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Orthopoxvirus variola infection of Cynomys ludovicianus (North American Black tailed prairie dog)

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A B S T R A C T

Since the eradication of Smallpox, researchers have attempted to study *Orthopoxvirus* pathogenesis and immunity in animal models in order to correlate results human smallpox. A solely human pathogen, *Orthopoxvirus variola* fails to produce authentic smallpox illness in any other animal species tested to date. In 2003, an outbreak in the USA of *Orthopoxvirus monkeypox*, revealed the susceptibility of the North American black-tailed prairie dog (*Cynomys ludovicianus*) to infection and fulminate disease. Prairie dogs infected with *Orthopoxvirus monkeypox* present with a clinical scenario similar to ordinary smallpox, including prodrome, rash, and high mortality. This study examines if Black-tailed prairie dogs can become infected with *O. variola* and serve as a surrogate model for the study of human smallpox disease. Substantive evidence of infection is found in immunological seroconversion of animals to either intranasal or intradermal challenges with *O. variola*, but in the absence of overt illness.

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Introduction

Homo sapiens are the only known natural or permissive host for smallpox (*Orthopoxvirus variola*). Heroic efforts in the 20th century eradicated smallpox from the human population and led to the eventual consolidation of *O. variola* stocks into two WHO sanctioned smallpox research collaborating programs in the US and Russia. Historically, although suckling mice succumb to infections with *O. variola*, no adult rodent or small mammal models using *O. variola* have been successfully developed (Mayr and Herrlich, 1960; Murti and Shrivastav, 1957). *O. variola* challenges of various non-human primate species were conducted in the 20th century (Hahon and Wilson, 1960; Brinckerhoff and Tyzzer, 1906; Noble and Rich, 1969), and some cause generalized rash, but rarely was mortality observed. In recent years, efforts to develop new therapeutics and vaccines for smallpox have relied on surrogate, non-*O. variola Orthopoxvirus* animal infection models and one *O. variola* model in non-human primates. Despite significant animal models of *Orthopoxvirus* infection (Chapman et al., 2010) to date the only lethal animal model using *O. variola* infection is a high dose intravenous, or intravenous/ aerosol challenge of non-human primates (*Cynomolgus macaques*)

(Jahrling et al., 2004). At highest challenge dose (10^9 pfu), disease onset is rapid, within 3–4 days post-infection, with some attributes of hemorrhagic disease and is often lethal by 7 days post infection. A lower dose (10^8 pfu), results in a more typical rash illness with rash onset occuring at 3–4 days post exposure and rash progression similar to smallpox. Studies based on this latter challenge model have used small number of animals, and overall mortality and disease progression has been variable (Jahrling et al., 2004). An *O. monkeypox* respiratory challenge model in non-human primates may provide a surrogate model for *O. variola* since clinical disease progression in this model is more similar to human smallpox than an intravenous challenge (Dyall et al., 2011; Goff et al., 2011; Nalca et al., 2010). However, human smallpox was not commonly associated with extensive pulmonary manifestations, as seen in the intrabronchial respiratory *O. monkeypox* challenges. Additional surrogate models have been researched using non-*O. variola* orthopoxviruses to mimic smallpox like disease in various animal models (reviewed in (Chapman et al., 2010)).

In 2003 a US outbreak of *O. monkeypox* resulted in the inadvertent infection of a North American species, *Cynomys ludovicianus* (Black-tailed prairie dog) as well as a substantial human outbreak (Reed et al. 2004). Subsequent research studies identified the susceptibility of this species to *O. monkeypox* and revealed a clinical course similar to ordinary (human) smallpox (Hutson et al., 2009; Reynolds et al. 2006). Evidence stemming from the 2003 US outbreak of monkeypox and

subsequent laboratory challenge studies of *O. monkeypox* in *Cynomys* confirm this susceptibility and lethality through intranasal *O. Monkeypox* challenge with relatively low viral doses (10^3 pfu). Similar

Fig. 1. Summary of clinical pathogenesis and stages of illness of smallpox in humans, and *monkeypox* in humans, prairie dogs and rhesus maquaces.

