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Translocation Of Yops By Yersinia Pestis Into Canine Cell Lines

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TRANSLOCATION OF YOPS BY *YERSINIA PESTIS* INTO CANINE CELL LINES

By

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This thesis, submitted in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Betsy Louise Young

July 2022

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ABSTRACT

Yersinia pestis, the causative agent of plague, secretes a set of virulence proteins called Yops when in contact with eukaryotic cells. A subset of these Yops is translocated directly into the cytosol of the host eukaryotic cells by the type III secretion system. Using a protein tag-based reporter system, this study is focused on the translocation of these Yops into the cytosol of canine derived cell lines: MDCK and DH82. The MDCK cell line is a canine derived epithelial cell line, and the DH82 cell line is a canine derived monocyte cell line. The reporter system uses a phosphorylatable peptide tag, called the Elk tag. Translocation of an Elk-tagged protein into eukaryotic cells results in host cell protein kinase-dependent phosphorylation that can be detected by phosphospecific antibodies. Canines are typically considered resistant to plague infections. Previous work has shown the requirement of receptor FPR1 for Yops translocation into mouse and human cells. Therefore, translocation of Yops into canine cells was examined because a lack of Yops translocation could explain plague resistance in canines. The translocation of Elk-tagged YopE was observed in the canine derived cell lines MDCK and DH82. This data suggests that there may be an underlying immune response allowing canines infected with *Yersinia pestis* to recover.

CHAPTER 1

INTRODUCTION

History of Plague

Yersinia pestis is the causative agent of the plague. This bacterium has been responsible for millions of deaths worldwide over the ages (1). *Y. pestis* is responsible for one of the earliest pandemics in the world. This pandemic would be the first of three major plague pandemics to occur throughout history (2). The first plague pandemic was referred to as the "Plague of Justinian," or the Justinian plague (3). It got its name from emperor Justinian who was ruler of the Byzantine empire in the sixth century when this pandemic occurred (3). The outbreak is thought to have begun in Egypt, where it moved along maritime trade routes to Constantinople (2). Once there, the infection killed thousands of residents. It is said that there were so many dead that the authorities had difficulties disposing of them. The people at the time believed that the plague was due to the wrath of God against a world full of sin. However, modern day science concludes that the bacterium was spread by infected domestic rats along trade routes (4).

The second, and most well-known, plague pandemic was the Black Death in Europe during the 14th century (5). Plague infections accounted for the death of around one-fourth of the entire population of Europe during the Black Death (2). For the next three centuries, plague outbreaks occurred throughout Europe (3). In the 1700s accounts of plague infections began to lessen, this is thought to be a result of progress in sanitation

and a change in domestic housing that excluded rats from human dwellings (6). However, plague infections never fully went away, throughout the 1800s and early 1900s outbreaks took place around the world (Fig 1).

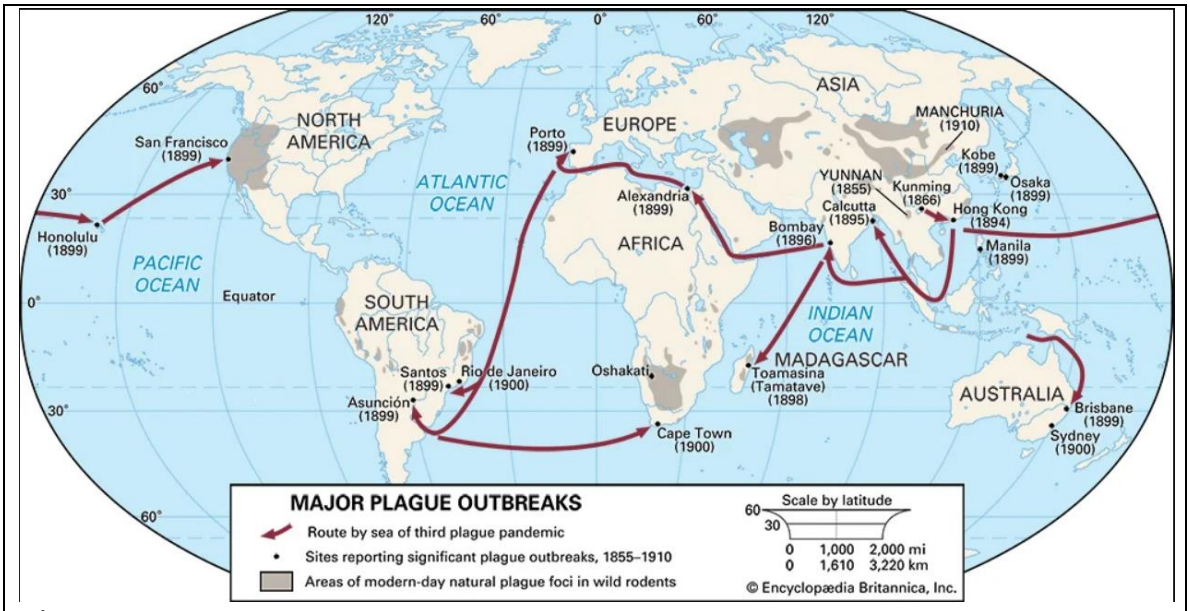

Figure 1
Locations of plague outbreaks in the 1800s and early 1900s. Locations of major plague outbreaks in the 1800s and early 1900s. Port cities became hubs for transmitting plague infections around the world. Used with permission (35).

The third plague pandemic allowed for advancements to be made in the scientific understanding of the disease (3). In the late 1800s, the germ theory of disease was recognized as true based on the work of Louis Pasteur, Joseph Lister, and Robert Koch (7). The third pandemic took place in the 1850s in the Yunnan province of southwestern China and spread across the world through port towns into the early 1900s (2). During this time, two scientists independently isolated the organism responsible for causing

plague (8). The bacteriologists, Alexandre Yersin and Kitasato Shibasaburo found bacteria in fluid samples taken from plague victims and injected them into animals and observed how the animals died quickly of plague. Yersin named the newly identified bacterium *Bacillus pestis* (9). Over the turn of the century, the bacterium would go through several name changes, from *Bacillus pestis* to *Pasteurella pestis* and finally to *Yersinia pestis* in the mid-1900s in honor of Yersin himself (9).

