responds to play overtures but does not initiate play activity
2	Nasal discharge on external nares	Alert but not playful
3	Mouth breathing	Not playful, not alert

Activity level and nasal symptoms were scored with a 0-1-2-3 scale as in Table 1. The activity and nasal symptoms of ferrets were monitored for 9 days. The mean scores and deviations for ferrets were calculated

were also used for the VI [4, 15]. The SYBR green RT-PCR was performed to amplify the CA7 virus fragment with 2 μ l of cDNA with the primer pair: SW-H1 F786: 5'-AATAACATTAGAAGCAACTGG-3', SW-H1 R920: 5'-AGGCTGGTGTATTATRGACACC-3'. A 153 bp fragment of HA gene of A/California/07/2009 strain was amplified. The amplification parameters were sequentially as follows: 3 min 94 $^{\circ}$ C for 1 cycle, 30 s at 94 $^{\circ}$ C, 30 s at 58 $^{\circ}$ C, 30 s at 72 $^{\circ}$ C for 35 cycles, and a final extension for 5 min at 72 $^{\circ}$ C.

Pathological Examination and Immunohistochemistry

The ferrets challenged with the A/California/07/2009 virus were also euthanized for pathologic examination. Excised tissues of the lungs were preserved in 10 % phosphate-buffered formalin. Tissues were processed for paraffin embedding and cut into 4- μ m-thick sections. The sections were stained with standard hematoxylin and eosin and immunohistologic staining (IH). For IH, the primary

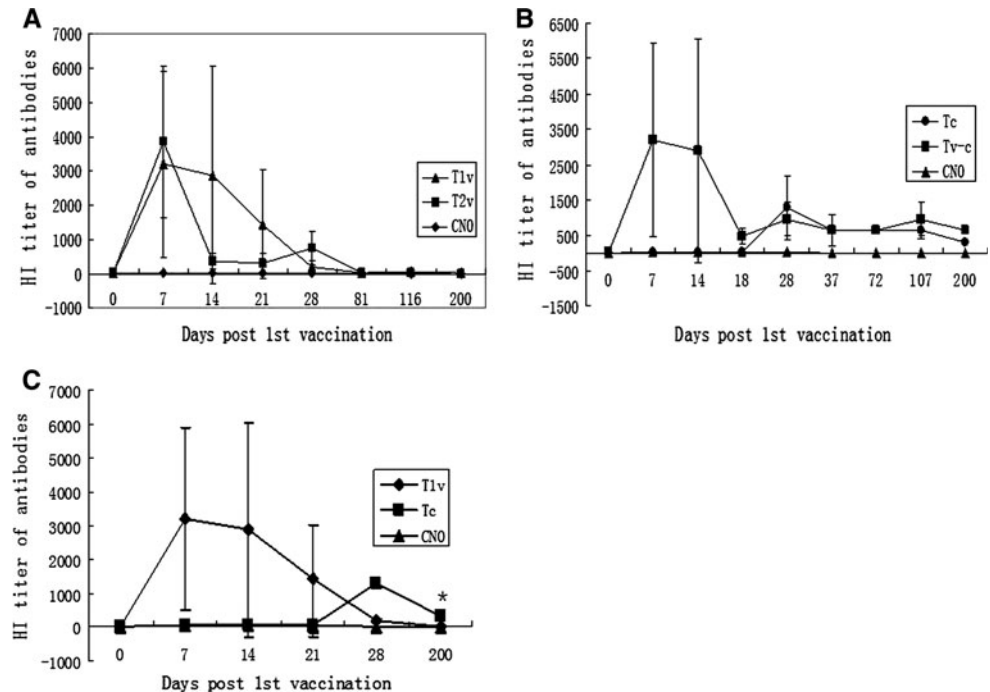
antibody (mouse monoclonal antibody anti-H1N1 influenza virus A/California/07/2009, a gift from Dr. Chen Honglin from Hongkong University) and the secondary antibody (rabbit anti-mouse IgG-HRP, Santa Cruz, USA) were diluted in the ratio of 1:1,000 in blocking Buffer. 50 μ l of the primary antibody and the secondary antibody were added sequentially into each section, and the specific antigen-antibody reactions were visualized by three, 3'-diaminobenzidine tetrahydrochloride staining (Zhongshan Golden Bridge Biotechnology Co. Ltd) [15].

