

Disease Progression and Cause of Death due to Primary Pneumonic Plague in Cynomolgus Macaques

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Abstract

Background: Testing vaccines and therapeutics for *Yersinia pestis* infections requires well-characterized large animal models. In this study we sought quantifiable secondary endpoints besides death and the cause of death in aerosol-infected macaques.

Methods: 21 unvaccinated, telemetered, adult cynomolgus macaques of Indonesian origin were exposed to aerosolized *Y. pestis* CO92 in doses ranging from 18 to 264 LD₅₀ equivalents. Groups of 3 to 5 animals were necropsied at 24h intervals after exposure and analyzed for tissue histopathology and cytokine production measured by Luminex assay. *Y. pestis* in blood and tissue was quantified by culture on Congo red agar and by highly sensitive *Y. pestis* rRNA-specific real-time RT-PCR. Cardiopulmonary dynamics were measured by arterial blood gases, telemetered electrocardiogram, and transthoracic echocardiography (ECHO) in unanesthetized animals.

Results: Irrespective of exposure dose, bacteremia was rarely observed prior to 72h post infection, confirmed by sensitive RT-PCR, in spite of high lung tissue levels of *Y. pestis*. Multiple measures of inflammatory response, all negative at 48h pi, were strongly positive at 72h pi, including histopathology, blood neutrophilia, 7 proinflammatory cytokines, and 5 chemokines. Normal arterial blood gases and O₂ saturations, even within hours of death, was contrasted with electrocardiographic abnormalities and reduced aortic ejection velocity and/or ejection fractions measured by ECHO, suggesting reduced myocardial performance beginning as early as 24h prior to death.

Conclusions: Primary pneumonic plague in cynomolgus macaques demonstrates many features of disease progression documented in the murine model, but bacteremia is not an early event. The data support a role for acute cardiac failure in addition to septicemia and pneumonia in causing death.

Exposure

Aerosol Exposure Needs to be Greater Than Fifty Multiples of the LD₅₀ to Assure Lethal Model

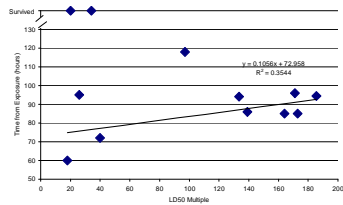


Figure 1. Exposure versus time until death comparison

Twelve animals from the first two exposure groups were chosen to represent exposure and death. Linear regression line was developed based on ten animals succumbing to infection.

Methods: *Yersinia pestis* was aerosolized in a Class III Biosafety cabinet using a Collison nebulizer. Animals in heads were placed into a head only exposure chamber allowing the air to pass the noses of the animal. An all glass impinger (AGI) was used to sample the air after passing the animal in order to determine bacterial concentration. Bacterial concentration was combined with real-time (during exposure) phlebotomy data to calculate inhaled bacterial amounts (LD₅₀ multiples). All moribund animals were euthanized between days three and five.

Results: Two cynomolgus macaques receiving twenty and thirty-four multiples of the LD₅₀ survived challenge. There is no relationship between exposure dose above forty multiples of the LD₅₀ and time until death.

Bacterial Load, Culture

Aerosol Challenge of *Yersinia pestis* Causes Bacteremia

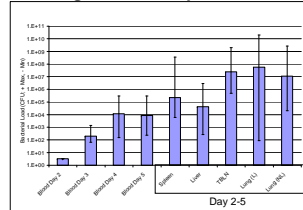


Figure 2. Bacterial load by culture

Culturable bacteria are represented by geometric mean colony forming units (CFU) with the maximum and minimum values for each tissue. Organ tissue was obtained from at necropsy.

Methods: Blood was drawn from chair-restrained unanesthetized animals. Tissues were obtained from both scheduled and moribund euthanasias for quantitative bacterial culture. Lung tissue, was obtained from both apparent gross lesion (L) and from non-lesion (NL) or normal appearing area.

Results: One animal had bacteremia two days after challenge. Bacterial levels in the blood were not different between days three and five.

Bacterial Load, Real Time RT-PCR

Comparison of Bacterial Culture and Real Time qRT-PCR Assays

Culture	qRT-PCR		Culture	qRT-PCR	
	+	-		+	-
+	3	0	+	21	0
-	2	7	-	7	32

Figure 3. Quantification of *Yersinia pestis* by Real Time RT-PCR and bacterial culture

Tissues include lung with gross lesion, non-lesion, tracheobronchial lymph node, liver, and spleen.

Methods: Aliquots from blood and tissue that was used to determine culturable bacteria was also used for *Yersinia pestis* specific RNA detection. Only one blood was cultured undiluted and diluted to achieve maximal sensitivity. Tissues were collected one, two, three, and four days post exposure.

Results: The assay detected more bacteria in the blood than the culture method. However, more blood samples will need to be analyzed from earlier time points to determine the usefulness of this assay as a rapid bioindicator of disease. The sample size for tissue is too small to make a comparison.

Host response

Tissue Chemokine Elevation

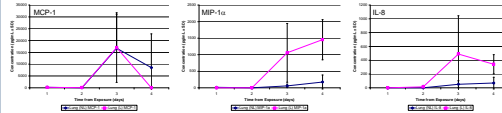


Figure 4. Lung tissue concentrations of selected chemokine

Blue circles are the mean concentration from non-lesion lung. Pink squares are the mean concentration from lesion lung. "a" is used to represent "c". Three samples for each time point.

