nature portfolio

Peer Review File

Pathogenesis of bovine H5N1 clade 2.3.4.4b infection in Macaques

Corresponding Author: Dr Heinz Feldmann

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Nature review
'Orogastric Exposure of Cynomolgus Macaques to Bovine HPAI H5N1 Virus Results in Subclinical Infection' from Rosenke et al describes an experimental infection of Macaques with a strain of HPAI H5N1. The strain and different routes of inoculation used, and the outcomes of these experiments are highly and urgently relevant with regards to the new risks for spillover to humans that occurs now that the virus is circulating in cattle in the USA. Therefore, I think this study is timely and relevant. Several HPAI infections of humans associated to cattle as well as poultry contacts with this strain have been detected. Of note HPAI H5 infection has been detected in at least one person (Missouri) for which the source of infection has remained unclear. This increases worldwide the concern that HPAI H5 infections in people might be or start circulating between people without being noticed. The manuscripts focus strongly on the results from the orogastric inoculation group, which I find somewhat off balance. I think the outcomes of the other groups are also important.
In general, I find that the experiment has been done thorough: it is combining clinical data, virological data, clinical pathology data and histopathology data. I do have two major concerns.

1. With regards to the methodology of the orogastric inoculation route: line 51 '107 TCID50 of HPAIV H5N1 clade 2.3.4.4b (A/bovine/OH/B24OSU342/2024', in line 52 the route is shortly described 'by the orogastric (oral cavity and gavage)'... 'mimicking oral ingestion'. Starting line 164 'The process of eating and drinking, however, is more complex exposing the oropharyngeal and esophageal mucosae prior to the mucosae lining the duodenum and stomach; therefore, virus was deposited in the oral cavity as well as in the stomach of the macaques in this study group. The local environment in the stomach is likely to inactivate enveloped viruses, such as HPAIV H5N1, dependent on pH, dose and time of exposure.' Previously experimental inoculation to mimic natural infection of cats and foxes with HPAI via the oral route was performed by making use of specific capsules, capsules contained liver homogenate of infected chickens (https://pmc.ncbi.nlm.nih.gov/articles/PMC3255817/) or feeding infected carcasses (Reperat et al, EID, 2008). It is expected that food intake, like meat or milk intake, could influence the amount of infectious virus passing the gastric barrier, and reaching the intestine. In my opinion the current description of the methodology raises questions on the exact methodology used and how well it mimicked intake of raw milk. To address this issue the methodology of the orogastric inoculation need further description. How was the virus dose administrated? (volume, medium, capsule use). How do authors expect the virus inoculation to have affected their results including comparing their route and outcome to other gastric routes and results performed previously?
2. Observations of outbreaks of severe disease and high mortality in carnivores (e.g. cats in Polen, large felids in Cambodia, cats on dairy farms https://www.nature.com/articles/s41586-024-07849-4) and individual carnivores e.g. foxes and seals after H5 HPAI infection of different strains, where the expected route of exposure was oral, I find the outcome of orogastric inoculation surprising. Cats died after oral inoculation (https://pmc.ncbi.nlm.nih.gov/articles/PMC3255817/), while Red Foxes (Reperat et al, EID, 2008) had mild disease and hardly detectable lesions. I miss discussion of these natural and experimental observations, on what authors expected based on those observations of H5 infection after oral exposure in mammals and explanations for the differences observed.
3. Overall, the focus of all the different analyses (clinical, pathological, virological) are on respiration, while neurologic disease, diarrhoea, conjunctivitis are also common presentations of avian influenza infections. It is not clear in what extend these were investigated. This especially striking when comparing a respiratory inoculation route with an oral inoculation route -where one might expect the start of the infection to be different.

Small comments:
In the introduction I would appreciate numbers on the number of people that (are estimated to) drink raw milk? What are USA legal regulations on selling raw milk?
Line 27: Number of affected dairy herds need to be updated? On Current H5N1 Bird Flu Situation in Dairy Cows | Bird Flu | CDC, 339 instead of 190. In my opinion the trend is also important to point out as numbers continue to increase and hence the outbreak increases in relevance. An option might be to highlight that the monitoring or surveillance allows for underreporting and that the problem is likely to be bigger.
Line 29: Please make clear in this sentence that it addresses risk exposure to humans (not clear, could also be risk for birds, domestic mammals, wild mammals that all have been exposed to this virus likely by drinking dairy). Are macaques here used to simulate human exposure?
Line 101: 'met the predetermined endpoint late on D4 and were euthanized at that time. The last animal in the intratracheal group and the orogastric and intranasal inoculated groups were euthanized at the predetermined endpoint (D5)' The way this is currently described is confusing to me as there are two different timepoints both described as being 'predetermined endpoints' while they refer to another type of endpoint (I think). Possibly authors could differentiate in their writing the reaching of the (predetermined) clinical/disease severity endpoint versus the methodologically (predetermined) endpoint more clearly.

For example: 'were generally free from evidence of pneumonia' However, 2 pulmonary lobes in one orogastric inoculated animal and 2 animals in the intranasal group (with one lobe each) exhibited mild signs of pneumonia and limited macrophage and neutrophil infiltration.' I struggle to follow the descriptions of these parts of the results. Maybe it would increase clarity to differentiate results in two (maybe in a table): 1. Infection presence /absence; 2. Infection/lesion severity.

