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Review article

Animal models for human Metapneumovirus (HMPV) infections

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Abstract – Since its detection in 2001 the human Metapneumovirus (HMPV), a member of the *Paramyxoviridae* family, was observed to be a serious pathogen in human respiratory infections during childhood. Meanwhile, several animal models have been established to study the virus-host interactions and pathogenic effects. Mainly, small laboratory animals like mice and cotton rats have been used, although the usage of these two species for HMPV research is controversially discussed and contradictious results were obtained by different groups. Further trials with ferrets, hamsters and non human primates were performed revealing different success in their individual usage. In this review we present the different animal models, summarize their advantages and disadvantages, and discuss the controversial results from different studies.

human metapneumovirus / respiratory viruses / animal models

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1. INTRODUCTION

In 2001 van den Hoogen et al. [20] detected a new respiratory virus. It was designated as human Metapneumovirus (HMPV) and is a member of the *Paramyxoviridae* family. Since the detection of HMPV, numerous studies conducted worldwide have shown that HMPV is the second most commonly detected virus in children suffering from acute respiratory tract infection, following respiratory syncytial virus (RSV). Although an increasing number of studies and case reports have been published on HMPV, many questions remain unanswered regarding the host-pathogen interactions of HMPV and the impact of these interactions on the severity and clinical course of the infection. These questions may be addressed by the use of sufficient animal models.

Almost five years after the first description of the virus, several reports of animal models for HMPV have been published, but the continuously increasing body of literature makes it difficult even for experts in the field to follow recent developments. For this reason we have summarized the recent studies describing animal models for HMPV infections and discuss the advantages and disadvantages of these models.

Several animal species have been determined to be permissive for HMPV infection [1, 4, 6, 9, 10, 12, 18, 23, 27]. These models, which are described in more detail below, include small animals such as mice, cotton rats, hamsters, guinea pigs, ferrets, and primates, including chimpanzees, rhesus macaques and African green monkeys. In most of the susceptible animals HMPV replicates to high titers and induces high levels of virus-neutralizing antibodies in the serum. Although HMPV infection in most of these models does not mimic the signs of human disease, experimental animal infections appear to be extremely useful for investigation of many characteristics of HMPV infection, including pathogenesis and antiviral immunity. Thereby the establishment of animal models of HMPV infection will also facilitate studies of both innate and adaptive immune responses, the characteristics of which are not very well understood.

2. ANIMAL MODELS FOR THE STUDY OF HMPV INFECTIONS

2.1. Rodent models

2.1.1. Mouse model (BALB/c)

In a recent report [1], HMPV infection of BALB/c mice led to an appearance of signs of illness, including body weight loss, ruffled coat, huddling, and heavy breathing from day 0 to day 7 post infection (p.i.). A decrease in body weight loss coincided with virus replication in lung tissues with a biphasic growth kinetics in which peak titers occurred at days 7 (10^8 PFU/g of lung tissue) and 14 p.i. (10^7 PFU/g of tissue). This unusual biphasic growth curve has not been observed for HMPV (or for that matter, RSV) in any other animal model by these or other investigators, and thus the biological significance is unclear. In addition to Alvarez et al. [1,2], Hamelin et al. [6] observed that the lung virus peaks somewhat earlier at day 5 p.i. in both BALB/c mice and cotton rats, an observation that is supported by Darniot et al. [4].

According to Alvarez et al. [1, 2], infectious HMPV could be recovered from the lung of the infected animals up to day 60, and viral RNA was present in lung tissues for ≥ 180 days (following depletion of T cells in the animals). Viable virus or viral RNA was not detected in the serum, spleen, kidneys, heart, trachea, and brain tissue. Substantial HMPV-specific antibody response was detected at day 14 p.i. and reached a maximum at day 28 p.i. Lung histopathology was modest and characterized by mononuclear cell infiltration in the

interstitium peaking day 4 p.i., and was associated with airway remodeling. The increased mucus production observed from day 2 was concordant with bronchial and bronchiolar infiltration. Subsequent experimental depletion of T and NK cells resulted in increased titers of HMPV in the lung, suggesting immune control of persistence.