Results

Split Vaccine and Live Virus Infection Alleviated Weight Loss and Activity Symptom Induced by Virus Challenge

The ferrets of vaccinated and infected which were challenged at day 207 (T1v, T2v, Tc, Tv-c, CNc) were observed daily. Initial observations indicated that all challenged ferrets developed influenza-like symptoms from day 1 post challenge (p.c.). The ferrets in T1v, T2v, Tc, Tv-c, and CNc group developed a transient increased fever from day 0 to day 2 p.c., and attained the peak at 24–36 h p.c. No statistic significant differences were found between the T1v and T2v group or Tc and Tv-c group. The weights of ferrets in T1v, T2v, Tc, Tv-c and CNc group declined with the lowest point at day 4 p.c., and the weight losses of ferrets in T1v, T2v, Tc, Tv-c group appeared an obviously less degree compared with CNc group. The clinical scores of ferrets indicated there were no differences between groups concerning nasal signs, but as for the activity, the

Fig. 2 The dynamics of antibodies induced by split vaccine and live virus. The antibodies were monitored for 200 days by HI assay. **a.** Kinetics of HI antibodies induced by split vaccine. *T1v*: one-dose vaccinated group, *T2v*: two-dose vaccinated group, *CN0*: blank control. **b.** Antibody dynamics of infected or vaccinated plus infected ferrets detected by HI assay. *Tc*: infected group, *Tv-c*: vaccinated plus infected group. *Error bar* indicates \pm standard deviation from different ferrets in the same group. **c.** Antibody titers of *T1v* and *Tc*. *Asterisks* referred the statistic significant difference between the titers of *T1v* and *Tc* at day 200



scores indicated that CNc group had a longer recovering time than T1v, T2v, Tc, Tv-c group (data not shown).

Live Virus Induced a Higher Antibody Titer than That of Split Vaccine within 200 Days

After a long time survey, antibodies of ferrets were measured by HI assay. The titer of T1v arrived to the peak value of 3,200 at day 7 post vaccination; as for the titer curve of T2v, the first peak was also seen at day 7 with titer of 3,840, and the second peak appeared at day 28 (7 days post the 2nd vaccination at day 21), then declined till day 81. From day 81 to day 200, the titers of both groups approximated to negative level, and there was no statistic significant difference between one-dose and two-dose vaccination group in a 200-day-period. (Fig. 2a).

When focused on the immunogenicity of natural infection, the titer of antibody had been monitored for 200 days. The HI titer of Tv-c group rose to the peak at day 7 after vaccination, and declined till day 18, when the ferrets were challenged with CA7 virus and the titer was boosted to higher level for at least 182 days. HI titer of Tc group was boosted by CA7 virus at day 18, and maintained a high level above 500 till day 200. Whether the ferrets had been primed or not with a dose of split vaccine before infection, there was no statistic significant difference between Tv-c and Tc group within 200 days (Fig. 2b).

The HI titer of antibody induced by natural infection could last a higher level than that of split vaccine within

200 days, and the P value in *t*-test for T1v and Tc was $P < 0.01$ (Fig. 2c).

Live Virus Infection Excelled That of Split Vaccine in Inhibiting Virus Shedding in Challenge After 207 Days

After intranasal challenge with the CA7 virus at day 207, all the ferrets survived the challenge, nasal washes of the un-sacrificed ferrets in T1v, T2v, Tc, Tv-c, CNc and CN0 groups were detected for CA7 virus by RT-PCR from day 1–8 p.c.. No viral RNA could be detectable for T1v group on day 5 p.c., for T2v on day 7 p.c., for Tc on day 2 p.c., for Tv-c on day 3 p.c., for CNc on day 7 p.c., while CN0 was blank control (Fig. 3a, b).