Methods: Lung tissue was taken from animals at scheduled necropsies at one, two, three, and four days after challenge. Non-lesion tissue was taken adjacent to lesion area. Each tissue type was homogenized and analyzed in a seventeen cytokine/chemokine Luminex panel.

Results: No lung tissue cytokine or chemokine proteins were found one and two days post exposure, consistent with the non-inflammatory phase as described in the mouse model. The cytokines, TNF and IL-6 showed the same pattern (data not shown) and IL-10 was not detected at any time.

Pathology

Rapidly Progressive Multifocal Pneumonia from Two to Three Days Post Exposure

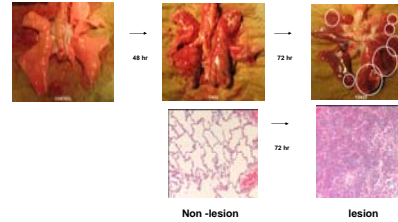


Figure 5. Gross and microscopic pathology.

Arrows in 48 hour photograph and circles on 72 hour demonstrate lesion areas.

Methods: Euthanized animals were necropsied on a downdraft table. Sterile instruments were used for each sample collection. Tissues were sectioned based on lesion area.

Results: Pneumonia was detected at necropsy. Histological analysis showed early interstitial infiltrates at 48 hours progressing to severe alveolitis at 72 hours post exposure.

Arterial Blood Gas

Tissue Oxygenation Demonstrates Effective Lung Function Throughout Disease Progression

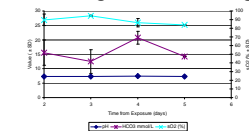


Figure 6. Arterial blood gas results from day two to five post exposure

Closed diamonds denote pH, closed triangles represent the partial pressure of oxygen (pO₂), closed circles signify the bicarbonate levels (HCO₃).

Methods: Arterial blood drawn from the tail of the chair-restrained unanesthetized was analyzed using an iSTAT. Blood was drawn into a syringe containing powdered heparin. Only samples filling cylinder, coming into contact with the heparin were analyzed. Animals are housed at the LRRI facility at approximately 5,000 feet above sea level.

Results: Arterial oxygenation and pH do not significantly decrease during the course of infection. Some macaques have normal arterial blood gas values even when moribund and unable to stand.

Electrocardiogram

Electrocardiogram Changes Indicate Cardiac Dysfunction



Figure 7. Telemetry-recorded electrocardiogram

Implanted telemeter sends radio signal to a software system where the information is analyzed and recorded.

Methods: Greater than two weeks prior to exposure animals were implanted with telemeters (Integrated Telemetry Services). Animals are allowed to acclimate to the facility for one week prior to exposure.

Results: The change in ECG, particularly ST segment depression and T inversion, prompted investigations into the cardiac component of death for pneumonic plague. Repolarization abnormalities are now used as one of the triggers for euthanasia.

Echocardiogram, Cardiac Dimensions

Myocardial Contractility Decreases in Pneumonic Plague

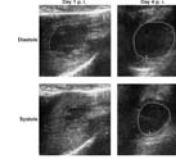


Figure 8. Two dimensional echocardiogram and results

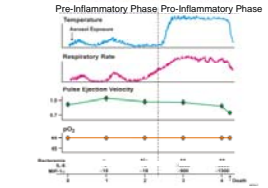
Ejection fraction (EF) is calculated from left ventricular volumes during diastole and systole. Volumes are calculated using endocardial wall tracings and the Method of Discs formula (Acuson software). $EF = (\text{systolic volume} - \text{diastolic volume}) / \text{diastolic volume}$

Methods: Animals received three injections of adjuvant or adjuvant and vaccine in three week intervals. Echocardiography (Acuson Sequoia 512) was performed prior to and daily for five days after exposure and at study termination.

Results: EF decreases in unvaccinated animals that died. The three vaccinated animals survived exposure and showed no decrease in EF. EF is a measure of myocardial contractility and circulating volume depletion; a role for the later cannot be ruled out.

Summary

NATURAL HISTORY OF PNEUMONIC PLAGUE IN NHP



Conclusions

- Confirmed that primary pneumonic plague in macaques is a two phase disease similar to the murine model.
- The pre-inflammatory phase lasts approximately two days and is characterized by lack of bacteremia, clinical signs, alveolitis, and absence of cytokine response in lung and blood.
- The pro-inflammatory phase appears rapidly between days two and three post exposure and is characterized by bacteremia, fever, tachypnea, tachycardia, severe focal alveolitis, and high levels of chemokines and cytokines in infected lung tissue.
- However, the lack of significant arterial oxygenation suggests that the cause of death is more than just severe pneumonia.
- Decrease in indices of myocardial contractility including ejection fraction and pulse wave velocity are consistent with a significant cardiac component to the cause of death. However, a component of hypovolemia cannot be ruled out.
- The origin of the myocardial compromise is unclear. Conventional bacterial sepsis does not appear to be operative because there is no significant acidosis, no thrombocytopenia, no coagulopathy (data not shown). Furthermore, the LPS of *Y. pestis* has anti-inflammatory properties.
- A combination of cardiac and pneumonic complications may be the primary cause of death in nonhuman primate pneumonic plague. Yet, we have not completed the data collection to answer this question.

Acknowledgements

- Western Regional Center of Excellence for Biodefense and Emerging Infectious Diseases
 - David Walker, Kim Schuenke
- Funding
 - US4-AIO-5616-05