From these results it seems that HMPV infection of BALB/c mice may be associated with lower pulmonary inflammatory responses compared to RSV infection of BALB/c mice. The studies in the BALB/c mouse have shown that primary HMPV infection elicits relatively weak innate and aberrant adaptive immune responses characterized by early (3 to 10 days p.i.) Th1-type cytokine response and late (after 7 days p.i.) Th2-type response, low levels of interferon-gamma (IFN-γ) production at all stages of infection, and delayed specific CTL response that coincides with persistent virus replication in the lung [2]. Weak interferon gamma production was also found in BALB/c mouse lung homogenates in other studies and peaked from days 5 to 7 [4–6]. Thereby, the work by Guerrero-Plata et al. [5] showed that HMPV is a stronger inducer of both alpha interferon (IFN-α) and IFN-γ responses than RSV. The variation in findings on the level of production may be explained by the viral strains used by the groups.

These responses as well as the presence of neutralizing antibodies may contribute to control HMPV replication but do not prevent the persistence of the virus in lungs. The findings showing that depletion of T cells or NK cells in infected BALB/c mice result in increased virus titers in the lungs are consistent with this hypothesis [1]. These latter data describing the inflammatory response in HMPV-infected mice [2] are consistent with the findings of a recent prospective study that demonstrated much lower levels of respiratory inflammatory cytokines in HMPV-infected infants than in those infected with RSV [11]. The authors suggested that HMPV and RSV either cause disease via different mechanisms or share a common mechanism that is distinct from innate immune activation.

The mechanisms leading to persistence of HMPV in BALB/c mice are not yet understood. Interestingly, prolonged shedding of the virus in respiratory secretions was also observed in high risk children [25], thus the observation of viral persistence in the HMPV infected mouse might be an important hint on the infection cycle also in humans. In this context, the results of a comparative analysis of the activity and regulation of IFN-α in BALB/c mice infected with HMPV or RSV are of interest [5]. In animals infected with either one of these viruses, the kinetics of IFN-α production was different. In HMPV-infected mice IFN-α remained at detectable levels between 6 h and day 5 p.i. with a peak between 12 and 24 h. In contrast, in RSV-infected mice, a peak of interferon production was observed at 24 h and production of this cytokine returned to undetectable levels by 72 h. If the mice were treated with interferon prior to infection, viral titers in the lungs were lower for HMPV than for RSV infected animals. If the animals infected by HMPV or RSV were treated 48 h p.i. with the inductor of interferon poly-ICLC, the production of IFN-α was completely suppressed. A limitation of these studies is the difference in permissiveness for viral infection in the BALB/c mice, which exhibited more than 10-fold lower replication of HMPV compared to RSV in the lungs. The persistence of HMPV and RSV in the lungs of infected mice might be attributable in part to an ability of these viruses to affect the interferon signaling pathways. It is possible that HMPV and RSV deploy different mechanisms to interfere with the production of IFN-α. For RSV such an inhibition has been linked to the effect of NS1 and NS2 viral proteins on IRF-3 [19]. For HMPV, which lacks the

NS proteins, a possible mechanism of immunomodulation remains unknown.