Line 83-84: 'This data indicates that all inoculation routes resulted in productive shedding, mainly from the oronasal mucosae.' These are quite relevant results which are not so much discussed or highlighted. For example seeing that the nasal swab data does not differ so much between the nasally inoculated and the orogastric inoculated group. From the nasal group this is an expected result as a large quantity of virus was deposited there – but still confirms that HPAI can replicate (a bit) in the upper respiratory tract – makes it more likely to spread? Also that this happens almost D1 to D7 after orogastric inoculation is surprising. Where does the virus come from? As stated above, that is quite concerning with regards to shedding in absence of disease.
Line 107: 'gastrointestinal tract to a limited extend' & 'Additionally infectious virus was found in the heart, liver, spleen, adrenal glands, kidney and the mediastinal and mesenteric lymph nodes' Is not clear to me what part of the gastrointestinal tract. I would rather read the specific observations then vague terminology like 'limited extend' or 'generally'. Was nervous system tissue investigated, what and how? In suppl fig 3 I see cerebellum brainstem and 'frontal' Please define frontal. Also were olfactory bulbs and trigeminal ganglia specifically sampled for IHC as they are expected to be the first to be infected if virus comes through neuronal route from the oral or nasal cavity?
Line 167: 'The shedding data indicate that onwards transmission following orogastric exposure may be limited due to low infectious titers shed but remain a possibility.'
The conclusion I draw based on the results is that there ís shedding of virus, it is not shorter but longer looking at nasal swab results D1 to D7, and equally long for oral swabs. This virus is maybe less high titers, but still potentially infectious, while animals do not show any disease, or less disease then the others. Healthy infected humans might be much more likely to have close contacts with other people and therefore can be more important in virus spread, then people with more and longer shedding that are ill. As outlined by some of the authors of this manuscript (Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. A Novel Coronavirus Emerging in China - Key Questions for Impact Assessment). To me this is an important result that needs more attention.

Line 169: 'This could explain the inefficient infection by the orogastric route seen in our study as well as the lack of rectal shedding following orogastric exposure. Nevertheless, our study suggests self-limiting infection after the consumption of contaminated food or liquid compared to droplet and aerosol exposure.'
In my opinion, depending on the details of the inoculation, it is important to address in this reasoning if the orogastric inoculation is or is not likely to have mimicked the intake of virus containing raw milk. If there are doubts about this, this needs to be addressed as it can have quite big implications on what the data suggest.

Figure 1C: the orogastric group has grey bars, but should be black?
Supplement:

Clinical analyses supplement table: I don't see the heading or legend. There is an asterisk for the possible rectal bleeding, but I don't see it explained? Looking at the excellent clinical scoring sheet from reference 18: why not provide all the data in the supplement as currently we see the endscore in figure 1 B, but don't see what determines the differences – makes detailed comparison here difficult? Or if too much data at least a more elaborate summary so that one can see what clinical signs make the differences shown in figure 1B of the main document. Especially as the current manuscript is so focused on the respiratory tract -dulness / wet stool /decreased appatite might be much more relevant clinical sign to address in the orogastric group then in a intranasal inoculated group.
Supplement figure 1: Both ALT and ALP levels have a strikingly different starting point (day -6) between orogastric group and the other two groups (ALT 40 versus 30) (ALP 100 versus 200). Do authors have any explanation for this?

Conclusion: well performed experimental study on a highly relevant topic. There seems relevant information missing with regards to the exact methodology of the orogastric inoculation, or I missed this? Would like to see the details and see discussed how this could influence their interpretation of the data. Is the experiment mimicking exposure of oral cavity to a drop of milk containing virus, or a glass of milk containing virus?

Referee #2

(Remarks to the Author)
The authors carried out experimental infections of cynomolgus macaques with a relatively high dose (10 7 log TCID50) of a bovine isolate of clade 2.3.4.4b virus via the intranasal (mucosal atomisation device) virus inoculum), intra-tracheal and oro-gastric routes. The high dose was chosen to mimic infectious titers detected in infected raw milk which has similar or even higher infectious titres.
In the orogastric inoculation, the virus was inoculated via and intragastric tube and also inoculated to the oro-pharyngeal cavity. Systematic clinical, biochemical and cytokine investigations were carried out. Animals inoculated via the orogastric route remained subclinical over the course of the study and had no lung pathology. Intranasally inoculated animals had mild disease course with slightly elevated clinical scores until study endpoint at D14 and mild radiological changes with minimal lung pathology. Intratracheally inoculated group had progressively severe respiratory disease with biochemical evidence of renal and hepatic dysfunction requiring euthanization before day 8. Severe lung pathology was observed with virus detected by immune-histochemistry in the alveolar cells. Broncho-alveolar brush virus only detected in intransal and intra-tracheal group, not in orgogastric group. Only interatracheal inoculated group (but not orogastric group) had any significant shedding in rectal swabs.
The oro-gastric inoculated group had virus shedding in oral and nasal swabs for 7-11 days and did sero-convert, as did the intranasal group. Interestingly, no comment is made about sero-conversion in the intra-tracheal group, presumably because that animals had to be euthanised prior to the planned time point for blood collection for seroconversion. It would be useful to spell this out, because otherwise it appears that the intra-tracheal group had not seroconverted (Figure 1i).
Overall, this is an important study documenting a) the course of clade 2.3.4.4b genotype B3.13 infection in cynomolgus macaques, b) a comparison of the intranasal, intra-tracheal and orogastric route, mimicking what may occur following ingestion of virus infected raw milk. The findings suggest that ingestion of infected raw milk may not lead to significant disease but lead to sero-conversion.