Results of virus isolation revealed that the virus shedding of T1v group lasted for 2.7 days, Tv-c lasted for 1 day, and T2v and CNc lasted for 4 days in average, while Tc did not shed any virus particles. CN0 was blank control (Fig. 4a, b, c). Statistic significant differences were showed between groups T1v and CNc, Tc and CNc, Tv-c and CNc (*t*-test, $P < 0.01$).

Virus Isolation from Organs

As for the organs, virus could be isolated only from small intestine of ferrets in T1v, T2v, Tc, Tv-c and CNc group, without significant difference among groups (data not shown).

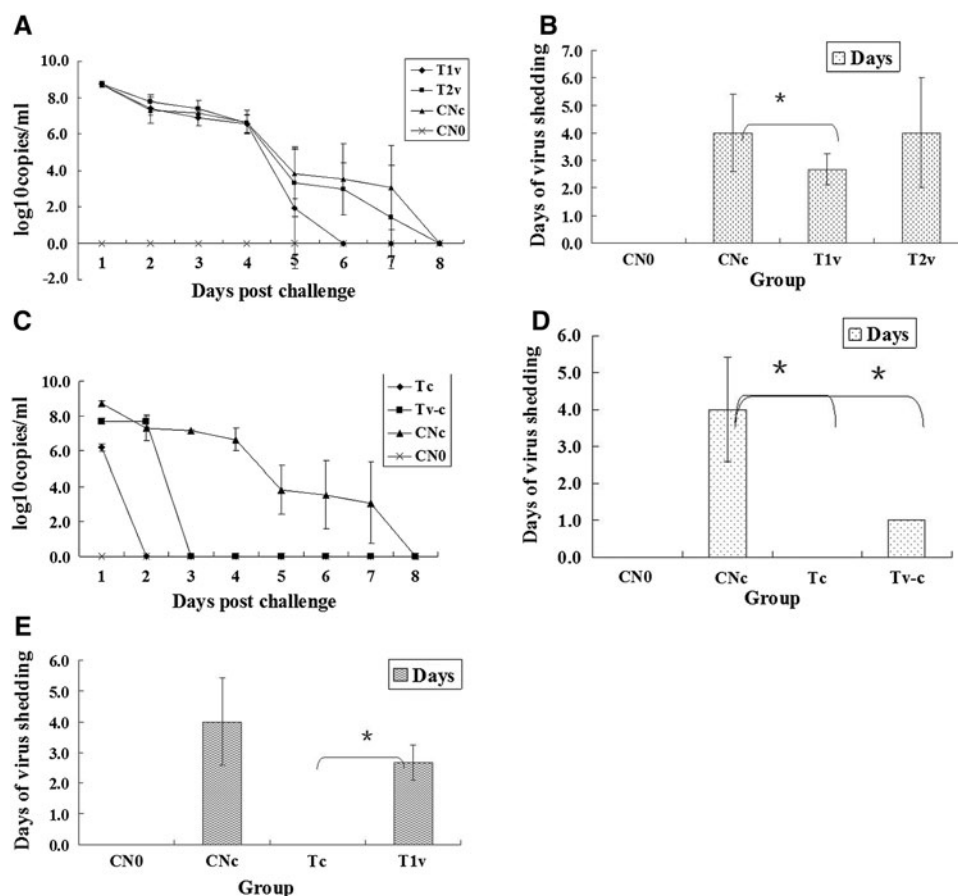


Fig. 3 Virus detections in nasal washes. The nasal washes were collected from day 1 p.c. to day 8 p.c., viruses in nasal washes were determined by RT-PCR or virus isolation (VI). **a.** The virus loads of one-dose and two-dose vaccinated ferrets, viral RNA was detected by RT-PCR, plotted values represented copy number (unit: \log_{10} copies/ml). **b.** Virus shedding from vaccinated ferrets determined by virus isolation (VI). 100 μ l of the nasal wash was used for CA7 virus isolation. **c.** Virus loads of nasal washes of infected or vaccinated plus

infected ferrets detected by RT-PCR. **d.** Viruses shedding of infected or vaccinated plus infected ferrets were measured by virus isolation (VI). **e.** Virus isolation from nasal washes of infected and vaccinated ferrets after challenge. Plotted values represented group average days of virus shedding, asterisks referred the statistic significant difference. Average values and error bars were shown. T1v: one-dose vaccinated group, T2v: two-dose vaccinated group, Tc: infected group, Tv-c: vaccinated plus infected group, CN0: blank control