In contrast to RSV, HMPV elicited a significantly lower inflammatory cytokine response [11]. This may indicate that the mechanisms of pathogenesis are different for both viruses. Alvarez et al. [1, 2] demonstrated that primary infection of BALB/c mice with HMPV is "associated with an indolent inflammatory response". The authors further showed, that the innate immune response was weak and accompanied by $CD4^+$ T-cell trafficking to the lung and low IFN-γ expression. Later during the infection, a Th2-type IL10 expression and a delayed CTL activity evolved that coincided with persistent virus replication in the lungs of infected animals. These findings are congruent with the earlier finding by Laham et al. [11], who observed an attenuated inflammatory response in infected children. In their interesting study on the persistence of HMPV in mice, Hamelin et al. [7] found that the persisting viral HMPV RNA was accompanied by a significant ongoing pulmonary inflammation until day 154 post infection, whereas the elevated secretion of mucus was observed only until day 12. They also found that the clinical severity, i.e. breathing difficulties, peaked at day five, whereas airway obstruction and hyperresponsiveness lasted until day 70. These latter observations along with earlier observation that HMPV may be associated to a subsequent history of asthma [13, 24] in children make the mouse model an excellent tool for the study of the earlier steps of asthma history.

However, it should be noted that prolonged replication or viral persistence of either HMPV or RSV was not observed in fully immunocompetent humans but in high risk patients [25], and that this phenomenon may occur only in mice. Furthermore, the BALB/c mice infected with HMPV exhibited symptoms that included ruffled fur, huddling behavior and weight loss [4]. Thus, BALB/c mice appear to be a good model for HMPV replication, immunity and protection, but do not exhibit respiratory illness similar to that of humans and do not constitute an exact "disease" model.

Furthermore, two recent reports made use of the BALB/c mouse model for peptide vaccination studies [9] and for the study of antiviral treatment with ribavirin [8]. The results of both studies were promising as it appeared that peptide vaccination is sufficient to protect mice from infection with HMPV [9] and that it is worth to consider ribavirin – which is also active against HMPV – as an antiviral drug for treatment of severe HMPV infections [8]. Thereby the latter study [8] also investigated on the use of glucocorticoid treatment as a supplement to the ribavirin therapy.

Nevertheless, although the studies summarized above revealed BALB/c mice as a permissive host and consequently as a sufficient animal model, there is an ongoing discussion whether the mouse model is the optimal solution for future studies. The reasons for this discussion were two earlier reports from Macphail et al. [12] and Williams et al. [23] that demonstrated that mice are not permissive for HMPV, although the usefulness of the model was supported by the data of Hamelin et al. [6–8] as well as the recent observations from Darniot et al. [4] who investigated immunological response against the HMPV infection in infected BALB/c mice [4, 6–8] and cotton rats [6], respectively. It is noteworthy that Darniot et al. [4] observed HMPV replication of 10^3 pfu/g in the lungs of BALB/c mice, similar to levels detected by Williams et al. [23] and thus substantially lower than the levels of lung replication detected in BALB/c mice by Alvarez et al. [1, 2] and Hamelin et al. [6–8]. A number of factors may account for these divergent findings. Due to long inbreeding in a single laboratory, a particular BALB/c strain may be permissive for

HMPV whereas in other labs it is not, although this may be a neglectable factor. A more likely reason for divergent findings regarding the levels of lung replication may be the different viral loads inoculated in mice, using titers of 10^8 TCID50 in Hamelin et al. [6] of a strain of subgroup A compared to lower titers used by MacPhail (1×10^6 pfu/mL) [12], Darniot et al. (3.3×10^5 pfu/mL) [4], and Williams et al. (1×10^5 pfu/mL) [23].

Furthermore, the sensitivity of HMPV detection methods is likely different in different studies. Another reason may be the different virus strains used in many of these studies, giving rise to the hypothesis that permissiveness of a model for HMPV replication depends on both the virus strain and the animal strain. Time point differences in the individual investigations may also have led to the controversial conclusions. However, based on the observations from Vicente et al. [21], it appears most likely that the different severity of the experimental infections is based on the different viral strains used. Vicente et al. [21] found that in infected children the clinical severity may vary between the genotype A and genotype B human metapneumovirus. Although the individual mechanism that is responsible for the disease severity in experimentally infected mice may be different, the same principle, i.e. a subtype specific illness severity, appears the most logical explanation. The resulting hypothesis that the clinical severity of the infection depends both on (hitherto unknown) viral factors and host factors is supported by the fact that Macphail et al. [12] also found cotton rats to be poorly permissive for HMPV replication, in contrast to the findings of Wyde et al. [27], Williams et al. [23], and Hamelin et al. [6–8]. Thereby, MacPhail et al. [12], Williams et al. [23], and Hamelin et al. [6–8] used virus from strain A but different isolates as inoculum for cotton rats, whereas Alvarez et al. [1,2] used an isolate from the HMPV lineage B that is supposed to be less pathogenic in children but replicates to high titers in mice.These discrepancies may thus indeed result from yet unknown differences in virus or rodent strains used, methods of inoculation and detection or the groups' varying experience with different animal models.