A few specific comments to be addressed:
While non-human primates are assumed to be closer to humans that other experimental models, can the authors comment on the alpha2-3 vs alpha 2-6 receptor distribution in the macaque and human respiratory tracts?
Viremia = viral RNA quantitation. Was culture done to ascertain of infectious virus was detected in blood?
It would have been interesting to also inoculate via conjunctiva, although the reviewer is aware of the challenges of obtaining sufficient macaques for such experiments.
Was the GI tract investigated histo-pathologically and immune-histochemically for viral antigen? It would be important to know of the virus was infecting the intestinal mucosa? The sero-conversion suggests that virus may replicate in the GI tract as well as respiratory tract in orogastric infected animals?
Similarly, was the brain investigated for virus and histopathology and IHC? Given the frequent neurotropism seen in ferrets (but not in humans) it would be interesting to know whether the virus has any neurotropism in macaques, even in the most severely ill intra-tracheal group
Figure 1C. The legend for the orogastric group should be grey to match what is shown in bar-chart.
Fig 1B should be headed “Clinical Scores in long term group” and 1H should be headed “Radiographic changes in long term group”
Line 51-52: Specify that the virus used was a genotype B3.13 virus.

Referee #3

(Remarks to the Author)
The authors describe infection of nonhuman primates with a human isolate of "bovine" H5N1 via three different routes - intranasal, intratracheal, and orogastric. Intranasal infection resulted in only mild disease, which matches with prior reports for other H5N1 viruses in nonhuman primates that used this route. In contrast with previous reports for other H5N1 viruses, intratracheal inoculation resulted in severe disease. Orogastric inoculation caused subclinical disease, suggesting that consumption of H5N1-contaminated dairy products might not cause severe disease. The article is well-written and clear. Some revisions are necessary to provide information (age, weight, sex, and geographic origin of all NHPs used), details on the scoring system used for clinical observations, and virus sequence, to confirm identity with the original isolate that was obtained and propagated prior to challenge. Some revision of the text is necessary to qualify statements made, as the number of animals is very small and statistical significance is lacking - the results are suggestive. The authors make a statement that intratracheal inoculation results in systemic disease but provide no proof, only indirect evidence. Either proof should be provided or the statements qualified to admit the data is merely suggestive, not definitive of systemic infection. Discussion of the results should include more of the previous work on NHP with H5N1 viruses, in particular previous studies with Vietnam/2004 given by intranasal and/or intratracheal inoculation which is only rarely lethal in cynomolgus macaques.
A few specific items
1. Was body temperature data collected? This seems a rather surprising omission, given the prominence of fever in H5N1 disease (in ferrets, macaques, and humans). If the data was collected, it should be included. Similarly, body weight data should be included. If the authors do not want to add figures to the main text, putting this information in the supplemental data would be adequate.
2. Line 35: Should be revised to "This study *suggests* that consumption of contaminated products, such as milk, may lead to self-limiting, subclinical infection in primates". Considering the low number of animals (n=3 that were allowed to go to study endpoint) and that the virus does not appear to have been inoculated in raw milk, the original statement is far too strong. Even if the virus was inoculated in milk, this statement is too strong given the number of animals.

3. Line 74: the statement that a decrease in blood albumin is indicative of systemic inflammation & infection needs to be qualified. Albumin levels could also be low as a result of pulmonary edema, which was reported in the 2017 Wonderlich et al paper.
4. Line 27: the number of infected herds should be updated (perhaps just state ">300")

Referee #4

(Remarks to the Author)
The study conducted by the Feldmann lab addresses an urgent question of public health concern, namely whether the currently circulating H5N1 HPAIV of the 2.3.4.4b clade pose a threat to humans.
Therefore, they use the closest surrogate marker for humans, cynomolgus macaques. They cross-compare key infection routes (orogastric (relevant if virus uptake occurs via contaminated food), intranasal (relevant if infection occurs via direct contact or airborne infection of the upper respiratory tract) and intratracheal (airborne infection of the lower respiratory tract)) for H5N1 in the macaque model. They show that orogastric H5N1 infection does not lead to a highly productive infection in line with a very mild disease outcome and rendering seroconversion. Intranasal H5N1 infection leads productive virus replication and a mild infection course. The most severe disease outcome is observed upon intratracheal infection of macaques resulting in high virus replication titers, viremia, inflammatory cytokine response and death on day 7 or 8 p.i..
The findings obtained in this study are of utmost importance for risk assessment for humans as they will guide future policies regarding pandemic intervention strategies. The study was conducted to highest standards and all conclusions drawn are supported by the experiments performed. However, there might a be a few points that could be addressed:

Specific points:
#1: Unfortunately, no information is provided regarding the bovine H5N1 isolate used regarding its receptor specificity (a2,3- versus a2,6-linked sialic acid preference) (van Riel et al., Science 2006) or replication efficiency in mammalian cells (PB2 E627K, PB2 D701N) (Subbarao et al., Science 1998; Gabriel et al., PNAS 2005). This should be at least discussed in the context of the mentioned literature. If mostly avian-like signatures are present in the used isolate (a2,3-linked SA, PB2 627E, 701D), the authors should consider sequencing isolates from the infected animals.

#2: It would be helpful to discuss the obtained results in the context of the humans infected with H5N1 clade 2.3.4.4b so far. What is known there regarding the infection route and correlation thereof with known molecular interspecies signatures (HA, polymerase)? Please discuss.

