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Short Communication

The use of plethysmography in determining the severity of lung pathology in a mouse model of minimally lethal influenza virus infection

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ABSTRACT

To characterize the impact on lung function, we assessed plethysmography parameters in a course of infection with mouse-adapted A/Pennsylvania/14/2010 (H3N2) influenza virus. Several parameters, represented by enhanced pause (penh) and ratio of inspiratory/expiratory time (Ti/Te), were observed that had early (1–7 dpi) and robust changes regardless of virus challenge dose. Other parameters, characterized by tidal volume (TV), breathing frequency (freq) and end inspiratory pause (EIP), changed later (7–15 dpi) during the course of infection and had a virus challenge dose effect. A third category of lung function parameters, such as peak inspiratory flow, had early, virus challenge-independent changes followed by later changes that were challenge dependent. These parameters changed in a similar manner after infection with a non-mouse adapted virus, although the time-course of many parameters was delayed somewhat when compared with mouse-adapted virus. Histopathological assessment of lung samples corresponded with changes in lung function parameters. This study demonstrates the utility of plethysmography in assessing disease in a mouse model of mild influenza virus infection.

Influenza virus (IAV) causes significant morbidity and mortality worldwide, although the majority of infections are mild and infected individuals generally recover after a couple weeks. Lethal rodent models are commonly used to better understand influenza disease and to identify potential therapeutics (Barnard, 2009; Boltz et al., 2010; Smee et al., 2012). In order to develop a lethal mouse model after the emergence of an IAV strain, the virus must often be adapted to mice. This is accomplished through serial passage, which includes genetic changes to the virus. These changes, including enhanced receptor binding by the virus, result in increased pathogenesis (Ilyushina et al., 2010; de Jong et al., 2013). It is generally unknown how mouse adaptation influences the translation of discoveries in mice to the realm of clinical intervention in man. Therefore, it would be useful to develop a mouse model of mild influenza using non-adapted clinical isolates of IAV, but such a model would require a more sensitive method for the evaluation of disease, as morbidity and mortality would be reduced or absent.

Pulmonary tissue damage is often a consequence of influenza infection and is a key component of disease in man (Marsolais et al., 2009; Sanders et al., 2013). Lung impairment as a result of influenza infection in rodents may be a useful parameter for assessing therapies for the prevention or treatment of disease associated with influenza infection. Noninvasive techniques, including the use of a plethysmograph, may be used to longitudinally quantify lung impairment in rodents after virus infection, as opposed to more traditional methods, which include necropsy to assess lung damage (Julander et al., 2011). A recent study demonstrated significant loss of type I pneumocytes with associated impairment of lung function after severe influenza virus A/Puerto Rico/8/1934 (H1N1) infection or with a mild infection after A/Aichi/2/1968 x A/Puerto Rico/8/1934 (H3N2) influenza virus (x31) infection (Sanders et al., 2013). Based on our previous results (Julander et al., 2011), measures of lung function are also useful in antiviral studies.

The purpose of the present study is to characterize mild disease caused by influenza virus in mice using plethysmography, including a comparison of adapted and non-adapted (2 passages in MDCK cells) viruses. We obtained the A/Pennsylvania/14/2010 (H3N2) influenza virus in 2011 from the Centers for Disease Control and Prevention (CDC, Atlanta, GA). The virus was adapted to mice by passaging 7 times in female 13–15 g Swiss Webster mice obtained from Charles River (Wilmington, MA). For passage, inoculated mice were sacrificed 3 days after virus challenge and clarified lung homogenates were administered to a subsequent group of mice in a volume of 0.1 ml. After 7 passages followed by one passage in Madin–Darby canine kidney (MDCK) cells, the virus titrated at