Clinical presentation and Treatment

Plague infections do not all present themselves the same way; presentation of the disease is dictated by what part of the body is infected and how long the host has been infected (3). Naturally occurring plague infections are spread by flea-borne transmission and usually exhibit the bubonic form of the disease (2). The bubonic plague is most associated with swelling of the lymph nodes due to excessive bacterial proliferation. The swollen lymph nodes, or buboes, seeping pus and bacteria can lead to other forms of the disease as the bacteria spreads to other parts of the body (10). Without treatment there is a 60% fatality rate from this type of plague infection, primarily through secondary sepsis and other complications. Thirty percent of flea-borne plague infections manifest as septicemic plague, a disease of the blood, without ever forming buboes. These infections are more difficult to diagnose due to lack of symptoms until organ failure begins to occur (10). Pneumonic plague is the most lethal form of the bacterial disease, with a nearly 100% fatality rate without treatment within 24 hours of onset of symptoms (11). Primary

pneumonic plague infections can begin to display symptoms in as early as 24 hours after initial exposure. Symptoms of pneumonic plague include fever, coughing, and difficulty breathing; death occurs from respiratory failure or sepsis (12).

All three forms of plague infections have a marked absence or severely limited immune response in the initial stages of infection. *Yersinia pestis* uses several different methods to avoid the host immune system. This allows the bacterium to grow to an extreme abundance, which can be seen in septicemic patients. It is thought that *Yersinia* uses this avoidance strategy for propagation; after growing to high enough numbers the bacteria can be effectively taken back into a flea vector through a blood meal (13). The death of the human host also forces the flea vector to seek other hosts, which propagates the bacterium (1).

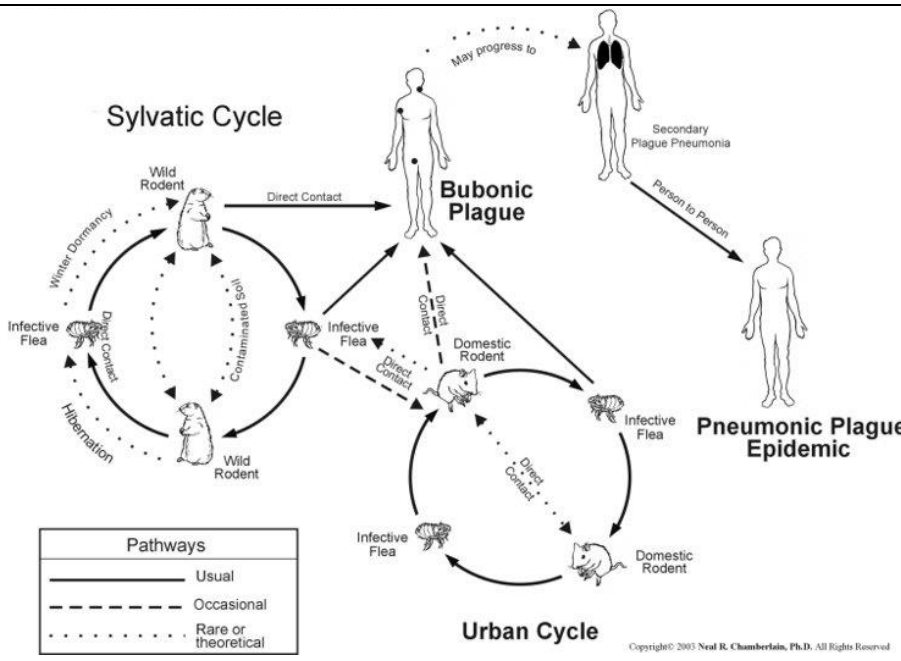

Figure 2
Life cycle and transmission of plague infections. Fleas and rodents are natural reservoirs of plague and can pass the infection to humans by direct contact. Humans once infected, can transmit infections to other humans as bacterial infections progress without treatment. Used with permission (36).

Unlike during the three major plague outbreaks throughout human history, nowadays, plague infections can be treated with broad-spectrum antibiotics such as tetracycline or streptomycin (14). Individuals infected with the bacterium should be isolated and exposed individuals should be quarantined depending on the form of plague infection (15). Modern plague infections, if treated early, can have a positive outcome. However, there are no current approved vaccines against *Yersinia pestis* infections (16).

Yersinia pestis and Virulence Factors

Yersinia are gram-negative bacteria that belong to the *Enterobacteriaceae* family (17). There are three human pathogens classified in the *Yersinia* genus: *Y. pestis, Y. enterocolitica,* and *Y. pseudotuberculosis* (14). Of these three bacteria, *Y. pestis* causes the most lethal disease manifestation, meanwhile *Y. enterocolitica* and *Y. pseudotuberculosis* cause the gastrointestinal infection, yersiniosis (18). *Y. pestis* is thought to have evolved from *Y. pseudotuberculosis,* which is closely related, as recently as 1,500 to 20,000 years ago (19). Y. *pestis* is a non-motile, non-spore forming coccobacillus (5). The bacteria display a bipolar staining with Giemsa, Wright's, or Waysin stain (5). *Yersinia pestis* can grow in a wide range of temperatures from 4˚C to 42˚C, however optimal growth is between 28˚C and 30˚C (20). Compared to *E. coli, Y. pestis* growth is slow, requiring 24 to 48 hours for colony formation on rich media. The generation time in defined media can be as short as 1.25 hours (15).

Yersinia pestis has been grouped into three biovars, which are variants that differ either physiologically or biochemically. In the instance of *Y. pestis,* the biovars are determined by the bacteria's ability to convert nitrate to nitrite and the fermentation of glycerol (21). Biovar Antiqua has been associated with the first major plague pandemic and can reduce nitrate to nitrite and ferment glycerol (15). Medievalis was the biovar associated with the Black Death plague and does not have the ability to reduce nitrate but could ferment glycerol (15). The third plague pandemic was associated with the biovar,

Orientalis, which could reduce nitrate to nitrite but could not ferment glycerol (15). However, recent analysis of samples from plague victims indicates that all three pandemics were linked to the Orientalis biovar (21). The KIM strain, Kurdistani Iraqi Man, or better referred to as the wild-type strain in this manuscript is of the Medievalis biovar (22).

Bacteria such as *Y. pestis* have evolved efficient strategies to negate host defense systems that allow the bacteria to invade, colonize, and multiply is host tissues (14). *Yersinia pestis* has also acquired a mechanism to survive inside the mid-gut of fleas when evolving from *Y. pseudotuberculosis* (23). The KIM strain of *Y. pestis* contains three plasmids: pPCP1 (encodes for pesticin, coagulase, plasminogen activator), pMT1 (encodes for murine toxin), and pCD1 (encodes for the T3SS) (15). The pMT1 plasmid encodes the murine toxin which is toxic to mice and rats, but not to dogs or rabbits (15). The murine toxin is part of the phospholipase D family that functions to block the flea gut from killing the bacterium during the vector stage of the *Y. pestis* life cycle (21). This is one of the reasons proposed to play a role in the bacteria's survival in the flea. The *pgm* (pigmentation) locus is a chromosomally located region that contains the *hms* (hemin storage) locus, which is responsible for blocking the flea mid-gut and allowing the bacteria's survival (15). The *pgm* locus also contains the yersiniabactin (Ybt) system which participates in iron acquisition (18). Iron is an essential micronutrient in mammalian proteins that is chelated to prevent the iron from being used by pathogens.