#3: Supplemental Figure 4: I couldn´t find an IHC for the intratracheal route. Is there any information on this? It would be helpful if the authors could discuss cell-types infected by H5N1 cross comparing the different infection routes.

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Gülsah Gabriel
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Dear Editor(s)

We appreciate reviewing our manuscript for its suitability for *Nature*. The comments provided by the reviewers and the editor(s) were helpful and certainly improved our manuscript. Below we have provided a point-to-point response to the comments and hope that the revised version satisfactorily addresses all concerns. We are looking forward to your response.

Sincerely,
Emmie de Wit, Vincent Munster & Heinz Feldmann

Point-To-Point Response

Referee #1 (Remarks to the Author):

Nature review
'Orogastric Exposure of Cynomolgus Macaques to Bovine HPAI H5N1 Virus Results in Subclinical Infection' from Rosenke et al describes an experimental infection of Macaques with a strain of HPAI H5N1. The strain and different routes of inoculation used, and the outcomes of these experiments are highly and urgently relevant with regards to the new risks for spillover to humans that occurs now that the virus is circulating in cattle in the USA. Therefore, I think this study is timely and relevant. Several HPAI infections of humans associated to cattle as well as poultry contacts with this strain have been detected. Of note HPAI H5 infection has been detected in at least one person (Missouri) for which the source of infection has remained unclear. This increases worldwide the concern that HPAI H5 infections in people might be or start circulating between people without being noticed. The manuscripts focus strongly on the results from the orogastric inoculation group, which I find somewhat off balance. I think the outcomes of the other groups are also important.
In general, I find that the experiment has been done thorough: it is combining clinical data, virological data, clinical pathology data and histopathology data. I do have two major concerns.
We have given the other routes of infection more visibility through expansion of the discussion. (lines 189-206)

1. With regards to the methodology of the orogastric inoculation route: line 51 '107 TCID50 of HPAIV H5N1 clade 2.3.4.4b (A/bovine/OH/B24OSU342/2024', in line 52 the route is shortly described 'by the orogastric (oral cavity and gavage)'... 'mimicking oral ingestion'. Starting line 164 'The process of eating and drinking, however, is more complex exposing the oropharyngeal and esophageal mucosae prior to the mucosae lining the duodenum and stomach; therefore, virus was deposited in the oral cavity as well as in the stomach of the macaques in this study group. The local environment in the stomach is likely to inactivate enveloped viruses, such as HPAIV H5N1, dependent on pH, dose and time of exposure.' Previously experimental inoculation to mimic natural infection of cats and foxes with HPAI via the oral route was performed by making use of

specific capsules, capsules contained liver homogenate of infected chickens (https://pmc.ncbi.nlm.nih.gov/articles/PMC3255817/) or feeding infected carcasses (Reperat et al, EID, 2008). It is expected that food intake, like meat or milk intake, could influence the amount of infectious virus passing the gastric barrier, and reaching the intestine. In my opinion the current description of the methodology raises questions on the exact methodology used and how well it mimicked intake of raw milk. To address this issue the methodology of the orogastric inoculation need further description. How was the virus dose administrated? (volume, medium, capsule use). How do authors expect the virus inoculation to have affected their results including comparing their route and outcome to other gastric routes and results performed previously?

The reviewer brings up an important point and one that is not easily addressed using the NHP model. We decided to infect the animals via the orogastric route by placing virus in the mouth and stomach. We think this is the best way to expose the oral mucosa, esophageal tract and stomach consistently. We opted to use the direct intragastric route with 6mls (8.3 x 106 TCID50s total dose) of inoculum in media (DMEM) followed by 5mls of buffered saline to flush the tube and help offset the acidity of the stomach. We also added 1ml of inoculum (1.7 x 10^6 TCID50s total dose) to the oral cavity and induced deglutition to expose both the oral mucosal and esophageal tract to infectious virus. We understand and agree with the reviewer that it is very difficult to measure how much virus made it through the gastric barrier and into the intestinal tract and there is no direct way to measure the success of our route selection. We think that the orogastric inoculation as performed best mimics the intake of liquid such as milk. As comparison of exposure route was one of the objectives of the study, we chose to use DMEM for dilution of the virus inoculum to not add another variable (milk versus DMEM). In response to the reviewer, we have provided more information in the Methods section and changed the wording in the text (lines 207-219).

2. Observations of outbreaks of severe disease and high mortality in carnivores (e.g. cats in Polen, large felids in Cambodia, cats on dairy farms https://www.nature.com/articles/s41586-024-07849-4) and individual carnivores e.g. foxes and seals after H5 HPAI infection of different strains, where the expected route of exposure was oral, I find the outcome of orogastric inoculation surprising. Cats died after oral inoculation (https://pmc.ncbi.nlm.nih.gov/articles/PMC3255817/), while Red Foxes (Reperat et al, EID, 2008) had mild disease and hardly detectable lesions. I miss discussion of these natural and experimental observations, on what authors expected based on those observations of H5 infection after oral exposure in mammals and explanations for the differences observed.

