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Preclinical vaccine efficacy studies are generally limited to certain read out parameters such as assessment of virus titers in swabs and organs, clinical signs, serum antibody titers, and pathological changes. These parameters are not always routinely applied and not always scheduled in a logical standardized way. We used computed tomography (CT) imaging as additional and novel read out parameter in a vaccine efficacy study by quantifying alterations in aerated lung volumes in ferrets challenged with the 2009 pandemic A/H1N1 influenza virus.

Vaccination protected from marked variations in aerated lung volumes compared to naive controls. The vaccinated group showed a daily gradual mean reduction with a maximum of 7.8%, whereas the controls showed a maximum of 14.3% reduction. The pulmonary opacities evident on CT images were most pronounced in the placebo-treated controls, and corresponded to significantly increased relative lung weights at necropsy.

This study shows that consecutive in vivo CT imaging allows for a day to day read out of vaccine efficacy by quantification of altered aerated lung volumes.

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1. Introduction

The field of influenza virus research is in particular an area of new emerging viruses that requires rapid development of animal models needed for pathogenicity studies and assessment of adequate vaccine candidates and antiviral therapies. This was recently illustrated by the emergence of the 2009 pandemic A/H1N1 influenza virus (pH1N1) [1,2]. Ferrets are being implemented extensively in human influenza virus research. However, influenza virus research is conducted in multiple separate laboratories all with their unique approach how to evaluate vaccine candidates within the ferret challenge model. Substantial differences can be found in all stages and aspects of challenge protocols, study set-ups and read-out parameters. A spectrum of recently published [1,3-12] infection/challenge protocols showing this diversity is listed in comparison in Table 1. In addition, obviously, different influenza strains are used as challenge virus instigated by the antigenic nature of the vaccine, or alternatively to evaluate efficacy to a heterologous influenza virus challenge. The routes of infection being intranasal, intratracheal or through virus transmission from experimentally infected and shedding ferrets show considerable differences in implementation and outcomes [13]. Different viral challenge doses are used, whether or not established in preceding dose-finding studies. However, the challenge doses are pivotal in the interpretation of a challenge outcome. Since, a too robust challenge may prove, false negatively, a poor efficacy of a human vaccine candidate in the ferret model, and vice versa. Furthermore, the duration of the challenge read out period varies, as well as the types of samples collected and frequency of sampling. Often the design of a challenge protocol is based on predefined end points and read outs, or may rely on results from historical experiments.

Because of these variations in the assessment of vaccine efficacy, the comparison of the outcomes of vaccine studies may be hampered, therefore a certain way of standardization could prove useful by providing clarity.

Abbreviations: ALV, aerated lung volume; RLW, relative lung weight.
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2.2. Immunisation

The animals \( (n=6) \) were immunised three times with a 3 week interval with an adjuvanted inactivated vaccine. 200 µl of vaccine was intranasally administered and divided equally over both nostrils. The controls \( (n=6) \) were similarly sham immunised with 200 µl PBS intranasally (referred to as placebo).

2.3. Challenge virus

All animals were challenged, 4 weeks after the last immunisation, intratracheally with \( 10^6 \) median tissue culture infectious dose (TCID\textsubscript{50}) of the 2009 pandemic influenza virus A/Netherlands/602/2009 (pH1N1) in 3 ml PBS, as described previously [2,12,14]. The virus was routinely propagated in MDCK cell cultures and infectious dose determined as described previously[15], and titres calculated according to the method of Spearman-Karber [16].

2.4. CT-scanning

All animals were scanned on \(-6, 1, 2, 3, \) and \( 4 \) d.p.i. (see also Table 1). A dual-source ultra fast CT-system (Somatom Definition Flash, Siemens Healthcare) was used (temporal resolution: 0.075 s, spatial resolution is 0.33 mm, table speed of 458 mm/s; ferret thorax acquisition time = 0.22 s). Enables accurate scanning of living ferrets without the necessity of breath-holding, respiratory gating, or electrocardiogram (ECG)-triggering) as previously described [11]. Briefly, during scanning the ferrets were in dorsal recumbency in a purposely built (Tecnilab-BMI) perspex biosafety container of 8.3 L capacity. The post-infectious reductions in aerated lung volumes were measured from 3-dimensional CT reconstructions using lower and upper thresholds in substance densities of \(-870 \) to \(-430 \) Hounsfield units (HU).