Yersinia pestis uses yersiniabactin to acquire iron and survive in the iron restricted environment in the host (24).

Once established inside the host, *Y. pestis* can avoid host immune defenses and spread to the draining lymph nodes (25). Expression of the plasminogen activator protein (Pla), which is encoded in the pPCP1 plasmid, is required for the dissemination of the bacteria from the site of a flea bite (18). Another important mechanism *Y. pestis* employs is the expression of the F1 capsule at 37˚C from the pMT1 plasmid (14). The F1 capsule forms an envelope around the bacteria that enable it to resist phagocytosis (18). The bacterium also possesses a type III secretion system that is used to deliver anti-phagocytic effector proteins into host cells (26).

Type III Secretion Systems

The type III secretion system (T3SS) is a protein-secretion machine used by many gram-negative bacteria to deliver virulence factors into eukaryotic host cells (26). The effectors that bacteria secrete into the host cell are specific to that pathogen, however, the T3SS is used in all cases to contribute to infection of the eukaryotic cell and allows the bacteria to communicate with the host cell (27). In the instance of *Y. pestis*, the effectors block phagocytosis by macrophages, suppress the production of pro-inflammatory cytokines, and disturb the dynamics of the host cytoskeleton (28). These traits allow for the survival and replication of the bacteria in lymphoid tissues (28).

The machinery is made up of a basal body that spans both bacterial membranes and an extracellular needle that protrudes from the bacterial surface (26). The T3SS is not constantly active, this mechanism is activated upon physical contact with a eukaryotic cell (29). Once contact with the host cell is achieved the bacterium will secrete two classes of proteins (28). The first being translocator proteins, YopB and YopD, which form a translocation pore on the surface of the host cell. The needle-cap protein, LcrV, binds to this translocation pore and allows for effector proteins to be translocated into the cytosol of the eukaryotic host (28). The type III secretion system exists to modulate host cell signaling in ways that benefit the bacteria (38). This task is completed through the translocation of "toxic" effectors called Yops (Yersinia outer proteins) from the bacterium into the eukaryotic host (38). Each Yop performs a specific task in the overarching goal of hijacking the host intercellular signaling (39).

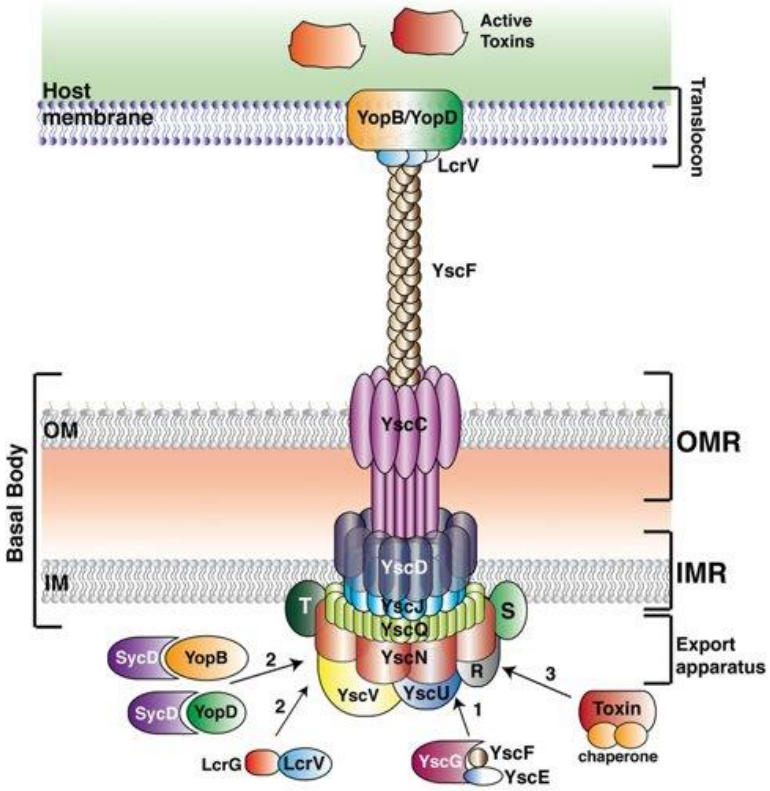

Figure 3
Key features of the type III secretion system needle-protein complex. A model showing the structural components of the type III secretion system needle-protein and how that needle-protein binds to the surface of a host cell and allows for Yop translocation. Used with permission (37).

Canine Immunity and Why Dogs Matter

Plague infections do not occur in all mammals. Unlike humans, rabbits, and rodents, canines such as wolves, dogs, coyotes, and foxes, are considered resistant to plague infections (40). Canines typically are not found to be infected by the plague because they display little to no symptoms even when they are infected (40). Canines also can recover from plague infections in the few instances when they become infected (40).

This plague resistance is hypothesized to manifest because canines have lost Fpr1 and the ability to respond to N-formylpeptides. Canines instead have a duplicated Fpr2 gene (41).

In humans and mice, Fpr1 is the gene that encodes N-formylpeptide receptor that is essential for the needle cap protein, LcrV, of the T3SS to bind to the surface of a host cell and is considered a plague receptor (41). Previous work has shown mice that are Fpr1-deficient have increased survival rates and a protective immune response against *Yersinia pestis (41).* Fpr1 has been co-purified with the *Y. pestis* proteins LcrV and YopD that are responsible for the binding of the T3SS needle to the surface of a eukaryotic cell (41). This demonstrates an interaction between Fpr1 and the T3SS needle. The lack of Fpr1 in canines, which express Fpr2, could be responsible for their plague resistance, unlike their human counterparts with Fpr1 (41). Multiple sequence analysis of the human Fpr1, human Fpr2, and the canine Fpr2 proteins identify several differences in amino acid expression amongst the three proteins. There are approximately thirty non-conservative mutations between the three receptor proteins (Fig 4). These amino acid differences could explain canine plague resistance.