2.1.2. Cotton rat model

The cotton rat model was established for the study of RSV-host interactions around 20 to 25 years ago [3, 14, 15] and since then has been widely used for the development of antivirals, vaccine research, and studies of immunity and immunopathogenesis (e.g. [14–17, 22, 26]). HMPV belongs to the same virus family as RSV and consequently several investigators have evaluated this well established model also for HMPV-host interactions. First, Wyde et al. [27] infected cotton rats with HMPV strains 26575 and 26583 from Canada (CAN98-75 (subtype B) and CAN97-83 (subtype A), respectively) and RL Bx from Gail Demmler, Texas, USA. It was observed that in general all viruses used in the study were able to replicate in rats, although significant differences in the degree of replication were detected, an observation that is in high agreement with the already mentioned observations by Vicente et al. [21] (see above paragraph).

In the infected animals the lung virus titer peaked at days 2–4 and decreased thereafter, with no virus detectable at days 10 to 14. Seroconversion was observed from day 19 p.i., but neutralizing antibody titers were dependent on the virus strain. Histological examination revealed peribronchiolar infiltration of inflammatory cells, bronchiolitis and even occasional sites of perivascular inflammation. Furthermore, thickened septal walls and numerous scattered patches of leukocytes (including PMN) were present. The histological changes were most apparent

between days 7 and 10 p.i. The virus was detected in the lung at day 7 p.i. or later, but not before day 7. Surprisingly, during the infection only a rather limited number of pneumocytes could be stained with α-HMPV antibodies [27]. Instead of pneumocytes the majority of cells stained with those antibodies were leukocytes, probably due to phagocytosis of the virus or viral replication within the leukocytes. As shown by Hamelin et al. [6], during the acute phase of the infection the expression of macrophage inflammatory protein 1α, gamma interferon, and RANTES was increased and peaked around day 5.

Nevertheless, it has to be taken into account that in rodents the innate and adaptive immune responses to HMPV infection or the virus itself may behave in a total different way which may be quite unrelated to the human infection. For example, it is commonly known that interferon antagonists can have marked host range effects. Thus, a human virus might have very different effects in monkeys or chimpanzees (which more closely model the human host) as opposed to rodents.

In another study Williams et al. [23] also tested cotton rats for permissiveness for HMPV replication [23]. All animals tested exhibited reasonable levels of viral replication in nasal turbinates to 10^6 pfu/g. This study also determined that the peak of viral replication in cotton rats was on day 4 p.i. and cleared by day 10. Cotton rats exhibited bronchiolitis-like histopathology in the lungs, with peri-bronchiolar mononuclear cell infiltrates and no alveolar or perivascular disease. Viral antigen was detected by immunohistochemistry only in ciliated respiratory epithelial cells throughout the respiratory tract, and previously infected cotton rats were protected against lung virus replication on challenge with high serum neutralizing antibody titers.