Table 1
Neutralization capacity of sera at day 21 post challenge. Sera collected at day 21 was analyzed for virus neutralization capacity using the array scan system. Titers reported are that which neutralized 50% of the virus titer.

Sample	50% neutralizing titer
	day 21
VRV-01 IN	< 1:40
VRV-02 ID	< 1:40
VRV-03 IN	50.45*
VRV-04 IN	< 1:40
VRV-05 IN	< 1:40
VRV-06 IN	106
VRV-07 ID	< 1:40
VRV-08 ID	153.6
VRV-09 ID	48.07
VRV-10 ID	154
VRV-11 IN contl	< 1:40
VRV-12 ID contl	< 1:40
Naïve/Prebleeds	< 1:40*
VIG	6103[a]

* Naïve sera and prebleeds from all animals tested < 1:40 dilution for 50% viral neutralization.
[a] VIG titer giving 50% viral neutralization; $R^2 = 0.9699$.

clinical features, and timecourse of illness, to that previously observed in ordinary human smallpox and in human monkeypox was observed in cynomys infected with *O. monkeypox* (Fig. 1). High susceptibility of this new world mammal species with an old world virus (*O. monkeypox*) promoted the question of whether or not *Cynomys* is susceptible to *O. variola*. In an effort to test this hypothesis, Black-tailed prairie dogs were obtained and subjected to infection with *O. variola* via two routes, intranasal and intradermal scarification.
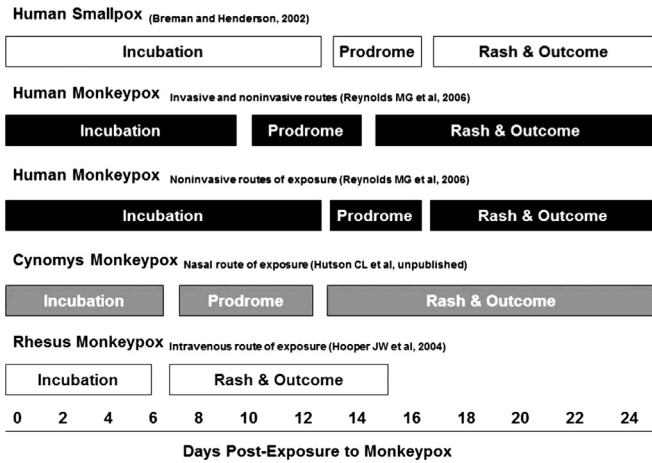
Results

Pathogenesis

To determine if prairie dogs are susceptible to *O. variola*, animals were infected by intranasal or intradermal routes with 6.6×10^6 pfu *O. variola* as described in materials and methods and monitored daily for signs of illness. Slight erythema was noted in all intradermally challenged animals at days 2–4 at the inoculation site but no observed lesion could be identified at day 7 (Fig. 2). In animals challenged intranasally, slight redness was noted at day 3 post challenge around the nares, but by day 7 no signs of infection could be identified (Fig. 2). Otherwise no signs of illness or infection were detectable upon gross physical examination. Body weight was taken every three days and indicates no significant weight loss post challenge for any animals (Fig. 3).

Immunology

ELISA testing for *Orthopoxvirus* specific antibody indicates seroconversion to *O. variola* in 9 out of 10 challenged animals at day 21. Of these 9, four were challenged intranasally, and five
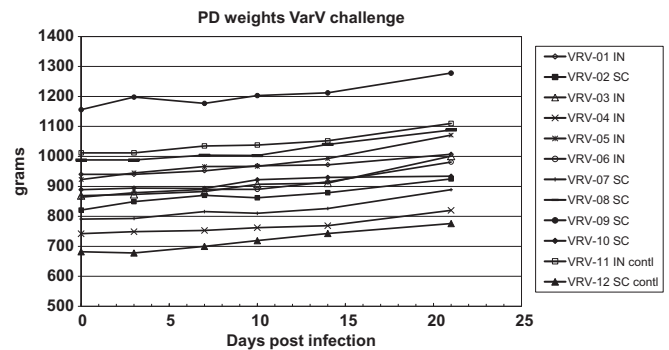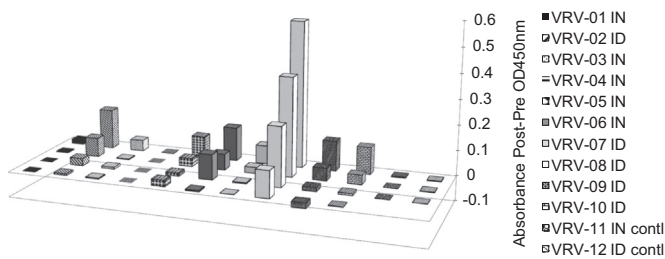