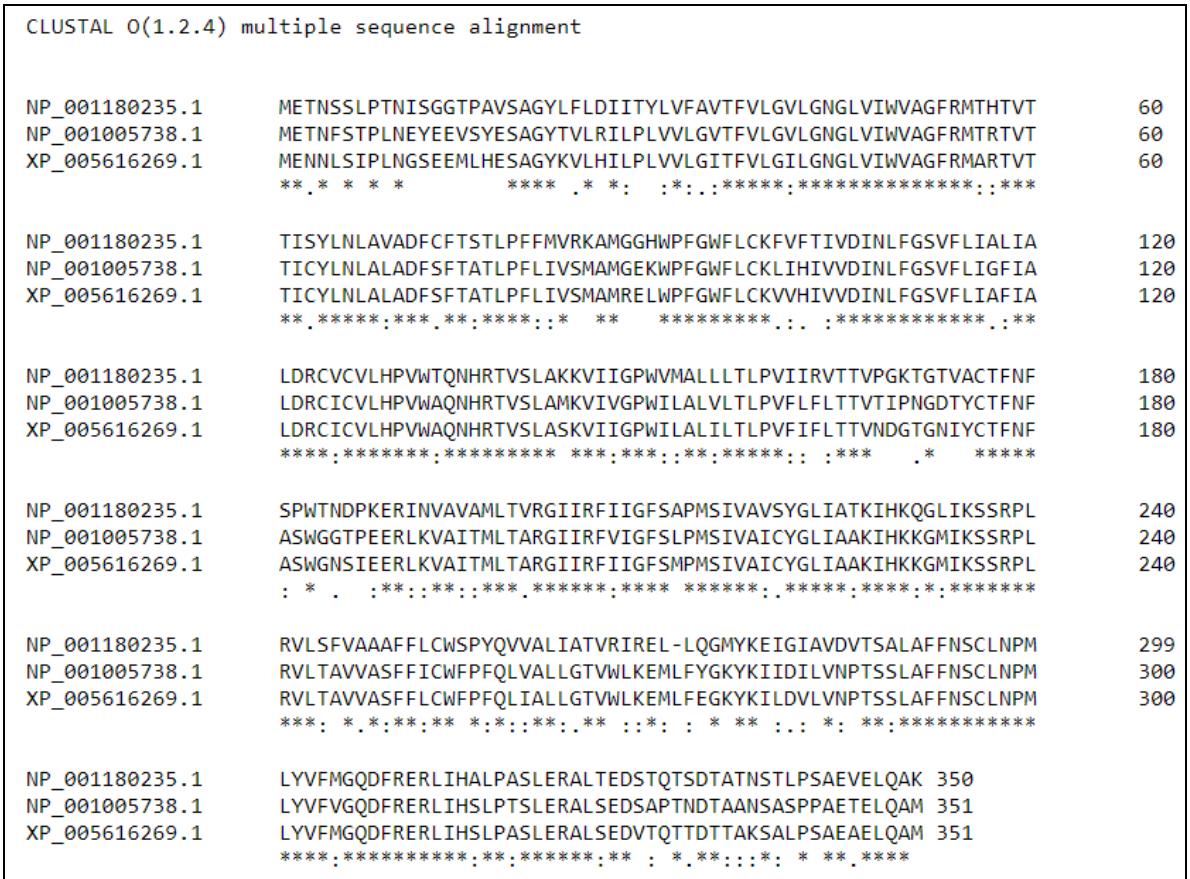

Figure 4
Multiple Sequence Analysis of Human Fpr1, Human Fpr2, and Canine Fpr2. All three sequences were taken from NIH BLAST and compared using the EMBOSS Cons program on the EMBL-EBI website. NP_001180235.1 is the sequence for human Fpr1. NP_001005738.1 is the sequence for human Fpr2. XP_005616269.1 is the sequence for canine Fpr2.

Although canines do not commonly become infected by plague, they can serve as a sentinel species in high-risk areas (42). Canines in high-risk areas such as Madagascar typically are free-range working dogs (40). These dogs are susceptible to two main types of exposures to plague infections. One such exposure is a dog consuming rodents infected with *Yersinia pestis* (40). The other would be a dog being bitten directly by an infected

flea (40). Typically rat populations are monitored in areas where plague infections are possible. However, by the time researchers can identify a shrinking rat population due to infection deaths, exposures and infections may already be circulating through the population (40). This is where dogs and other canines can be an incredibly useful tool in monitoring plague outbreaks. The dogs that already are near humans in these high-risk areas could be monitored for infections (42). By monitoring the canine population in conjunction to the rat populations, scientists may identify outbreaks before they become an issue for the human population (42).

CHAPTER II

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids

The *Y. pestis* and *Escherichia coli* strains used in this study are listed in Table 1. All bacterial strains were stored at -80°C in 50% (v/v) glycerol. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (Difco, Thermo Fisher Scientific, Waltham MA) or on tryptose blood agar (TBA, Difco, Thermo Fisher Scientific, Waltham MA) plates. *Y. pestis* strains were grown at 26°C on TBA plates or in TMH medium (33). TBA plates contained antibiotics as needed. Streptomycin and ampicillin were used at a concentration of (100 µg/ml). Plasmids used in this study are listed in Table 1. Plasmid DNA was isolated using a QiaPrep Spin kit (Qiagen, Inc, Studio City, Calif.). Cloning methods were as described previously (43). Electroporation of DNA into *Y. pestis* was done as described previously (44).

Table 1: Strains and plasmids used in this study

Strain	**Relevant properties**	**Source or reference**
E. coli	Host used in plasmid cloning	Gibco-BRL
D27XSm	*Yersinia pestis* attenuated wild type	(30)
D27XSm Δ*yopB*	*Yersinia pestis* YopB mutant	(32)
Plasmids		
pYopE-Elk	Encodes YopE-Elk chimera, Ap^r	(45)
p141 CB	*araBAD* promoter fused to *yopE*-Elk1, Ap^r	(32)

Eukaryotic cell lines, culture conditions

The eukaryotic cell lines used in this study are listed in Table 2. The cell lines HeLa, MDCK, and DH82 were maintained in DMEM (Corning, Manassas, VA) supplemented with Penicillin/Streptomycin (Gibco, Life Technologies), non-essential amino acids (Gibco, Life Technologies), 25 mM Hepes buffer (Gibco, Life Technologies), and BSA 7.5% (Gibco, Life Technologies). The cell lines HeLa and MDCK were supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies). The DH82 cell line was supplemented with 15% (v/v) heat inactivated FBS. All eukaryotic cell lines were incubated at 37°C under a 5% CO2 atmosphere.