The Vaccination and Sublethal Infection in the Early Stage Could Alleviate Lung Lesions in the Challenge at Day 207

The pathologic changes were observed for the organs of sacrificed ferrets. In gross observation, focal pulmonary consolidation was observed in CNc group ferrets, and no obvious macroscopic lesions were found in T1v and T2v group ferrets. On histopathological, the major lung lesion of CNc group was diffuse interstitial pneumonia, and focal interstitial pneumonia for T1v and T2v group (Fig. 4a, panel b, c). Immunohistochemistry results showed that the antigen of CA7 virus were positive in partially alveolar epithelial cells of CNc group (Fig. 4a, panel e), whereas negative for T1v and T2v group. No lesions were seen in extrapulmonary organs.

The lung lesions of ferrets were classified with severe pneumonia, slight pneumonia and normal (Fig. 4b). The severe pneumonia was characterized by the obviously dilated pulmonary vessels, oedematous venous wall, flooding of alveolar lumina with inflammatory cells, edema fluid, exudated or leaked erythrocytes and the necrosis and shedding of bronchial and alveolar epithelial cells. For slight pneumonia (Interstitial pneumonia), alveolar wall was thickened and a few inflammatory cells were infiltrated in it, no obvious damage in the vascular. Severe pneumonia and slight pneumonia were observed in the CNc group. Slight pneumonia was showed with T1v, T2v, Tc and Tv-c group. In the quantitative histological scoring, the percentage of slight pneumonia was T1v < T2v, Tc < Tv-c, but the differences between groups without statistic significance (Fig. 4b, c).