Similar to previous studies of RSV in the cotton rat model, cotton rats infected with HMPV in these studies did not display any sign of an overt disease [6,23,27]. Thus, like the mouse model, the cotton rat is a model of replication, lung histopathology and protection, but not a true "disease" model. However, since the histopathology of HMPV infected cotton rat tissue strongly resembles the histopathology of monkey tissue and since the model is well established also for other paramyxoviruses (i.e. human parainfluenza virus type 3, measles virus, and RSV), the differences in the pathogenicity of the different paramyxoviruses can be easily studied in this model. It should be noted that efficacy studies of the RSV monoclonal antibody palivizumab in cotton rats provided the foundation for clinical studies of this antibody [17], which is now approved for RSV prophylaxis in high-risk infants [16]. Taking into account the most recent data of Hamelin et al. [6] that demonstrate that there are only slight differences to the BALB/c mouse model the cotton rat model will be of great use for future HMPV research.

2.2. Other small rodent models

Williams et al. [23] also tested other small animal species for permissiveness for HMPV replication, including guinea pigs, hamsters, and nine different inbred strains of mice. All animals tested exhibited reasonable levels of viral replication in nasal turbinates, ranging from 10^3 pfu/gram tissue to 10^6 pfu/g. However, the permissiveness of different species for HMPV replication in the lungs was quite variable, from none detected in guinea pigs and some mice to high levels (10^5 pfu/g) only in hamsters and cotton rats.

In an earlier investigation, Kuiken et al. [10] as well as MacPhail et al. [12] tested various animal models in order to evaluate if they support in vivo replication of HMPV. In the study of Macphail et al. [12] those authors demonstrated as well that hamsters, and additionally ferrets and

green monkeys supported virus replication efficiently and produced high antibody levels.

Interestingly they found that hamsters vaccinated with subgroup A were protected from challenge with group A or B, probably giving implications for future vaccine design. The Syrian golden hamsters as well as ferrets supported HMPV replication to high titers [12] although neither fever nor any signs of illness were observed. However it appeared that both models are sufficient tools for the study of HMPV-host interactions, although it is rather likely that the mouse and cotton rat model will be used in most future studies for the reason of the availability of secondary reagents like antibodies and probes.

2.3. Primate models

2.3.1. Small nonhuman primates

During the past decades small monkeys were frequently used as primate models for human viral disease. The Dutch group that initially described HMPV is highly experienced in the use of non human primates and was also involved in the detection of and studies on the SARS coronavirus. Thus, Kuiken et al. [10] consequently infected 6 cynomolgus macaques (*Macaca fascicularis*) experimentally with HMPV. The animals were clustered into 3 groups and 2 animals of each group were euthanized at days 5, 9, or 14 respectively. Thereby a large set of parameters was investigated. It was found that viral excretion peaked at day 4 p.i. and decreased under all detection limits at day 10 p.i., an observation that is congruent to the findings from the rodent models.

The replication of HMPV was restricted to the respiratory tract and was associated with minimal to mild multifocal erosive and inflammatory changes in conducting airways. Kuiken et al. [10] observed an increased number of macrophages in alveoles, an observation that may be congruent with the assumption from Wyde et al. [27] that the clearance of the infection may be mediated by phagocytosis. In the infected macaques the viral replication was dominant in the apical surfaces of ciliated epithelial cells throughout the respiratory tract and less frequent in type 1 pneumocytes and alveolar macrophages [10]. This latter observation supports the alternative hypothesis of Wyde et al. [27] that the virus is also able to replicate in leukocytes. However, the macrophages that stained positive for viral antigen may simply have ingested debris from apoptotic or sloughed infected epithelial cells. It is not yet known whether HMPV is capable of infecting macrophages directly in vivo or in vitro.

Amongst the six infected animals, three individuals displayed a mild rhinitis that was characterized by the loss of ciliation in the epithelium, an architectural disruption, intra- and intercellular edema, transmigration of a few neutrophils, edema and infiltration with a few neutrophils in underlying submucosa. In these animals Kuiken et al. [10] also observed minimal multifocal lesions in conducting airways that were variable in size and were detectable from the larynx to the bronchioles, as well as epithelial lesions (i.e. loss of ciliation, architectural disruption, erosion, intercellular edema, transmigration of neutrophil infiltration with a few neutrophils in underlying submucosa). Thereby the lumen of some bronchi contained few sloughed cilial epithelial cells, mixed with scant cellular debris and mucus [10], and some also contained a few alveolar macrophages, rare multinucleated giant cells and neutrophils that were accompanied by scant cellular debris and fibrin. In general the authors concluded that the pathogenesis of the HMPV infection is similar to the pathogenesis of the RSV infection at least in macaques. From their observations

Kuiken et al. [10] conclude and summarize that viral replication is short-lived, polarized to the apical surface, and occurs primarily in ciliated respiratory cells.