Table 2: Eukaryotic cell lines used in study

Strain reference	**Relevant properties**	**Source or**
HeLa CCL-2	Human derived epithelial cell	ATCC #CRM-
MDCK 34	Canine derived epithelial cell	ATCC #CCL-
DH82 10389	Canine derived monocyte cell	ATCC #CRL-

Infection assay

Prior to infection, eukaryotic cells were subcultured to a density of 2.5 x 10^5 cells per well into 35-mm-diameter six-well tissue culture plates in DMEM-FBS at 37°C in a 5% CO2 atmosphere for 48 hours. The DH82 cells were activated with LPS (*E. coli* 026:

B6, Millipore Sigma) at a concentration of 100 ng/ml 24 hours prior to infection. Cells were washed twice with warmed L15 (Corning, Manassas, VA) media lacking FBS immediately prior to infection. *Y. pestis* was cultivated at 26°C in TMH media overnight prior to infection. The optical density at 620 nm (A_{620}) of the bacterial strains were measured and *Y. pestis* strains were added directly to wells containing L15 medium and eukaryotic cells to a multiplicity of infection (MOI) of fifteen. 1 A_{620}•ml of *Y. pestis* is 3 x 10^8 bacteria/ml. Plates were then allowed to incubate at 37°C with humidification for 4 hours. After incubation, each well was photographed using light microscopy with 20x magnification using Hoffman modulation contrast. After infection, each well was washed twice with cold PBS. 10 µl Protease inhibitor cocktail (Sigma-Aldrich #P8340) and 10 µl phosphatase inhibitor cocktail (Sigma-Aldrich #P2850) were added to each well. 2X Laemmli sample buffer was added to each well to lyse cells (46). Sample buffer added was adjusted so samples represented equivalent amounts of the original bacterial cultures (0.01 OD_{620}•ml/µl). Cells were scraped off the surface of plate and added to 1.5ml microfuge tubes. Samples were boiled for 5 minutes, vortexed to shear DNA, and centrifuged for 10 minutes at 10,000 x *g* before being analyzed by SDS-PAGE and immunoblotting.

Protein electrophoresis and immunodetection

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 15% (wt/vol) polyacrylamide gels (Bio-Rad, Hercules, CA) as indicated,

according to the method described by Laemmli (46). Samples were loaded such that lanes containing different culture fractions represented equivalent amounts of the original bacterial cultures (0.05 OD_{620}•ml/well). Proteins separated by SDS-PAGE were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.), using transfer buffer (47). Specific proteins (Elk-1, Phospho-Elk-1, and YopE) were visualized on the membranes by using the following rabbit polyclonal antibodies specific for the proteins at the indicated dilutions: Elk-1 antibody (1:1000; Cell Signaling #9182), Phospho-Elk-1 antibody (1:1000; Cell Signaling #9181), and YopE antibody (1:20,000;). Bound primary antibodies were detected with alkaline phosphatase conjugated to secondary antibodies (goat anti-rabbit immunoglobulin G; 1:2,000; Cell Signaling #7054) followed by color development with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (NBT-BCIP) (Thermo Fisher Scientific, Chicago, IL).

CHAPTER III

RESULTS

Yersinia pestis induces MDCK and DH82 cell cytotoxicity.

The epithelial cell line, HeLa, has been characterized to display a "rounding up" effect when exposed to *Yersinia pestis* (34). For the sake of these experiments this change in the eukaryotic cell morphology was a positive control when analyzing the MDCK and DH82 cell lines. HeLa cells were infected to an MOI of 15 and contact time with the bacterium was allowed for 4 hours. As has been previously studied, HeLa cells infected with the wild-type strain of *Y. pestis* displayed a "rounding up" effect (Fig 5). A deletion mutant of the wild-type strain of *Y. pestis* was used as a negative control for this "rounding up" effect. This strain had the inability to produce YopB, which is a necessary protein for the binding of the T3SS apparatus to the surface of a eukaryotic cell (32). Thus, when eukaryotic cells are infected with this strain of *Yersinia* cannot be intoxicated by translocation of Yops into the cytosol of the cell (Fig 5).

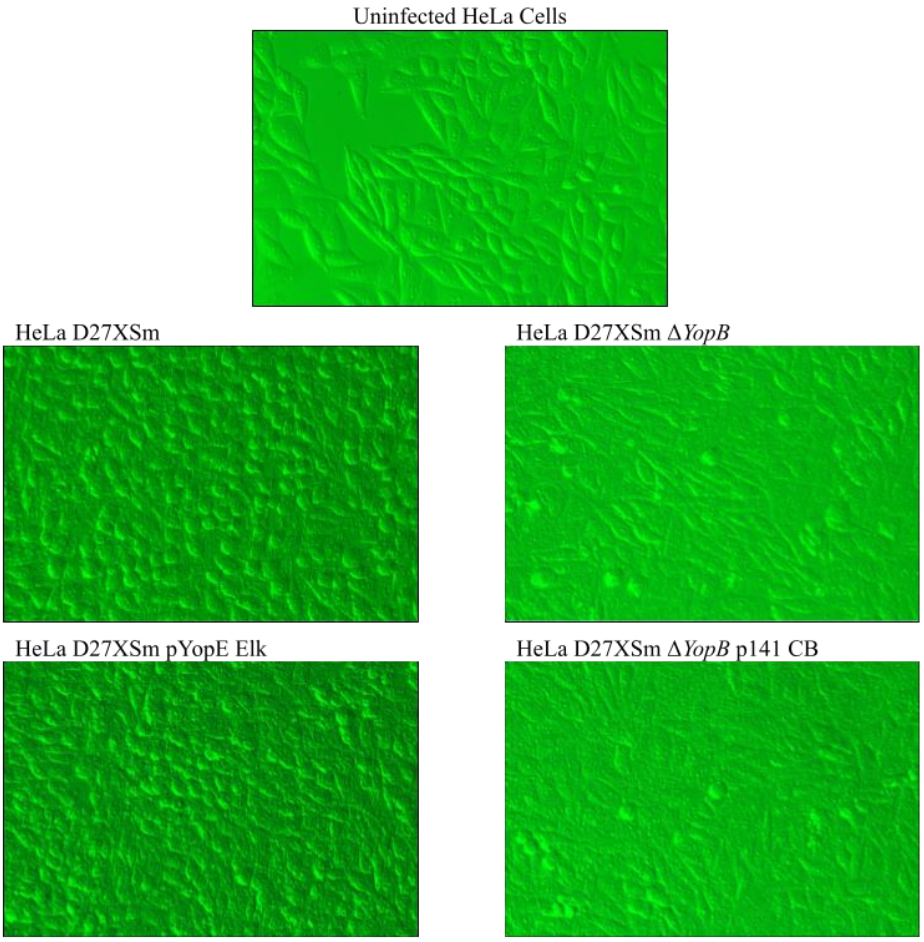

Figure 5
HeLa cell infections by *Yersinia pestis* wild-type strain and YopB mutant. HeLa cells were infected at an MOI of 15. Images were captured 4 hours post infection on an Olympus IX50 inverted microscope fitted with a Nikon D70 digital camera (magnification 200x) to document cell cytotoxicity. These images are representative of repeated experiments.