We have added a discussion on the oral route of infection in natural and experimental settings. A final conclusion, however, is difficult. We think that the route we have chosen for orogastric exposure best mimics consumption of contaminated liquid by an animal, while naturally exposed carnivores most likely exposed through eating which affect the duration of exposure, as well as the inactivation of virus in the stomach. (lines 207-219)

3. Overall, the focus of all the different analyses (clinical, pathological, virological) are on respiration, while neurologic disease, diarrhoea, conjunctivitis are also common presentations of avian influenza infections. It is not clear in what extend these were

investigated. This especially striking when comparing a respiratory inoculation route with an oral inoculation route -where one might expect the start of the infection to be different.
Animals were monitored at least twice daily for any clinical signs according to the clinical scoring form (see Extended Data Table 1). Additionally, complete clinical exam was performed on anesthetized animals at the indicated times. Thus, clinical signs of disease would have been noticed and reported (Figure 1B & Extended Data Table 1&2). Viral loads were determined for many tissues, swabs and blood (Figures 1 & 2; Extended Data Figure 4). The findings support systemic infection at least for the animals infected intratracheally and likely also intranasally. Clinical disease signs were driven by virus replication in the respiratory tract organs, which is supported by the viral load data and histopathology. No other organ analyzed showed pathologic changes that would account for additional clinical signs others than respiratory signs. Especially, there was no evidence for viral replication or pathology in the brain (Extended Data Figure 4). Overall, the study paid a large deal of attention to general clinical signs, which were thoroughly investigated (Figure 1B; Extended Data Table 1&2; Extended Data Figure 4).

Small comments:

In the introduction I would appreciate numbers on the number of people that (are estimated to) drink raw milk? What are USA legal regulations on selling raw milk?
4.4% of U.S. adults reported consuming raw milk at least once in the past year, with 1.6% reporting frequent consumption of raw milk (once per month or more often) and 1.0% reporting consumption once per week or more often. The individuals who consumed raw milk in the previous 12 months were more likely to be younger, living in a rural area, and living in a state in which retail sale of raw milk is legal. (Lando AM et al. Characteristics of U.S. Consumers Reporting Past Year Intake of Raw (Unpasteurized) Milk: Results from the 2016 Food Safety Survey and 2019 Food Safety and Nutrition Survey. J Food Prot. 2022 Jul 1;85(7):1036-1043). This has now been added to the introduction. (lines 51-53)

Line 27: Number of affected dairy herds need to be updated? On Current H5N1 Bird Flu Situation in Dairy Cows | Bird Flu | CDC, 339 instead of 190. In my opinion the trend is also important to point out as numbers continue to increase and hence the outbreak increases in relevance. An option might be to highlight that the monitoring or surveillance allows for underreporting and that the problem is likely to be bigger.
The text has been changed to reflect the ongoing outbreak. (lines 30-31)

Line 29: Please make clear in this sentence that it addresses risk exposure to humans (not clear, could also be risk for birds, domestic mammals, wild mammals that all have been exposed to this virus likely by drinking dairy). Are macaques here used to simulate human exposure?
This has been clarified as indicated. (line 33)
Yes, we used macaques as a surrogate model for humans. (lines 55-57)

Line 101: ‘met the predetermined endpoint late on D4 and were euthanized at that time. The last animal in the intratracheal group and the orogastric and intranasal inoculated groups were euthanized at the predetermined endpoint (D5)’ The way this is currently described is confusing to me as there are two different timepoints both described as being ‘predetermined endpoints’ while they refer to another type of endpoint (I think). Possibly authors could differentiate in their writing the reaching of the (predetermined) clinical/disease severity endpoint versus the methodologically (predetermined) endpoint more clearly.
This has been clarified. (lines 117-122)

For example: ‘were generally free from evidence of pneumonia’ However, 2 pulmonary lobes in one orogastric inoculated animal and 2 animals in the intranasal group (with one lobe each) exhibited mild signs of pneumonia and limited macrophage and neutrophil infiltration.’ I struggle to follow the descriptions of these parts of the results. Maybe it would increase clarity to differentiate results in two (maybe in a table): 1. Infection presence /absence; 2. Infection/lesion severity.
We have changed the text accordingly to make it more clear to the reader. (lines 130-140)

Line 83-84: ‘This data indicates that all inoculation routes resulted in productive shedding, mainly from the oronasal mucosae.‘ These are quite relevant results which are not so much discussed or highlighted. For example seeing that the nasal swab data does not differ so much between the nasally inoculated and the orogastric inoculated group. From the nasal group this is an expected result as a large quantity of virus was deposited there – but still confirms that HPAI can replicate (a bit) in the upper respiratory tract – makes it more likely to spread? Also that this happens almost D1 to D7 after orogastric inoculation is surprising. Where does the virus come from? As stated above, that is quite concerning with regards to shedding in absence of disease.
The orogastric route inoculation included dropping a portion of the inoculum into the mouth exposing the oropharyngeal mucosae. This is likely the source of the virus in the upper respiratory tract of the animals inoculated by the orogastric route. (see description in Methods)

Line 107: ‘gastrointestinal tract to a limited extend’ & ‘Additionally infectious virus was found in the heart, liver, spleen, adrenal glands, kidney and the mediastinal and mesenteric lymph nodes’ Is not clear to me what part of the gastrointestinal tract. I would rather read the specific observations then vague terminology like ‘limited extend’ or ‘generally’. Was nervous system tissue investigated, what and how? In suppl fig 3 I see cerebellum brainstem and ‘frontal’ Please define frontal. Also were olfactory bulbs and trigeminal ganglia specifically sampled for IHC as they are expected to be the first to be infected if virus comes through neuronal route from the oral or nasal cavity?
We have added an additional table (Extended Data Table 3) to the extended data section to clarify the IHC in specific tissues, including the cell type infected (mainly macrophages). The nervous system (frontal lobe, brainstem and cerebellum) was included in our analysis, but no infectious virus was detected nor was there viral antigen

staining observed in any neural tissues. (lines 147-151) The olfactory bulb in macaques is too small to remove as a single structure, it is included in the brain section marked as frontal lobe.