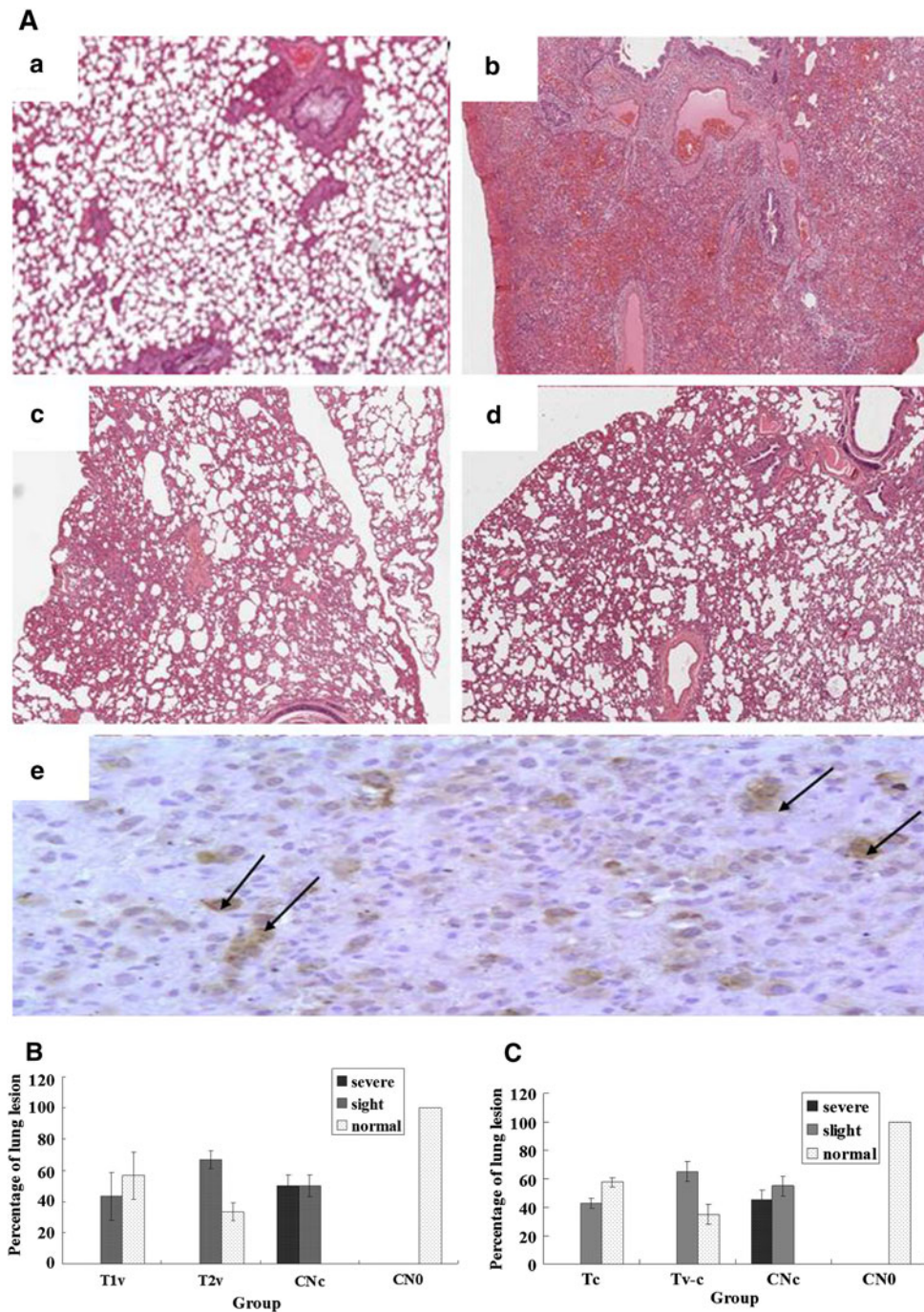


Fig. 4 Histopathology of lung. **A** Histopathologic changes of ferrets' lung after virus challenge at day 207. *a*: CN0 group: normal ferret lung as blank. *b*: CNc group: severe pneumonia. The pulmonary vessels were dilated obviously and the venous walls were oedematous. The alveolar lumina were infiltrated with inflammatory cells, edema fluid, exudated or leaked erythrocytes. The necrosis and shedding of bronchial and alveolar epithelial cells were also observed in ferrets, $\times 40$. *c*: T1v group: focal interstitial pneumonia. The lesion area covered less than 1/3 of the whole lung area, $\times 40$. *d*: T2v group: Focal interstitial pneumonia. The lesion area was less than 1/3 of the whole lung area, $\times 40$. *e*: Immunohistochemistry (IH) of CNc group: The antigen of CA7 virus was positive in partially alveolar epithelial

cells (arrow), $\times 200$. The IH results of Tc and Tv-c group were negative for CA7 virus antigen (Pictures not shown). **B** Percentage of lung lesion in T1v and T2v ferrets. The lung lesions of ferrets sacrificed at day 211 were scored according to severity classified by severe pneumonia, slight pneumonia and normal. There were no significant differences between T1v and T2v group, Tc and Tv-c group or T1v and Tc group. Average values and standard deviations were shown in the plot. **C** Lung lesions of infected and vaccinated plus infected ferrets in the virus challenge. The percentages of severe, slight pneumonia and normal lung were shown in the plot. *Plotted values* were average percentages and *error bars* reflected standard deviation

The infection at day 18 showed the ability to alleviate lung lesions in the re-infection at day 207. But the immunization at day zero was found to no additional effect on mitigating lung lesion.