The MDCK cell line was infected with the same two strains of *Y. pestis*, a wild-type strain and the YopB mutant (Fig 6). The MDCK cells infected with the wild-type strain of *Yersinia* displayed the same "rounding" effect as infected HeLa cells (Fig 6). Alternatively, the MDCK cells that were exposed to the *Y. pestis* YopB mutant did not

experience a change in morphology (Fig 6). The DH82 cell line displays a mixed morphology of cell types, both rounded cells and skinny-irregular shaped cells, under normal growing conditions. When exposed to *Yersinia pestis* the DH82 cell lines behave similarly to the MDCK cell line (Fig 7). The wild-type exposed DH82 cells lost the morphology of skinny-irregular shaped cells, all DH82 cells became rounded after 4 hours of exposure (Fig 7). The DH82 cells that were exposed to the YopB mutant did not experience a change in morphology compared to normal growing conditions (Fig 7).

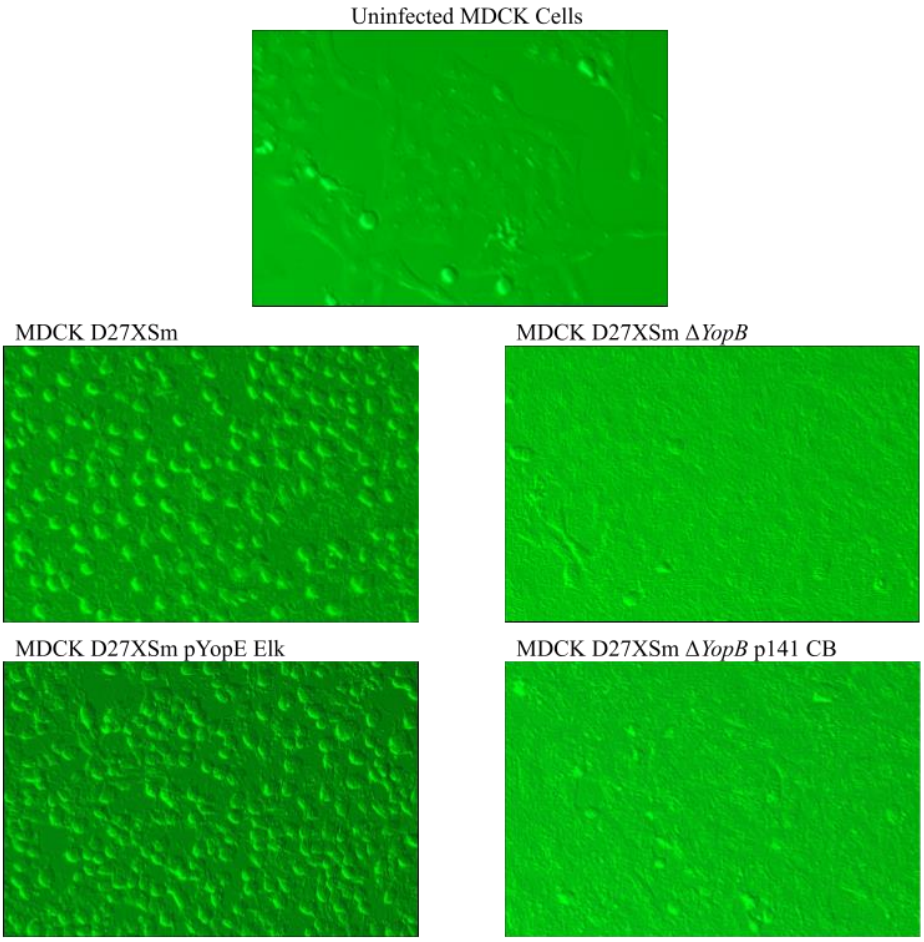

Figure 6
MDCK cell infections by *Yersinia pestis* wild-type strain and YopB mutant. MDCK cells were infected at an MOI of 15. Images were captured 4 hours post infection on an Olympus IX50 inverted microscope fitted with a Nikon D70 digital camera (magnification 200x) to document cell cytotoxicity. These images are representative of repeated experiments.

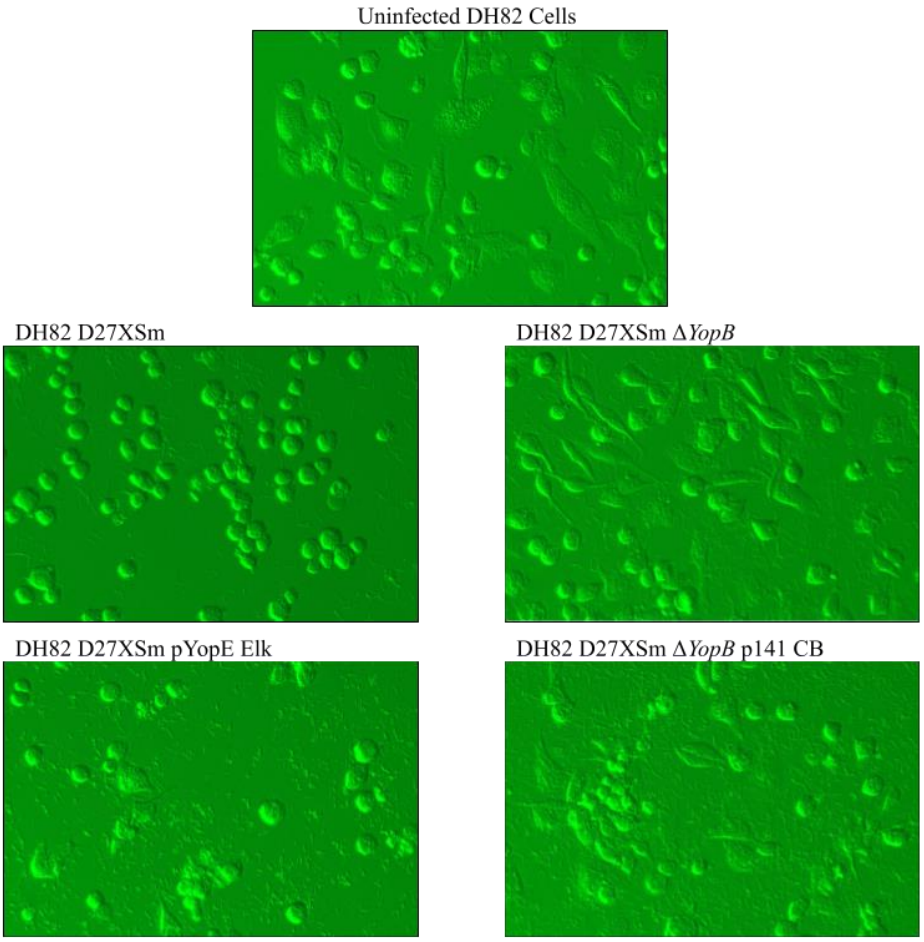

Figure 7
DH82 cell infections by *Yersinia pestis* wild-type strain and YopB mutant. DH82 cells were infected at an MOI of 15. Images were captured 4 hours post infection on an Olympus IX50 inverted microscope fitted with a Nikon D70 digital camera (magnification 200x) to document cell cytotoxicity. These images are representative of repeated experiments.