Fig. 3. Weights of animals (in grams) post challenge, measured every 3 days.

Fig. 2. Photos of inoculation sites day 7 post inoculation (a) intranasally and (b) intradermally.

Fig. 4. ELISA of anti-Orthopoxvirus antibody in animal sera. Values are reported as post challenge (day 21) minus pre challenge (day 0) absorbance at 450 nm.

intradermally (Fig. 4). Control animals were negative by ELISA. Serum viral neutralization titers were observed in 5 of the challenge animals; two intranasally and 3 challenged intradermally. The highest 50% neutralizing titer (1:154) was observed in two intradermal challenge animals. Positive control (VIG) resulted in a 50% neutralizing titer of 1:6103 (Table 1).

Viral DNA detection

Real time PCR was performed for *O. variola* specific sequences (A36R), and each sample was tested in triplicate. CT values above 40 are considered negative (see methods). As such, all samples tested were considered negative. A description of the analysis is as follows. PCR results from control animals were negative in all oral swab and tissue samples although certain necropsy samples at day 21 did have PCR results which crossed the threshold after cycle 40 (intranasal control: CT > 42 for both lung and spleen; and intradermal control: CT > 42 in liver and gonad). Three inoculated animals also showed PCR results which crossed the threshold after cycle 40 in oral swabs. At day 3, oral swabs from two intranasally challenged animals (VRV-04 female, VRV-05 male) and one intradermally challenged animal (VRV-07 female) exhibited CT values ≥42, yet they were not reproducibly seen (< 3 of triplicate reactions crossing the threshold). Day 7 oral swab from the intradermal challenged animal (VRV 07) had two of triplicate PCR assays cross the threshold (CT values of 43.53, and 43.77). All other oral swab samples remained negative throughout the study. Tissues collected on day 21 post challenge were also tested for the presence of viral DNA. All tissues did not have PCR assays which crossed the threshold with the exception of three (VRV-07 female, VRV-08 male, VRV-09 male) intradermally challenged animals in lung, spleen, and gonad samples (CT values ≥42 in one of three runs per sample in all tests). The intradermal challenge site (skin) of one animal (VRV-07) exhibited a CT≥42 in all three replicate reactions from day 21 post challenge. No viable virus was cultured from oral swabs or tissue samples (including inoculation site) following two weeks of incubation on cell culture. Although CT levels above 40 are considered negative, the analyses of the results are discussed (below).

Discussion

Historically, laboratory research efforts have tested several animal species for susceptibility to *O. variola*. To date, using adult animals, only non-human primate models have exhibited overt illness in a manner comparable to smallpox (Hahon and Wilson, 1960; Jahrling et al., 2004; Noble and Rich, 1969). However, the recent studies showing the most significant clinical disease require the intravenous injection of a high infectious dose, $(1 \times 10^8 – 1 \times 10^9$ pfu). The discovery of a novel, more permissive/representative animal model system may facilitate the development of next-generation smallpox vaccines and therapeutics.