Line 167: 'The shedding data indicate that onwards transmission following orogastric exposure may be limited due to low infectious titers shed but remain a possibility.'
The conclusion I draw based on the results is that there ís shedding of virus, it is not shorter but longer looking at nasal swab results D1 to D7, and equally long for oral swabs. This virus is maybe less high titers, but still potentially infectious, while animals do not show any disease, or less disease then the others. Healthy infected humans might be much more likely to have close contacts with other people and therefore can be more important in virus spread, then people with more and longer shedding that are ill. As outlined by some of the authors of this manuscript (Munster VJ, Koopmans M, van Doremalen N, van Riel D, de Wit E. A Novel Coronavirus Emerging in China - Key Questions for Impact Assessment). To me this is an important result that needs more attention.
We agree with the reviewer to an extent, the presence of infectious virus means transmission may occur. However, macaques are not an established transmission model for influenza A virus, and we do not know whether they would be a good predictor for transmission in humans. Thus, without performing transmission studies we cannot address this question appropriately. (lines 220-228)

Line 169: 'This could explain the inefficient infection by the orogastric route seen in our study as well as the lack of rectal shedding following orogastric exposure. Nevertheless, our study suggests self-limiting infection after the consumption of contaminated food or liquid compared to droplet and aerosol exposure.'
In my opinion, depending on the details of the inoculation, it is important to address in this reasoning if the orogastric inoculation is or is not likely to have mimicked the intake of virus containing raw milk. If there are doubts about this, this needs to be addressed as it can have quite big implications on what the data suggest.
We have made changes to the text in the corresponding paragraph. (lines 207-219)

Figure 1C: the orogastric group has grey bars, but should be black?
This has been corrected.

Supplement:

Clinical analyses supplement table: I don't see the heading or legend. There is an asterisk for the possible rectal bleeding, but I don't see it explained? Looking at the excellent clinical scoring sheet from reference 18: why not provide all the data in the supplement as currently we see the endscore in figure 1 B, but don't see what determines the differences – makes detailed comparison here difficult? Or if too much data at least a more elaborate summary so that one can see what clinical signs make the differences shown in figure 1B of the main document. Especially as the current manuscript is so focused on the respiratory tract -dulness / wet stool /decreased appatite might be much more relevant clinical sign to address in the orogastric group

then in a intranasal inoculated group.
Supplement figure 1: Both ALT and ALP levels have a strikingly different starting point (day -6) between orogastric group and the other two groups (ALT 40 versus 30) (ALP 100 versus 200). Do authors have any explanation for this?
We have added heading and legend for Supplemental Table 1. In addition, we have added the ACUC approved clinical scoring sheet that we used here (Extended data Table 1) to explain/support the scores provided in Extended Data Table 2 and Figure 1B. With the spelled out clinical signs in Extended Data Table 1 and 2, this should make clear what scores were given for what clinical signs.
Regarding the ALT and ALP levels, the changes seem animal -dependent and we don't have an explanation. The changes, however, are unlikely to be related to infection as the differences are already visible prior to H5N1 challenge.

Conclusion: well performed experimental study on a highly relevant topic. There seems relevant information missing with regards to the exact methodology of the orogastric inoculation, or I missed this? Would like to see the details and see discussed how this could influence their interpretation of the data. Is the experiment mimicking exposure of oral cavity to a drop of milk containing virus, or a glass of milk containing virus?
We appreciate the general concluding remark by the reviewer. We have addressed the methodology of the orogastric inoculation to the best of our ability. The oral cavity was exposed to 1mL of inoculum which is more than a drop but less than a glass of milk.

Referee #2 (Remarks to the Author):

The authors carried out experimental infections of cynomolgus macaques with a relatively high dose (10 7 log TCID50) of a bovine isolate of clade 2.3.4.4b virus via the intranasal (mucosal atomisation device) virus inoculum), intra-tracheal and oro-gastric routes. The high dose was chosen to mimic infectious titers detected in infected raw milk which has similar or even higher infectious titres.
In the orogastric inoculation, the virus was inoculated via and intragastric tube and also inoculated to the oro-pharyngeal cavity. Systematic clinical, biochemical and cytokine investigations were carried out. Animals inoculated via the orogastric route remained subclinical over the course of the study and had no lung pathology. Intranasally inoculated animals had mild disease course with slightly elevated clinical scores until study endpoint at D14 and mild radiological changes with minimal lung pathology. Intratracheally inoculated group had progressively severe respiratory disease with biochemical evidence of renal and hepatic dysfunction requiring euthanization before day 8. Severe lung pathology was observed with virus detected by immune-histochemistry in the alveolar cells. Broncho-alveolar brush virus only detected in intransal and intra-tracheal group, not in orgogastric group. Only interatracheal inoculated group (but not orogastric group) had any significant shedding in rectal swabs. The oro-gastric inoculated group had virus shedding in oral and nasal swabs for 7-11 days and did sero-convert, as did the intranasal group. Interestingly, no comment is made about sero-conversion in the intra-tracheal group, presumably because that animals had to be euthanised prior to the planned time point for blood collection for

seroconversion. It would be useful to spell this out, because otherwise it appears that the intra-tracheal group had not seroconverted (Figure 1i).
This has been spelled out. (lines 115-116)

Overall, this is an important study documenting a) the course of clade 2.3.4.4b genotype B3.13 infection in cynomolgus macaques, b) a comparison of the intranasal, intra-tracheal and orogastric route, mimicking what may occur following ingestion of virus infected raw milk. The findings suggest that ingestion of infected raw milk may not lead to significant disease but lead to sero-conversion.
We appreciate the positive assessment by the reviewer.