***Yersinia pestis* translocates Yops into the cytosol of MDCK and DH82 cells.**

Yersinia pestis can translocate Yops into the cytosol of HeLa cells, which is indicated by the rounding of HeLa cells after being exposed to the bacterium. Logically, we wanted to analyze the cytosol of the eukaryotic cells to confirm Yop translocation.

This was done by electroporating a plasmid containing an Elk tag, which can be phosphorylated inside eukaryotic cells, into the *Y. pestis* strains of interest. The wild-type strain of *Yersinia* was transformed with a plasmid that encoded the YopE-Elk-1 chimera under control the native *yopE* promoter. The YopB mutant strain was transformed with a plasmid that encodes the YopE-Elk-1 chimera controlled by the arabinose inducible $araBAD_p$ promotor. This plasmid would prevent the bacteria from intoxicating eukaryotic cells unless arabinose was present in the cell culture media. Phosphorylation of the YopE-Elk-1 chimera would allow us to know if Yops from *Y. pestis* were entering the cytosol of the eukaryotic cells. In this way, analyzing the immunoblots were quite straight-forward. The Elk tag from the plasmid can only be phosphorylated by eukaryotic cell-specific protein kinases. A band on the immunoblot probed with the Elk-1-PO_4 specific antibody would indicate that Yops did enter the eukaryotic cell through translocation.

Once again, the HeLa cell line would be used as a control for the two canine cell lines, MDCK and DH82. As expected, the uninfected HeLa cells showed no signs of Yops from *Yersinia pestis* (Fig 8a, 8b, 8c). The HeLa cells infected with the wild-type strain of *Yersinia* displayed Yop expression for all three proteins assessed: YopE-Elk-1, phosphoYopE-Elk-1, and YopE (Fig 8a, 8b, 8c) and translocation of YopE-Elk-1 was detected by the presence of phospho-YopE-Elk-1 (Fig 8b). The HeLa cells that were infected with the YopB mutant of *Y. pestis* expressed YopE and YopE-Elk-1 (Fig 8a and 8c) while no expression of phospho-Elk-1 (Fig 8b) was detected. Yop translocation into

the MDCK cell line was expected because the MDCK cells behaved similarly to the HeLa cells when looking at the cell cytotoxicity. When looking at the YopE antibody blot (Fig 8c), the MDCK cells infected with the wild-type strain of *Y. pestis* showed there was YopE-Elk-1 expression in the cell. However, the MDCK cells infected with the YopB mutant strain did not experience any Yop expression (Fig 8c). This trend continued for the MDCK cells with both the Elk-1 antibody and the phospho-Elk antibody (Fig 8a, 8b). In both instances the MDCK cells displayed Yop expression with the wild-type strain of *Yersinia* but did not display Yop expression with the YopB mutant. The DH82 cell line also was probed with these three antibodies: YopE, Elk-1, and phospho-Elk. The DH82 cells that were infected with the YopB mutant of *pestis* did not experience Yop expression (Fig 8a, 8b, 8c). Yop translocation and expression was noted in the DH82 cells that were infected with the wild-type strain of *Yersinia pestis* (Fig 8a, 8b, 8c).