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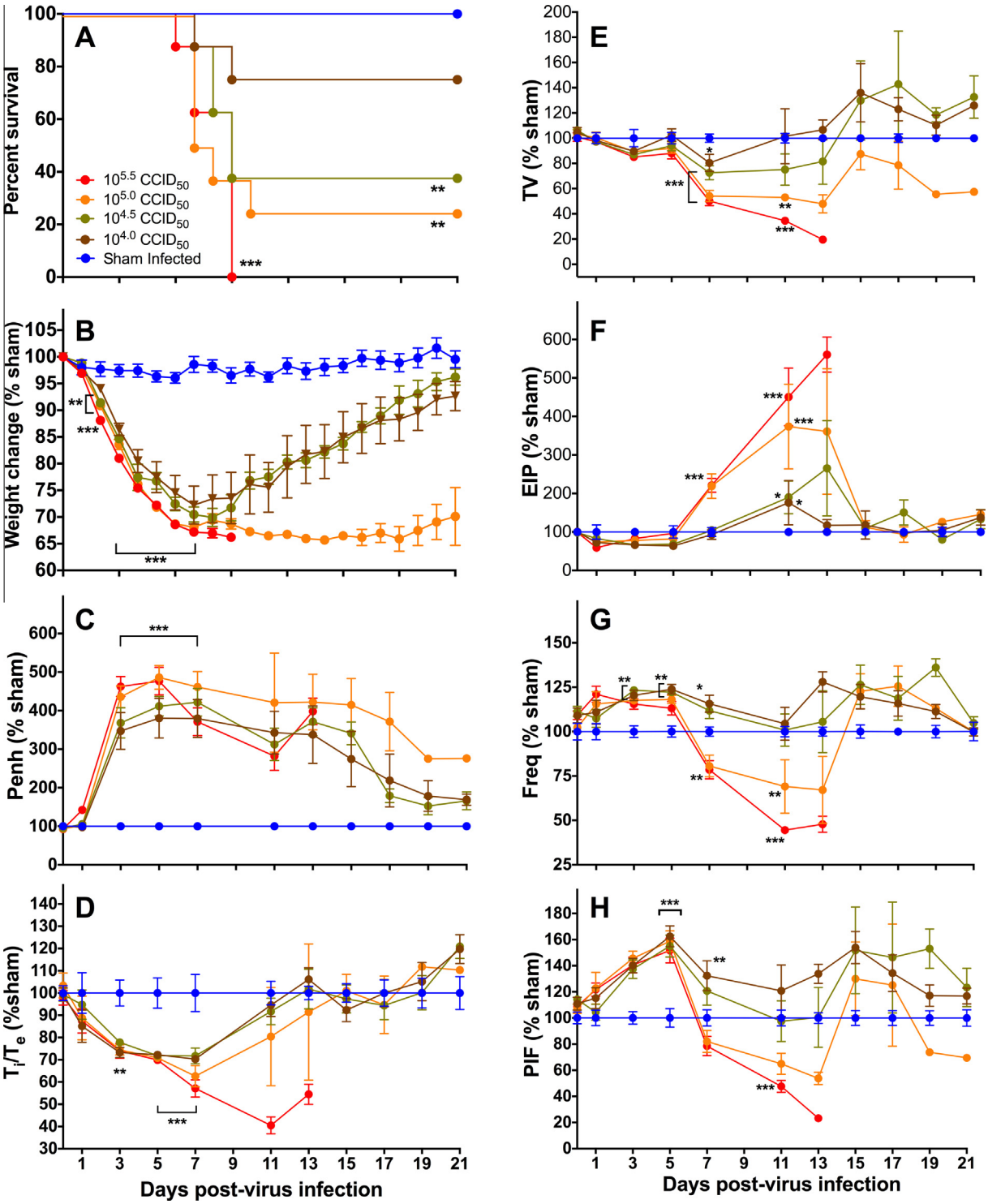

Fig. 1. Mortality (A) and mean weight change (B) of mice (n = 8) infected with various infectious doses of adapted A/Pennsylvania/14/2010 (H3N2) influenza virus are shown. The virus that was used had undergone 7 sequential passages through mouse lung. The representative lung function parameters of penh (C) and Ti/Te (D) show changes soon after virus challenge, while TV (E) and EIP (F) show dose-responsive changes later during the course of infection. The Freq and PIF measurements are representative of parameters that show early non-dose dependent changes followed by later virus challenge dose-dependent changes. Kaplan–Meier survival curves were analyzed by the log-rank test followed by a pairwise comparison using the Gehan–Breslow–Wilcoxon test. Mean weight change and plethysmography curves from each group were compared using a two-way ANOVA with a mean column effect analysis. Error bars represent the standard error of the mean (SEM) ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, as compared with mock-infected controls).