The absence of overt illness in Black-tailed prairie dogs suggests that this host is not permissive for clinical infection by *O. variola*. However, seroconversion of 9 out of 10 challenged animals to *O. variola* indicates recognition of the virus by the host immune system and perhaps some level of viral replication. The highest serologic response was observed in an intradermally challenged animal (VRV-08), with all intradermally challenged animals serocoverting, while 4 of 5 intranasally challenged animals seroconverted (Fig. 4). PCR results are negative for all samples tested and all samples were negative for viral growth in tissue culture. PCR values above the threshold (CT > 40) in control animals suggests that these are not reflective of viral DNA, but perhaps some other factor causing low signals. In fact, 9/10 challenged animals seroconverted while unchallenged controls did not, suggesting that cross contamination of controls is unlikely and supports CT values above 40 as being negative. Attempts to correlate seroconversion to sex of animals, challenge route or evidence of viral replication (PCR or culture) lack support. Immune induction in 9/10 challenged animals may be considered a measure of viral replication, or viral gene expression to some degree, but the ability to establish viral replication in this host remains speculative since viral DNA was not detected and cultures from animals were negative. And while cynomys species are extremely susceptible to some Orthoxpoxviruses, under the challenge conditions presented here they do not appear to be permissive for overt illness when challenged with *O. variola*.

Multiple immortalized cell lines are permissive to in vitro infection with *O. variola* but in vivo host restriction indicates some biological barrier to productive infection (Pirsch et al., 1963). In *C. ludivicanus*, an increase in challenge dose and/or altered challenge route may facilitate a more robust infection, as observed in non-human primate models. However, our attempt was to develop a model with natural routes of infection and moderate dosing to mimic human smallpox disease onset and sequelae. Additional testing of *O. variola* in this species to develop a model of human smallpox is not currently being considered. However, understanding the different host responses to *O. variola* or *O. monkeypox* infection may enable our understanding of virus host interactions. The results of this study suggest that Black-tailed prairie dogs are not readily susceptible to clinically relevant *O. variola* infection through traditional and natural routes of infection.

Materials and methods

Laboratory

All work with live *O. variola* was conducted within the maximum containment laboratory (biosafety level 4) under the Terms of Reference of the WHO Collaborating Center for Smallpox and Other Poxvirus Infections at the World Health Organization (WHO) collaborating center in Atlanta, GA USA. The facility is reviewed for safety and biosecurity practices by independent U.S. and WHO teams on a frequent basis. The work was pre-approved by, and presented to, the WHO Advisory Committee on Variola Virus Research.

Animals

Twelve wild-caught (Colorado) black-tailed prairie dogs (6 females (VRV-02, VRV-04, VRV-06, VRV-07, VRV-10, and VRV-12), 6 males (VRV-01, VRV-03, VRV-05, VRV-08, VRV-09, and VRV-11) were used in this study. The animals were approximately one year old. The animals were topically treated with permethrin prior to transport to the CDC, and were cared for in accordance with the CDC

Institutional Animal Care and Use Committee (IACUC) under an IACUC approved protocol (♯ 1729KARPRAC-A2). Animal handling and husbandry was performed by properly trained personnel using biosafety level 4 personal protective equipment (PPE).

Pathogen screening

Animals were screened for *Yersinia pestis*, *Francisella tularensis*, *Bartonella spp.* and *Rickettsia spp.* by polymerase chain reaction (PCR) as previously described (Stevenson et al., 2003; Kugeler et al., 2006; Sackal et al., 2008). Briefly, these assays use primer sets to target the *Bartonella* and rickettsial citrate synthase gene (*gltA*), the *F. tularensis pdpD* gene, and the *Y. pestis pla* gene. In addition, serum from these animals taken prior to study start was tested by Enzyme-Linked Immunosorbant Assay (ELISA) to detect IgG antibodies to *Orthopoxvirus* species.

Diet

The prairie dogs were provided with a commercially available diet (Exotic Nutrition, Newport News VA) and water; both ad libitum. The food is a high fiber formula developed within GMP guidelines to replicate the prairie dogs natural high fiber intake in the wild and contains vitamin D-3, A and E supplements. One or more of the following was provided approximately three times a week for dietary enrichment: either Sugar Beet Treats (Exotic Nutrition), Fruit Kabobs (Exotic Nutrition) containing, fresh sweet potatoes, alfalfa or Timothy Hay (Exotic Nutrition). In addition, the animals were provided with Monkey Biscuits (Exotic Nutrition) as treats on a daily basis as they are an easily measured indicator of inappetance if refused by the animals.