A few specific comments to be addressed:
While non-human primates are assumed to be closer to humans that other experimental models, can the authors comment on the alpha2-3 vs alpha 2-6 receptor distribution in the macaque and human respiratory tracts?
The distribution of alpha2,3 vs alpha2, 6 receptors throughout the respiratory tract of cynomolgus macaques (or other nonhuman primates) as determined by lectin stains is not available from the literature. We have attempted to perform these stains on tissues from uninfected cynomolgus macaques in response to this question but have so far been unsuccessful. We could try to optimize these stains, but we cannot do it within the time set for submitting our revised manuscript. However, there is literature showing that seasonal (H1N1 and H3N2), pandemic (1918 and 2009 H1N1), and avian influenza viruses (HPAI H5N1 and LPAI H7N9) can infect macaques and cause respiratory disease similar to that observed in humans, suggesting that receptor distribution might be similar.

Viremia = viral RNA quantitation. Was culture done to ascertain of infectious virus was detected in blood?
Yes, we have added the virus infectivity data on blood. (see Figure 1F and lines 101-105)

It would have been interesting to also inoculate via conjunctiva, although the reviewer is aware of the challenges of obtaining sufficient macaques for such experiments.
We agree with the reviewer but unfortunately, we had only a limited number of animals for this study. This should be addressed in a follow-up study.

Was the GI tract investigated histo-pathologically and immune-histochemically for viral antigen?
Yes, we did investigate histopathology. The data is presented as Extended Data Table 3 in the revised manuscript.

It would be important to know of the virus was infecting the intestinal mucosa? The sero-conversion suggests that virus may replicate in the GI tract as well as respiratory tract in orogastric infected animals?

Virus was titered in the GI tract and infectious virus was found in a few animals at low levels. Histologically, we did not find evidence of replication in the intestinal mucosa. This is highlighted in lines 147-151 (Extended Data Table 3; Extended Data Figure 4)

Similarly, was the brain investigated for virus and histopathology and IHC?

The brain section analyzed were negative for infectious virus and pathological changes, including immunohistochemistry staining for NP. (Lines 147-151; Extended Data Table ; Extended Data Figure 4)

Given the frequent neurotropism seen in ferrets (but not in humans) it would be interesting to know whether the virus has any neurotropism in macaques, even in the most severely ill intra-tracheal group.
We did not see any pathology in the brain sections analyzed neither did we detect any virus in the brain. (Extended Data Figure 4; Extended Data Table 3)

Figure 1C. The legend for the orogastric group should be grey to match what is shown in bar-chart.
This has been corrected.

Fig 1B should be headed “Clinical Scores in long term group” and 1H should be headed “Radiographic changes in long term group”
This has been corrected.

Line 51-52: Specify that the virus used was a genotype B3.13 virus.
Added to line 56 and in Methods

Referee #3 (Remarks to the Author):

The authors describe infection of nonhuman primates with a human isolate of "bovine" H5N1 via three different routes - intranasal, intratracheal, and orogastric. Intranasal infection resulted in only mild disease, which matches with prior reports for other H5N1 viruses in nonhuman primates that used this route. In contrast with previous reports for other H5N1 viruses, intratracheal inoculation resulted in severe disease. Orogastric inoculation caused subclinical disease, suggesting that consumption of H5N1-contaminated dairy products might not cause severe disease. The article is well-written and clear.
Some revisions are necessary to provide information (age, weight, sex, and geographic origin of all NHPs used), details on the scoring system used for clinical observations,
Thanks for the positive assessment of our manuscript.
We have provided the requested data on the animals and the scoring system. (lines 57-58; Methods; Extended Data Table 1 and 2)

and virus sequence, to confirm identity with the original isolate that was obtained and propagated prior to challenge.

The inoculum used was identical to the deposited sequence. This has been added to the Methods section. (lines 409-410)

Some revision of the text is necessary to qualify statements made, as the number of animals is very small and statistical significance is lacking - the results are suggestive. The authors make a statement that intratracheal inoculation results in systemic disease but provide no proof, only indirect evidence. Either proof should be provided or the statements qualified to admit the data is merely suggestive, not definitive of systemic infection.
We have added virus isolation data for blood supporting that at least intratracheal and likely also intranasal inoculation can cause systemic infection whereas orogastric inoculation does not. We have adapted the text accordingly. (lines 35-37 & 101-105 & 198-203)