$10^{8.9}$ CCID$_{50}$/ml. A sample of the virus was submitted to CDC for genetic analysis where it was confirmed to be the A/Pennsylvania/14/2010 (H3N2) virus (data not shown). We titrated the passage 7-adapted virus in mice using survival, weight change, and longitudinal plethysmography measurement of lung function over the course of virus infection.

A commercially available plethysmograph, acquisition software, and mouse-sized plethymograph chambers (emka Technologies,

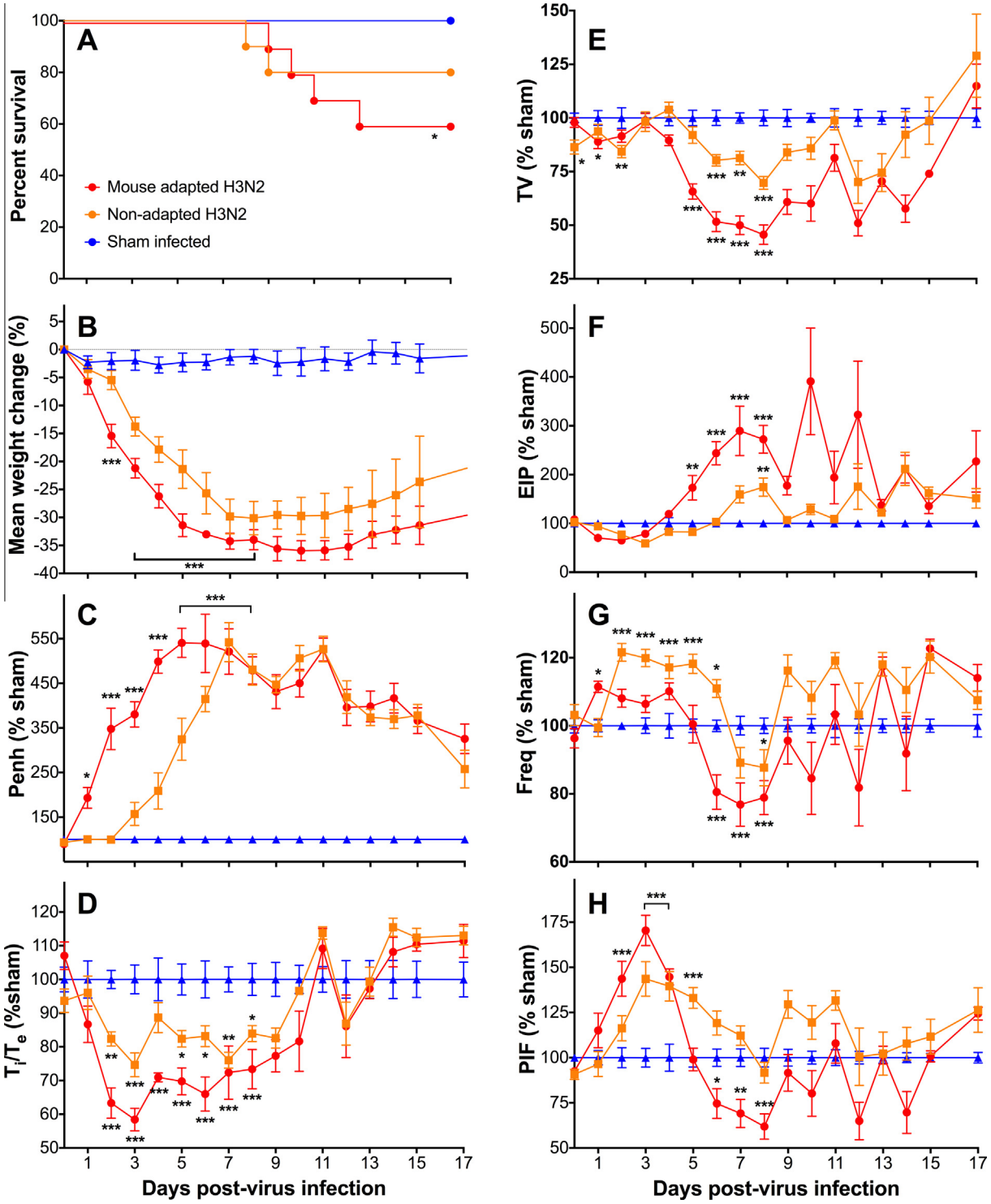

Fig. 2. Time-course of disease in mice ($n = 10$) after challenge with mouse-adapted (via 4 sequential passages through mouse lung) and non mouse-adapted A/Pennsylvania/14/2010 (H3N2) influenza virus including mortality (A), mean weight change (B) and various parameters of lung function (C–H). Statistical analysis was performed as in Fig. 1. Error bars represent the standard error of the mean (SEM).

Falls Church, VA) were used for lung function analysis. This system allows measurement of the differential pressure within the chambers due to breathing of the animal. Lung function parameters that were analyzed included enhanced pause (penh), tidal (TV), minute (MV), and expiratory (EV) volumes, times of inspiration (Ti), expiration (Te), and relaxation (RT), peak inspiratory (PIF) and expiratory (PEF) flow, Frequency of breath (Freq), 50% expiratory flow (EF50), and end inspiratory (EIP) and expiratory (EEP) pauses. There may be variability from day to day when taking measurements by plethysmography, so mean readings from infected mice in a group were normalized to mean readings from mock-infected controls each day.