Animal infections and sampling

All animal manipulations were performed on animals, which were sedated using 5% inhalant isoflurane. Control animals ($n=2$) were sham infected for each route of infection and observed for possible non-specific signs (e.g., inoculation site trauma). Intranasal infection was performed in ($n=4$) animals with approximately 6.6×10^6 pfu of purified *O. variola* strain Solamain (BSH-74-Sol) in total volume of 10 ul (5 ul per nostril) administered with a pipette. Intradermal infection was performed via scarification in ($n=4$) animals by administration of 6.6×10^6 pfu in total volume of 10 ul to the skin on the back between the shoulder blades followed by ten intradermal sticks with a tuberculin syringe needle. The animals were observed daily for 21 days for inappetance and general health. For sample collection, animals were anesthetized using 5% isoflurane gas anesthetic in a chamber with maintenance of anesthesia via nosecone and kept on heating blanket to maintain core temperature in accordance with IACUC-approved protocol. The hind limb/groin area was sprayed with 70% isopropanol. A 28-gauge needle was used to collect 1 ml of blood from the saphenous vein. The femoral vein was used only when blood could not be obtained from saphenous vein. Blood was distributed to a serum separation tube (Fisher Scientific) for serum collection. Sera was gamma irradiated at 4.4×10^6 rads (1 kill dose for *Orthopoxvirus*) prior to being tested by ELISA and neutralization testing. Animals were bled for serum pre infection and again at day 21. Oral swab and weight of animals were taken pre infection and post challenge on day 3, day 7, day 10, day 14, and day 21. At the conclusion of the study (day 21), all animals were euthanized and necropsy performed to collect lung, liver, gonads and spleen tissues for immunopathology analysis. Animal carcasses were autoclaved and incinerated for disposal.

Real time-PCR and tissue infectivity culture of virus

Samples were assayed by RT-PCR using forward primers, reverse primers and probe complementary to the conserved *O. variola*A36R (a viral envelope protein) gene. The primer/probe sequences were selected from a conserved region of A36R (*O. variola* BSH75_banu, GenBank L22579) with Primer Express (version 1.5; Applied Biosystems). These included A36R forward primer (5′–TTC AGA CTA CCA GTT ATT CTC GGA-3′), A36R reverse primer (5′–AGC CAG GTA GTC AAG ACA TCA GA-3′), and A36R probe (5′FAM-CA AAT TGC GCC ACA GAA TCA TCA AC-BHQ13′). The target sequence is identical in all sequenced strains of *O. variola* with the exception of a single nucleotide polymorphism within the forward primer sequence of the *O. variola* CHN48_horn strain. Primers and probe were synthesized in the Biotechnology Core Facility (CDC, Atlanta GA), utilizing standard phosphoramidite chemistry. The detection probe contained 5′ reporter molecule (FAM) and 3′ aminomodifier (Glen Research, Sterling, VA). A 3′ quencher molecule, BHQ1 (Molecular Probes, Eugene, OR), was conjugated to the 3′ amino group after synthesis. PCR assay conditions were optimized according to standard protocols (protocol 04304449, Applied Biosystems, Foster City, CA) by adjusting primer and probe concentrations, and thermal cycling temperatures/duration. Each reaction (30 μL) contained $1\times$ TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 1 μl of 25 μmol/μL each primer, 1 μl of 25 μmol/μL TaqMan probe, and 1 μL of template DNA. Thermal cycling conditions for the ABI7900 (Applied Biosystems, Foster City, CA): one cycle of 95 °C for 6 min; followed by 45 cycles of 95 °C for 5 s, and 60 °C for 25 s. PCR amplification is based on fluorescent emission after annealing/elongation (60 °C). *O. variola* BSH75_banu was used as a positive control. Based on positive controls, CT values were correlated to genome copies with a CT value of 39.3 being equivalent to 20 genome copies and CT value of 42.6 equivalent to 2 genome copies. For this assay, CT values above 40 are considered negative based on reproducibility (among triplicate testing and in duplicate testing) and absence of viral growth in tissue culture (BSC40 cells with 10% RPMI media). Any sample which caused the fluorescent signal to cross the threshold (even if it was after cycle 40) was evaluated for viability by growth in tissue culture.