2.5. Pathology

Following euthanasia by exsanguination all animals were submitted for necropsy. The lung lobes were inspected and lesions
were assessed while the lung was inflated. The trachea was cut at the level of the bifurcation and the lungs were weighed. The relative lung weight was calculated as proportion of the body weight on day of death (lung weight/body weight × 100).

![CT images showing lung consolidation](image1)

**Fig. 2.** Changes in aerated lung volume after infection with H1N1 A/Netherlands/602/2009. The aerated lung volume was calculated using lower and upper thresholds in substance densities of −870 to −430 Hounsfield units (HU) for the analysis of 3D-reconstructions of the lung. The percentage change of aerated lung volume was calculated using the individual baseline aerated lung volumes of day 6 against the aerated lung volumes of the different days after infection. These data are expressed as mean ± SEM. Animals were intratracheally challenged with 10^5 TCID_{50} H1N1 A/Netherlands/602/2009 on day 0.

### 3. Results and discussion

All animals from both groups were scanned 6 days prior to virus inoculation to define the uninfected baseline status of their respiratory system. Consecutive in vivo imaging with CT scanning showed that ferrets intranasally immunised with the vaccine candidate were largely protected against the appearance of pulmonary ground-glass opacities, as is shown by means of transversal CT images in Fig. 1. The ALVs measured from 3D CT reconstructions likewise showed that the immunised ferrets were protected against major alterations in ALV (group mean ALV ranging from 0.95 to −7.8%) and did not show a temporal increase in ALV on dpi, which was observed in the placebo group (group mean ALV ranging from 17.3 to −14.3%) (Fig. 2). This sudden and short increase of 17.3% (Mann–Whitney test, two-tailed, P = 0.035) in the unprotected placebo-treated animals may result from a virally-induced acute respiratory depression with compensatory hyperinflation. A compensatory increase in respiratory tidal volume by means of hyperinflation is a pathophysiologic phenomenon known to occur in respiratory viral infections [17,18]. However, CT scanning could not discern possible emphysema due to ruptured alveoli as cause of ALV increase. The relative change of ALVs on days 2, 3 and especially 4 after infection did not show significant differences between the two groups. One possible explanation is that over-expansion of the thorax and lungs allows for increased alveolar flooding in excess of baseline aeration resulting in approximately unaltered ALVs between the two groups. Another explanation is that the inflamed and oedematous areas were aerated less than normal, but because the unaffected areas of lung were aerated more

![CT images showing lung consolidation](image2)

**Fig. 1.** Consecutive transversal lung CT images after infection with H1N1 A/Netherlands/602/2009. Two columns of consecutive (top to bottom) transversal lung CT images of one representative immunised ferret (left) and one representative placebo-treated ferret recorded in vivo compared with their gross aspect at necropsy (bottom). At day 6 before infection, the lungs showed the clear aerated baseline condition, from 1 dpi with the new pandemic H1N1 influenza virus onwards marked almost diffuse ground-glass opacities appear that show a gradual increase with a plateau on 3–4 dpi. The two photographs taken at necropsy on 4 dpi depict the ventral aspect of the lungs, with the hearts removed. The lungs of the placebo-treated animal (bottom right) show diffuse reddish consolidation indicative of acute inflammation that essentially match the opacities on the CT images taken just before necropsy; non-affected aerated lung tissue from the immunised animal is light pink in colour (bottom left).
than normal (hyperinflation or emphysema), the overall ALV values remained approximately unaltered.

Nevertheless, these ALV profiles provide more detailed knowledge about the influenza-induced respiratory disease development than confined data obtained from a single predefined read out. Moreover, survival and recovery from challenge infection can be included in this set-up and with the opportunity to still measure the development of serum antibody responses upon challenge infection.

Upon necropsy, the relative lung weights (RLWs) of the intranasally immunised ferrets was about 2-fold lower (Mann–Whitney, two-tailed, \( P<0.0047 \)) as compared to those of the placebo-treated animals (Fig. 3), which is in agreement with the absence of pulmonary ground-glass opacities. Usually, more severely affected and inflamed lungs with increased amounts of fluid are heavier compared to normal or less affected lungs. This translates within the ferret model in influenza research to RLWs \( \geq 1.0 \) associated with non- to minimally affected lungs and RLWs \( >1.0 \) associated with severe pulmonary inflammation with oedema [12,19,20].

In conclusion, the implementation of consecutive CT imaging enables repeated in vivo measurements of lung aeration as parameter to evaluate vaccine efficacy in preclinical protocols. Consecutive day to day imaging overcomes the limitations entailed by necropsy at a predefined time point after infection, and the lung capacity can be repeatedly quantified in real-time.

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