Lungs from mice challenged with adapted or non-adapted virus were fixed by gravity perfusion with paraformaldehyde on 3 or 7 days post-virus inoculation (dpi), and submitted (blinded to the pathologist) for histological examination. Hematoxylin and eosin-stained slides were scored from 0 to 4 depending on severity according to several pathological findings. Data were analyzed

Table 1
Histological analysis of lung samples from mice infected with a mouse-adapted or non-adapted influenza A virus (H3N2).

IAV (H3N2) challenge	dpi[a]	Bronchi	Bronchioles	Alveoli
Mouse-adapted virus	3	0.8 ± 1.0 (2/4)[b]	1.0 ± 0.8 (3/4)	1.1 ± 0.6 (3/4)
	7	1.0 ± 1.7 (1/3)	2.0 ± 1.7 (3/4)	1.7 ± 0.8 (4/4)
Non-adapted virus	3	– (0/5)	0.8 ± 0.3 (5/5)	0.6 ± 0.5 (3/5)
	7	0.2 ± 0.4 (1/4)	0.6 ± 0.9 (2/5)	1.8 ± 0.5 (5/5)
Sham infection	3	– (0/3)	– (0/3)	– (0/3)
	6	– (0/3)	– (0/3)	– (0/3)

[a] Days post-virus instillation.
[b] Histologic lesions were scored from 0 to 4 depending on severity: (0) no lesion; (1) minimal lesion; (2) mild lesion; (3) moderate lesion; (4) severe lesion. An average score ± SD is shown for each group. The number of animals with evidence of pathology/total slides read is shown in parentheses.

with appropriate statistical methods using Prism 6.0 (GraphPad Software, San Diego, CA).

Female 18–20 g BALB/c mice, obtained from Charles River Laboratories, were inoculated intranasally with serial half $\log_{10}$ dilutions of mouse-adapted (i.e., passaged 7 times in mice) virus from $10^{5.5}$ to $10^{4.0}$ $CCID_{50}$ after an acclimation period of at least 48 h. This range of virus infectious dose was selected to cover a range of severity from 20% to 100% lethality.