Discussion of the results should include more of the previous work on NHP with H5N1 viruses, in particular previous studies with Vietnam/2004 given by intranasal and/or intratracheal inoculation which is only rarely lethal in cynomolgus macaques.
The reviewer is correct that mortality from inoculation with HPAI H5N1 viruses including A/VN/1203/04 does not often lead to observed mortality. However, our study is one of the few studies with a survival group, while most published studies had preset euthanasia timepoints with the latest timepoint being 7 days post infection (dpi). In one study (Muramoto et al, JVI 2014) where cynomolgus macaques were inoculated with A/VN/3040/04 (identical in aa sequence to VN1203) via combined intratracheal, intranasal, ocular and oral routes, one animal succumbed to infection on 9 dpi. In a separate study (Baskin et al., PNAS 2009), one animal succumbed to infection with A/VN/1203/04 6 dpi via combined tracheal, nasal, conjunctival, and tonsillar routes. The remaining animal in the same inoculation group was euthanized on a predetermined timepoint at 7 dpi and showed similar pulmonary lesions, indicating that this animal may have died if it had not been euthanized. These examples highlight that preset euthanasia timepoints at 7 dpi as used in the majority of published studies may have prevented some mortality. Together with the variation in inoculation routes and doses used in the published literature, this makes it very difficult to compare the pathogenesis of older HPAI H5N1 viruses like A/VN/1203/04 to that of the bovine isolate we used in our study without performing a side-by-side comparison. We have expanded the paragraph on the use of NHP models in H5N1 studies in our revised discussion and added references. (lines 189-206)

A few specific items
1. Was body temperature data collected? This seems a rather surprising omission, given the prominence of fever in H5N1 disease (in ferrets, macaques, and humans). If the data was collected, it should be included. Similarly, body weight data should be included. If the authors do not want to add figures to the main text, putting this information in the supplemental data would be adequate.
We have included body weights and temperature over time (Extended Data Figure 1). The data is now discussed in the text. (lines 74-79)

2. Line 35: Should be revised to "This study *suggests* that consumption of contaminated products, such as milk, may lead to self-limiting, subclinical infection in primates". Considering the low number of animals (n=3 that were allowed to go to study endpoint) and that the virus does not appear to have been inoculated in raw milk, the original statement is far too strong. Even if the virus was inoculated in milk, this statement is too strong given the number of animals.
We adapted the suggestion by the reviewer and have changed the statement accordingly. (lines 39)

3. Line 74: the statement that a decrease in blood albumin is indicative of systemic inflammation & infection needs to be qualified. Albumin levels could also be low as a result of pulmonary edema, which was reported in the 2017 Wonderlich et al paper.
We have added the suggested alternative explanation of pulmonary edema for the decrease in albumin levels. We also added the suggested reference. (lines 89-91)

4. Line 27: the number of infected herds should be updated (perhaps just state ">300")
We have updated the sentence. (lines 30-31)

Referee #4 (Remarks to the Author):

The study conducted by the Feldmann lab addresses an urgent question of public health concern, namely whether the currently circulating H5N1 HPAIV of the 2.3.4.4b clade pose a threat to humans.
Therefore, they use the closest surrogate marker for humans, cynomolgus macaques. They cross-compare key infection routes (orogastric (relevant if virus uptake occurs via contaminated food), intranasal (relevant if infection occurs via direct contact or airborne infection of the upper respiratory tract) and intratracheal (airborne infection of the lower respiratory tract)) for H5N1 in the macaque model. They show that orogastric H5N1 infection does not lead to a highly productive infection in line with a very mild disease outcome and rendering seroconversion. Intranasal H5N1 infection leads productive virus replication and a mild infection course. The most severe disease outcome is observed upon intratracheal infection of macaques resulting in high virus replication titers, viremia, inflammatory cytokine response and death on day 7 or 8 p.i..
The findings obtained in this study are of utmost importance for risk assessment for humans as they will guide future policies regarding pandemic intervention strategies. The study was conducted to highest standards and all conclusions drawn are supported by the experiments performed. However, there might a be a few points that could be addressed:
We thank the reviewer for the positive assessment and the critique.

Specific points:
#1: Unfortunately, no information is provided regarding the bovine H5N1 isolate used regarding its receptor specificity (a2,3- versus a2,6-linked sialic acid preference) (van Riel et al., Science 2006) or replication efficiency in mammalian cells (PB2 E627K, PB2

D701N) (Subbarao et al., Science 1998; Gabriel et al., PNAS 2005). This should be at least discussed in the context of the mentioned literature. If mostly avian-like signatures are present in the used isolate (a2,3-linked SA, PB2 627E, 701D), the authors should consider sequencing isolates from the infected animals.
We have performed sequencing on lung samples and bronchoalveolar samples collected from animals euthanized on D4 or D5 post infection. We detected amino acid substitutions in only 3 samples: one lung samples from an intratracheally inoculated animal, one brush sample from an intranasally inoculated animal and one brush sample from an intratracheally inoculated animal. These substitutions did not correspond with known substitutions that facilitate replication in a mammalian host (PB2 (Q591K, E627K, D701N), PB1(H99Y, K577E), PA (T97I), HA (Q226L, D225G/N, N158D). (162-176)

#2: It would be helpful to discuss the obtained results in the context of the humans infected with H5N1 clade 2.3.4.4b so far. What is known there regarding the infection route and correlation thereof with known molecular interspecies signatures (HA, polymerase)? Please discuss.
Human cases are either subclinical or show relatively mild clinical signs including conjunctivitis. This would compare to what we have seen here with the orogastric and intranasal groups. It is, however, difficult to compare as we don't know the exposure routes for most of the human cases.

#3: Supplemental Figure 4: I couldn´t find an IHC for the intratracheal route. Is there any information on this? It would be helpful if the authors could discuss cell-types infected by H5N1 cross comparing the different infection routes.
This is now included in the revised version (Extended Data Table 3)