Discussion

To assess the immune efficacy of CA7 H1N1 split vaccine and live virus infection in virus challenge in long term, a series study were carried out. As for the T1v and T2v group, the antibody could last a moderate level till day 81, with peak value at day 7 p.c, and then declined to a very low level (<1:40) from day 81 to day 200. Though the antibody titers approximate to a negative level at day 200, a moderate protection still could be appeared, judging from the alleviated clinical signs, the partially inhibited virus shedding and slight lung lesions. And there was no significant difference between T1v and T2v group (Figs. 2a; 3a, b; 4a, b).

As for the infected ferrets, the HI titer of antibody lasted a high level for at least 182 days (Fig. 2b). After the challenge, viruses shedding of the ferrets were inhibited obviously (Fig. 3c, d). The lung lesions were slight and similar with that of vaccinated ferrets (Fig. 4c). There was no obvious difference between Tc and Tv-c group.

But judging from the high antibody level, inhibited virus shedding and slight lung lesion, the live virus infection was better than inactive split vaccine in protecting ferrets against challenge at day 207, especially in preventing virus shedding (Figs. 2c, 3d).

Consistent with previous report, the matched inactivated vaccine demonstrated moderate protection against a pandemic H1N1 2009 virus, the antibody mounted by vaccine could last at least 60 days without significant reduction [2], and was found to lessen clinical signs and lung lesion to some extent, whereas with a high virus replication levels in the upper respiratory tract of vaccinated ferrets [4]. In addition, one single dose vaccination without adjuvant was sufficient to elicit potentially protective antibody response in the host for short term immunization [6, 8].

Furthermore, our study had also provided more important evidences not reported for pandemic 2009 influenza.

Firstly, natural CA7 virus infection (infection-only and vaccinated plus infection) stimulated a stable higher level antibody than that of vaccine at least for 182 days, while the vaccinated could maintained a moderate level for 81 days, which was not reported in ferret till now. In addition, it was reported that the anti-pandemic H1N1 virus antibodies were first observed on day 10 post vaccination in human serum, lasting for 180 days without significant

reduction [12], but for ferret, our study demonstrated that the antibody also rose to its peak titer at day 7 post vaccination, lasting for 81 days without significant reduction.

Secondly, immunity induced by live virus excelled that of split vaccine in inhibiting virus shedding in challenge after 207 days. Both split vaccine and infection could inhibit the virus shedding, split vaccine displayed partial inhibition while live virus infection did complete inhibition to the virus shedding after challenge. The complete inhibition might ameliorate clinical signs individually, and prevent cross-infection among ferrets.

Thirdly, our study provided direct and reliable evidence about the protective immunity induced by CA7 vaccine and infection against challenge in long term, not only based on antibody analysis, but also on the clinical signs, the virus shedding, pathological pulmonary damages. It could be concluded that mucosal immune induced by CA7 live virus provided longer protection than the system immune induced by split vaccine against virus challenge in long term.

Fourthly, histopathology results indicated that slight pulmonary damages were found in ferrets vaccinated or infected or vaccinated plus infected, but IH data demonstrated there were no CA7 viruses in lung of T1v, T2v, Tc, Tv-c group. On the other hand, CA7 virus was not isolated from other organs except small intestine across all animals, but the lesion was not observed in other organs except lung. This suggested that the lesion might not be the direct damage induced by CA7 virus (Fig. 4a, b).

Finally, pulmonary lesions in T1v animals were a little slighter than in T2v animals (no statistical difference), and both group animals were shown slighter than CNc group. So in the long term, a second dose of vaccine might be saved and unnecessary, because another dose of vaccine might elicit harmful response to host. In addition, T1v and T2v ferrets exhibited slighter illness than CNc group animals even when the HI titer decreased to a level lower than 1:40 (Figs. 2a, 4a, b), this indicated that cellular immunity might exert protection when the antibody decreased to a negative level (<1:40).

As a whole, our study assessed the risk of recurrence for the vaccinated, vaccinated-infected and infected individuals, bridged the gaps between the immunity induced by split CA7 vaccine and live virus in preventing against re-infection in the long term, lighting the hope to develop live virus vaccine against the recurrence of pandemic 2009 influenza. However, future studies are indispensable to overcome a few pitfalls and to develop one new effective vaccine.

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