Survival and weight change of infected mice corresponded with challenge dose of the adapted virus (Fig. 1A and B), as did lung weights, lung hemorrhage score and lung virus titers (data not shown). Infection with non-mouse-adapted as compared with adapted (4 mouse lung passages) virus resulted in increased severity and more rapid change relative to mock infection (Fig. 2A and B). The time-course of lung function parameters could be divided into three major groups; (1) those parameters that increased or decreased rapidly irrespective of virus dose, (2) those that maintained levels similar to mock-infected controls for several days followed by a virus dose-responsive increase or decrease after 5 dpi, and (3) parameters that increased rapidly, (as in 1), followed by a dose-responsive changes beginning after 5 dpi (as in 2). The lung function parameters listed above fit in one of these three groups.

The first group parameters that had an early increase or decrease as compared with mock-infection controls are typified by the parameters penh, a measure of airway resistance, and the ratio of Ti/Te, a measure of compensatory respiration or airway restriction, respectively (Fig. 1C and D). The parameters EEP and RT showed similar curves to penh and Ti/Te, respectively (data not shown). Changes in these parameters occurred irrespective of virus challenge dose and gradually returned to normal levels between 11 and 21 dpi. These changes likely reflect disease of the upper airway after virus colonization. Early reduction in Ti/Te ratio was due to a more rapid inspiratory phase, possibly as compensation for upper airway restriction. Indeed, histological analysis of samples obtained from infected mice on 3 dpi show pathological changes in the airways, including minimal to mild bronchitis and bronchiolitis with necrosis of the respiratory epithelium as well as minimal multifocal neutrophilic alveolitis (Table 1). Non mouse-adapted virus showed a delay from 1 to 2 days in the curves of these parameters as compared with adapted virus (Fig. 2C and D), which is likely due to altered virus infection and replication kinetics. Despite the delay observed in non mouse-adapted strains, the amplitude of change was similar when compared with changes after infection with adapted virus.

The second group of changes to lung function included decreases in various volumetric parameters, which occurred later during the course of infection around 5 dpi. This group was typified by TV (Fig. 1E). In addition, Freq, EIP (Fig. 1F and G), and Te also changed in a similar manner. As opposed to those parameters described in the preceding paragraph, the magnitude of change shown with these parameters corresponded with virus challenge dose. As opposed to early, virus dose-independent changes described above, these changes in volumetric parameters likely reflect lung damage that causes decreased lung capacity. Histopathology on 7 dpi showed more prominent injury and inflammatory reaction in the more distal lung structures, with mild to moderate neutrophilic, lymphocytic and histiocytic bronchiolitis and alveolitis (Table 1). Similar changes were also observed after infection with non mouse-adapted virus (Fig. 2E–G), which when compared with plethysmography output of mice infected with adapted virus behaved as though a lower virus challenge dose was given, despite a similar amount of virus being administered.

Finally, a combination of early and late changes was observed in the remaining lung function parameters. Initial increases in PIF occurred regardless of virus challenge and were followed by dose-responsive decreases after 5 dpi (Fig. 1H). Almost identical changes were also seen in the PEF and EF50 parameters. It is likely that an increase in flow due to a more rapid respiratory rate would possibly account for the early increase in these parameters, and as deep lung structures become involved, the respiratory rate slows in a virus dose-responsive manner. Such changes were also evident in a non mouse-adapted H3N2v IAV, although the initial increase in flow rates was delayed as compared with infection with adapted virus (Fig. 2H). After 5 days, these parameters did not decrease as drastically as observed in mice challenged with adapted virus.

The present and previous studies (Sanders et al., 2013) demonstrate a correlation between lung pathology and changes in lung function. Although the dynamic range of lung function parameters is diminished with a mild infection, the sensitivity of the plethysmograph allows ante mortem tracking of disease during the course of infection. Previous work (Julander et al., 2011) and current studies that are being performed will further determine the utility of plethysmography for use in monitoring mild disease during antiviral studies, and it is anticipated that this technology will be an important advance to the field.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.002.

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