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Blood Meal Source Identification of the Human Flea, *Pulex irritans,* from Rural Villages in Madagascar Using Polymerase Chain Reaction

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Abstract

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By: Nick An

Plague is a zoonotic disease caused by the bacterium *Yersinia pestis* and spread through flea vectors. Flea species vary in their potential to spread plague to their hosts. This dynamic is further complicated as the blood sources consumed by fleas can impact their ability to transmit plague efficiently. Although the human flea, *Pulex irritans,* has commonly been found infesting homes in Madagascar, little is known about its role in the plague transmission cycle and its feeding patterns. This study compared the efficacy of two polymerase chain reaction (PCR) techniques to conduct blood meal source identification for wild caught *P. irritans* from rural villages in Madagascar; singleplex PCR with Sanger sequencing and multiplex PCR with avian and mammalian primer sets. Singleplex PCR was prone to contamination with 20% of samples not matching positive controls. With the avian primer set, 81.12% out of 376 samples could be identified by multiplex PCR, with 81.12% positive for humans, 5.59% for avian, and 0.50% for non-human mammals DNA. With the mammalian primer set, 78.49% out of 93 could be identified by mutiplex PCR, 78.49% positive for humans, 12.90% positive for pigs, 6.45% positive for cows, and none were positive for dogs or goats. These results indicate that although the primary host preference for *P. irritans* was humans, they also fed on pigs, cows, and birds. These findings are an important first step toward understanding *P. irritans* host preference and range, which can better inform this vectors behavior and potential role in the plague transmission context.

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Introduction

Madagascar is one of the few countries in the world where plague is still prevalent (WHO, 2017). Plague is caused by the bacterium, *Yersinia pestis*, that typically infects fleas and small mammals (WHO, 2017). Humans and other mammals can be infected with plague through bites from infected fleas, but flea species vary in their ability to transmit plague efficiently (Eisen et al., 2012). The relationship among the flea, *Y. pestis*, and the host is complex and can involve numerous hosts with various susceptibility and vectors with diverse vectorial capacity (Eisen et al., 2012). Although the human flea, *Pulex irritans*, has frequently been found in homes in Madagascar with active human plague infections, its potential role in interhuman transmission of plague has not been well-studied (Ratovonjato et al., 2014). However, one study indicates that *P. irritans* could be used as an indicator to assess the risk of plague because their density was positively associated with plague frequency in villages in Tanzania (Laudisoit et al., 2007).

Fleas are strict hematophagous insects and their host range and feeding preference may vary between species. One way to better understand the epidemiological importance of *P. irritans* is to study its feeding patterns. Although *P. irritans* is known to feed on humans, they can also be found on a wide range of animals, such as rodents, birds, and domestic animals (Buckland et al., 1989). Blood meal source identification is important to understanding vector competence since blood source can impact a flea's capability to transmit plague efficiently (Miarinjara et al., 2021). For instance, one study investigated *P. irritans* transmission potential with different blood meal sources and found that rat blood-fed fleas can transmit more bacteria (Miarinjara et al., 2021). Other researchers have hypothesized that fleas may digest blood from different host species at differential rates, which may lead to varying rates of clearing *Y. pestis* bacterium from the gut, influencing their ability to transmit plague (Eisen et al., 2008).

Therefore, understanding flea feeding patterns is a critical step in evaluating the vector competence of different flea species for plague transmission.

Polymerase chain reaction (PCR) is a molecular method that creates high numbers of copies of DNA sequences of interest (Kuslich et al., 2019). To study hematophagous insect blood meal sources, PCR protocols have been developed with primers that target host DNA (Townzen et al., 2008, Field et al., 2019, Kent et al., 2005, Maleki-Ravasan et al., 2009). Another molecular technique that has been used for blood meal identification is the enzyme-linked immunosorbent assays (ELISA), but this technique is time-consuming and can lack sensitivity to detect the small quantities of blood present from insects with small abdomens (Maleki-Ravasan et al., 2009). PCR-based techniques are more suitable for identifying blood meals within insects due to greater accuracy of the technique and ability to analyze for multiple species within the same DNA sample (Maleki-Ravasan et al., 2009). Although PCR protocols exist for many insect species, such as mosquitoes, there are no PCR protocols for *P. irritans* within the plague transmission context. Thus, the goal of this research is to develop such a PCR protocol for flea blood meal detection.

This study aims to test two different types of PCR protocols: singleplex and multiplex. Singleplex PCR allows for amplification of a single target sequence of DNA (Townzen et al., 2008). One study used singleplex PCR to analyze mosquito blood meals to determine hosts mosquitos fed on using a generalist *Cytochrome b* vertebrate primer, which is the most studied gene to identify vertebrate blood meals, followed by DNA sequencing to identify the organism (Townzen et al., 2008). This approach is useful for identification of a broad array of potential hosts through comparison of amplified products to publicly available sequences in repositories, such as GenBank (Townzen et al., 2008). However, this approach may not allow for

identification of multiple hosts from the same experiment, even though fleas are likely to feed from more than one animal source (Bitam et al., 2010). This PCR approach can be modified to amplify more specific sequences, but this would require running multiple protocols on the same sample to amplify multiple hosts, which can be time consuming (Kent and Norris, 2005). In contrast, multiplex PCR amplifies species-specific fragments of varying DNA sizes, allowing for simultaneous detection of multiple targets amplified within a single reaction well by including primers for each target (Kent and Norris, 2005; Kent et al., 2007).