In addition to the Rotterdam's group of Osterhaus, MacPhail et al. also investigated the use of small primates as animal models for HMPV replication. They observed only marginal replication in rhesus monkeys [12], but found that African green monkeys supported virus production. Neutralizing antibodies developed in African green monkeys to high titers, and antibodies to subgroup A also neutralized HMPV subgroup B. This interesting observation further hints that a serological correlate to the existence of genotypes may not be present. MacPhail et al. [12] concluded that rhesus macaques were not permissive for the HMPV subtypes used in their study (prototype for A: HMPV/NL/1/00; B: HMPV/NL/1/99), although the conflicting data on permissivity of animals as well as of cell cultures suggests that the permissivity depends on the viral subtypes used for inoculation as well as on the host. However, none of the models tested by MacPhail et al. mimicked signs from a human HMPV infection [12]. As discussed earlier, none of the small animals that have been shown to be permissive for HMPV exhibit bronchiolitis or pneumonia resembling human illness, and thus all of these are models of replication, lung pathology and immunity rather than true "disease" models. This does not preclude their usefulness to study mechanisms of immunity, antivirals and therapeutic interventions against HMPV, with lung replication and lung histopathology as the outcomes of interest.

2.3.2. Chimpanzees

Yet, to the best of our knowledge, there is only one report of experimental infection of chimpanzees with HMPV. Besides the results of experimental infection of hamsters which were highly confirmative to the observations summarized above, Skiadopoulos et al. [18] presented the results of experimental infections of captive chimpanzees (*Pan troglodytes*). In our view, the most impressive results of the study was that out of the 31 animals ranging between 1.5 and 3 years of age 61% ($n = 19$) were seropositive for antibodies to HMPV, thus there were only 12 seronegative animals left that were included in the study. These surprising data indicate that chimpanzees may also be naturally infected with HMPV thus leading to the assumption that there is no species barrier between humans and chimpanzees regarding HMPV. Most important, seronegative animals displayed symptoms of respiratory illness post infection whereas the seropositive animals were protected from reinfections independent of the genotype with which they were infected. All animals shed only small amounts of virus. Thereby the shedded number of infectious virus from seropositive animals was the lowest, a finding that vice versa indicates that virus shedding may be a marker for the illness severity. This finding remarkably was neglected for diagnostic purposes since there is a rather limited number of reports on clinical studies that made use of quantitative detection of HMPV in clinical samples.

The study from Skiadopoulos et al. [18] further shows that immunization of the chimpanzees protected the animals from challenge irrespective of the subtype used thus the lack of serotypes is also supported by this study. For obvious ethical and economical reasons, the use of the chimpanzee model is rather limited, although it seems to be the only model in which "real" infection occurs.

3. CONCLUSION

In summary, a number of small animal and primate models for HMPV

have been investigated, including hamsters, African green monkeys, rhesus, cynomolgus macaques, and chimpanzees. Most of these animals were more or less permissive for HMPV replication in the respiratory tract, and were shown to be protected against challenge either with homologous or heterologous virus, suggesting that previous infection with the virus belonging to one major HMPV group (type) may confer protection against reinfection by the virus from another group (type). These findings have evident implications for HMPV vaccine research and enable future therapy studies. However, it also appears that the chimpanzee infected with HMPV represents the only "real" infection model; the small rodent models with mice and rats seem to be the tool of choice for most future preclinical studies.

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