Enzyme-linked immunosorbant assays (ELISA)

ELISA was used to screen animal sera for the presence of orthopoxvirus specific antibodies as previously described (Hutson et al., 2009). Briefly, microtiter plates (Immulon II) were coated with 100 μl of *O. vaccinia* (Dryvax purified) at 0.01 μg/well in carbonate buffer overnight at 4 °C followed by formalin inactivation on the plate. Blocking buffer was used (PBS 0.01 M, pH 7.4 (GibcoBRL cat. ♯93-0223 DK)+0.05% tween-20, 5% dried skim milk (DIFCO, ♯232100), 2% normal goat serum (CDC ID♯ CP0523), and 2% bovine serum albumin (Sigma, A-7030)) and test samples were tested using 2 fold dilutions of 1:100, 1:200, 1:400 and 1:800 in assay diluent and incubated for 1 h at 37 °C. A 1:30,000 dilution of Immunopure A/G conjugate (Pierce ImmunoPure® Protein A/G/Peroxidase conjugated, ♯ 32490) in assay diluent was added for 1 h at 37 °C. Detection was performed with TMB microwell peroxidase substrate (KPL ♯50-76-05) 1-component substrate was added followed by addition of stop solution (KPL ♯50-85-05) Raw absorbance was determined at OD 450 nm. Values reported represent the average of duplicate wells of each dilution run with paired prebleed sample dilution values subtracted. Standard deviation in raw absorbance values between dublicate wells in all samples was < 0.035. Positive and negative control sera were used as assay controls.

High content screening-green fluorescent protein (HCS-GFP) neutralization assay

Neutralizing antibody titers against *O. vaccinia* were measured using a previously validated and described GFP-based assay (Johnson et al., 2008). In brief, Vero E6 Cells were diluted with 10% DMEM from a stock suspension to 1.7×10^5 cells/mL, seeded into a 96 well flat bottom plate and overnight in a 37 °C CO_2 incubator to form a cell monolayer. Serial dilutions of the prairie dog serum samples were made with 2% DMEM. Tubes containing 180 µL of virus (virus only, no serum), and 12 tubes containing 360 µL of media only (no virus or serum) were used as assay controls. WR-GFP *O. vaccinia* was thawed on ice for 1 h followed by three rounds of sonication and dilution in 2% DMEM. This viral was added (180 µL) to samples and used as plate viral only control. Samples were briefly rocked and placed in a 37 °C CO_2 incubator for 2 h, and rocked once every 30 min. The media was carefully removed from the cell monolayer (96 well plate) and replaced with test samples and controls in triplicate (3 wells per sample). After a 2 h incubation, the inoculum was aspirated and replaced with 100 µl of 2% DMEM +46 µg/mL cytosine arabinoside (Ara-C) and then placed in a 37 °C CO_2 incubator for 18 h. The cells were formalin fixed and stained with DAPI (3 mM). Two channel analysis was performed for DAPI and GFP signals. The plate was analyzed on the Array Scan HCS Reader with the target acquisition software from Cellomics (Thermo Scientific: Cellomics, Pennsylvania). Prism 5.0 (GraphPad) was used to confirm the Gaussian distribution of our data. Because the data passed the D'Agostino-Pearson omnibus test, which measures the deviation from a predicted Gaussian distribution by skewness (asymmetry) and kurtosis (shape), further statistics were performed assuming a Gaussian "normal" distribution. The column statistics option was used to determine means, medians, standard deviations, and standard error from the mean measurements of our data. Additional statistics utilized in this paper include one-tailed t-tests.

Acknowledgments

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