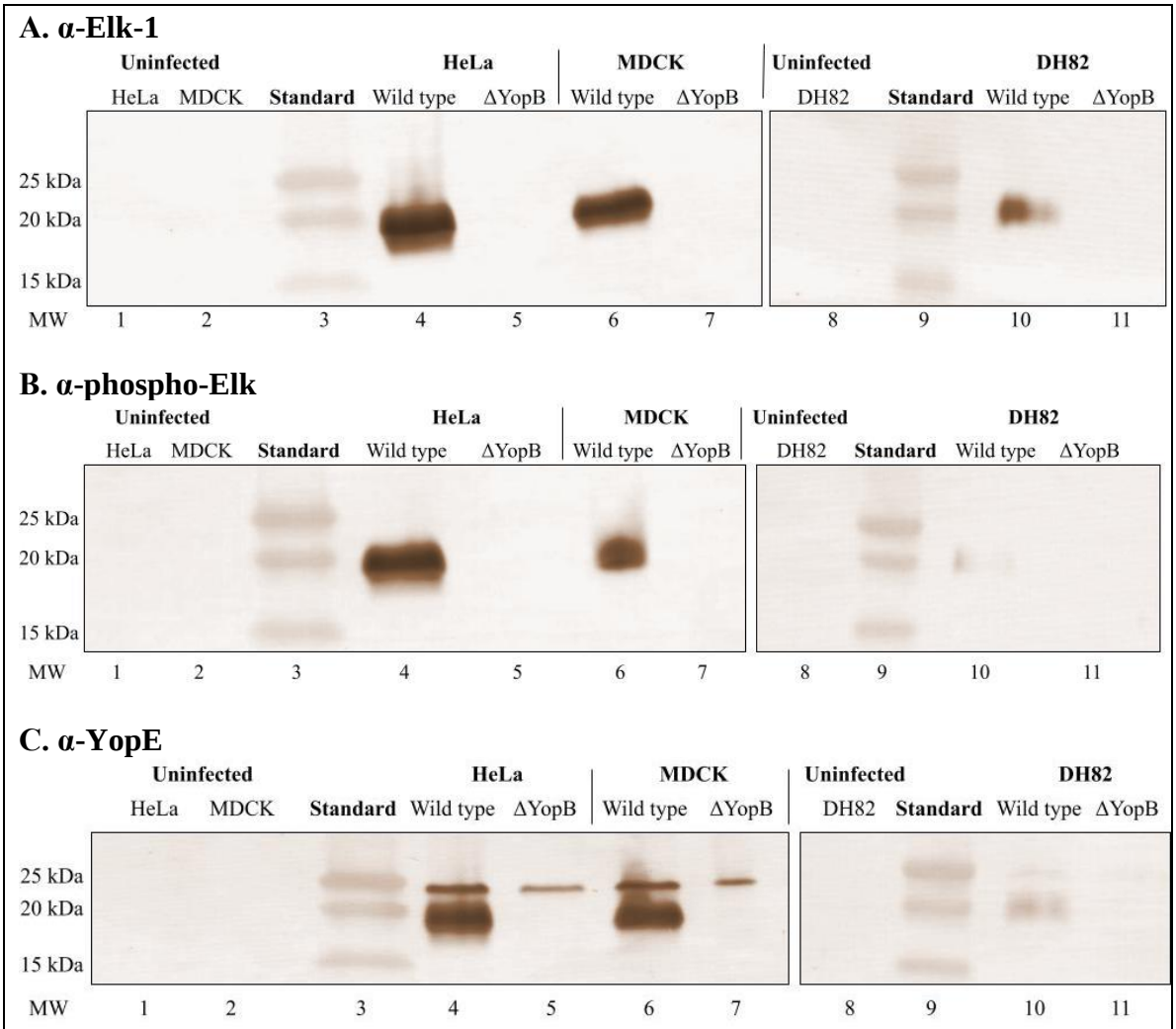

Figure 8

Expression and translocation of YopE-Elk in HeLa, MDCK, and DH82 cells.
Translocation and phosphorylation of YopE-Elk in infected HeLa, MDCK, and DH82 cells. Proteins were analyzed by probing with α-Elk-1, α-phospho-Elk, and α-YopE primary antibodies. Proteins were visualized by probing with alkaline phosphatase conjugated antibodies and developed with NBT-BCIP.

CHAPTER IV

DISCUSSION

Yop translocation occurs in MDCK and DH82 cells

In this study, we evaluated the hypothesis that MDCK and DH82 cells can be infected by *Yersinia pestis.* We compared Yop translocation in the human epithelial cell line, HeLa, to the canine derived cell lines MDCK and DH82. As can be seen in Figure 6, the MDCK cells exhibit the same cell cytotoxicity as HeLa cells when exposed to the bacterium *Y. pestis.* Figure 7 displays this same cell cytotoxicity in the DH82 cells. This change in cell cytotoxicity has been used previously as an identifier of Yop translocation in HeLa cell studies (34). In that same way, the cell lines MDCK and DH82 can be considered to experience Yop translocation into the cytosol. Yop translocation can be confirmed through the immunoblot analysis. The phosphorylatable Elk-tag that was expressed by *Yersinia pestis* was used to identify the presence of the YopE-Elk-1 chimera in the cytosol of the canine cells. The detection of phosphorylated YopE-Elk-1 demonstrates translocation of Yops (45). The blots that were probed α-phospho-Elk (Fig 8b) show that YopE-Elk-1 chimera entered the cytosol of the eukaryotic cells. This YopE-Elk conjugate can only be phosphorylated by cell specific protein kinases inside the host cell. The α-YopE blots were probing directly for the protein YopE (8c). The eukaryotic cells infected with the wild-type strain of *Y. pestis* were positive for both bands that were expected being probed with this antibody. The lighter, top band is YopE

and the dark and lower band represents the Elk-tagged YopE. Although bands on different gels cannot be directly compared, the main purpose of this series of experiments was to determine whether Yops can be translocated into the cytosol of the canine derived cell lines. The samples added to each lane for the immunoblots were normalized using the same number of bacteria (using A_{620}•ml of bacteria as a surrogate for bacterial numbers) added to the eukaryotic cells during infection.

Moving forward, the DH82s hold the key

Previously, the canine's ability to be plague resistant was postulated to the absence of the protein Fpr1 (41). However, the data collected in these experiments indicate the canine derived cell lines MDCK and DH82 can be infected by *Yersinia pestis* and experience Yop translocation. Knowing that canine cells can be intoxicated by the bacterium, several key points come to mind. First involving canines expressing the protein Fpr2 which is different than the human expressed Fpr1 and Fpr2. Multiple comparison analysis of the three protein amino acid sequences shows there are non-conserved regions which differ between the human and canine proteins. These amino acid differences could contribute to an overall structure and function change of the protein. Further analysis of the association found between human Fpr1 and LcrV should be completed.

Canines infected by *Y. pestis* typically display little symptoms or are completely asymptomatic. This brings to question the possibility of an innate immune response that

canines have to be able to fight off plague infection. This is where the DH82 cell line becomes important. The DH82 cells are a monocyte cell line that were activated into macrophages for the purpose of these experiments using LPS. Macrophages are used by the host cell to eliminate foreign pathogens through phagocytosis. The next logical step in this research is to look at whether the DH82s are being infected by *Y. pestis* or if the DH82 cells are ingesting *Y. pestis* by phagocytosis. This could be examined using a gentamicin protection assay, a standard assay to detect intracellular bacteria. The assay would compare the DH82 cell line to a human derived monocyte cell line, THP-1, human and murine macrophages are not infected by *Y. pestis* or taken up by phagocytosis to any significant degree as the bacteria remains extracellular. Gentamicin is an antibiotic that does not have the ability to enter the eukaryotic cell. The assay can be used to differentiate between bacteria that are in the cytosol of a host cell and those that remain extracellular. This would help to shed a light on whether the DH82 cells are being infected by the bacteria or are undergoing phagocytosis of the invading bacteria. An assay blocking phagocytosis in the macrophages, by disrupting actin using cytochalasin D, would aid in determining whether the DH82 cells are truly infected by *Y. pestis* or if DH82 cells can phagocytose *Y. pestis.*

If there is an activation of the innate immune system when canines are exposed to *Yersinia pestis*, running flow cytometry to determine which macrophage types are being induced would provide vital information. M1 and M2 macrophages are activated by different cytokines and are associated with two different activities in cells. M1

macrophages inhibit cell proliferation and cause tissue damage to the surrounding cells, while M2 activity promotes cell proliferation and tissue repair. Understanding which subtype of macrophage is being activated in infected DH82 cells can give key insights into how canines can recover from *Y. pestis* infections.

Canines in locations where plague outbreaks may occur can serve as a sentinel species for identifying outbreaks before they occur. The more we understand how canines are able to fight off a pathogen seemingly perfect at evading host immune responses, the closer we become to applying the knowledge towards human use. These experiments took a step towards understanding what makes canines different than humans when it comes to *Y. pestis* infections. Not only do plague infections still occur around the world, but the bacterium is considered a dangerous bioweapon. Understanding how infections are overcome in key groups could be the difference in protecting people from further harm from this famous pathogen.

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