Considering the potential role of *P. irritans* in transmitting plague, and the lack of entomological studies of this flea species in Madagascar, this thesis aims to elucidate the host range of *P. irritans* by comparing singleplex and multiplex PCR methods from the literature to determine the best method for identifying blood meal sources of wild-caught *P. irritans* from rural villages in Madagascar. I hypothesize that the PCR protocols will reveal the feeding preferences of *P. irritans* to be humans, while also identifying other animals that *P. irritans* feed on that could reveal important information about the plague transmission cycle.

Materials and Methods

Singleplex PCR

To validate PCR protocols prior to use on wild-caught *P. irritans*, protocols were tested on rat fleas (*Xenospylla cheopis*) obtained from the NIH Rocky Mountain Laboratory. Fleas were fed blood from known sources to evaluate the efficacy of singleplex PCR and sequencing to correctly identify sources. Fleas were fed for one hour via an artificial feeding apparatus with a stretched parafilm membrane at the bottom of the device to mimic animal skin (Miarinjara et al., 2021). Blood sources included mouse, rat, or chicken blood purchased from Lampire Biological Laboratories. In addition, mosquito samples (*Aedes aegypti* and *Anopheles gambiae*) fed with

known blood were obtained from CDC and the Prokopec Lab at Emory University. These mosquitoes were fed with human, pig, cow, or rabbit blood, with male mosquitos as a control since they never feed on blood. DNA was extracted from 80 insect samples using Nucleospin DNA Insect Kit and Qiagen DNeasy® Blood & Tissue Kit. To perform this extraction, fleas were crushed and lysed so that their DNA was suspended in solution. DNA was then binded using solutions and columns from extraction kits so that only highly pure DNA remained. Extracted DNA samples included fleas fed on different blood sources to observe how accurately sequencing from singleplex PCR would detect hosts when there is more than one blood source.

Singleplex PCR was performed on the extracted DNA based on a protocol from a study amplifying *cytochrome b* gene sequences from mosquito blood meals (Townzen et al., 2008). The *cytochrome b* primer sequences were obtained from the same study (Townzen et al., 2008). Mix composition of the PCR reactions were made for a total of 50µl solutions that included the following: 1µl of forward primer, 1µl of reverse primer, 25µl of Invitrogen™ Platinum™ SuperFi™ PCR Master Mix, 20µl of nuclease free water, and 3µl of extracted DNA. These samples were amplified using a Thermocycler with the following PCR conditions 95 °C for 1 min; 35 cycles of 95 °C for 30 s, 48–52 °C for 50 s, 72 °C for 1 min; and a final extension cycle at 72 °C for 5 min. The amplified PCR products were visualized using electrophoresis on a 1% ethidium bromide-stained agarose gel. The samples ran under voltage conditions of 90V for 30 minutes using small gels that fit 12 samples at once (25 mL). Products that successfully amplified as indicated by bands on the gel (n = 25 samples) were sent for Sanger DNA sequencing (Azenta Life Sciences, NJ).

DNA sequences were edited using the MEGA software to remove portions of DNA at 3’ and 5’ ends unusable or not accurate enough after visualizing each sequence electropherogram.

(Kumar, et al., 2008). This was done to obtain accurate sequences by removing or replacing nucleotides that did not show conclusive electropherogram peaks. Forward and reverse sequences were then aligned for each sample to get a consensus sequence, which provides more accuracy since two complementary sets can be compared for a single sample. These edited sequences were uploaded into the Basic Local Alignment Search Tool (BLAST) tool to identify the species and the percent accuracy of match results. BLAST can do this by comparing the uploaded sequences with known sequences in its database and using statistical significance to identify percent similarity (U.S. National Library of Medicine, n.d). Sequenced samples that matched known blood sources from the fleas were kept as positive controls.

Collection of P. irritans from the field

Fleas were collected by Malagasy researchers from households in four rural villages in plague-endemic regions of Madagascar in July and August of 2022. These villages included Nanda, Soafandry, Ambohipanalinana, and Alakamisy Ambohimaha. Fleas were sampled using candle traps, which consisted of a tray filled with soapy water and a candle burning in the middle of the tray. The fleas are attracted to the light of the candle and get caught in the trap as they fall in the soapy water and cannot escape. These candle traps were placed in households of interest (n = 30-33) in each village. Dead fleas were collected the following morning using tweezers and conserved in tubes containing absolute ethanol or RNA later. All fleas from a single trap within a household were placed in individual tubes with solutions of either RNAlater or ethanol.

Fleas were sorted by species and sex using a stereo microscope. *P. irritans* were identified based on their distinct morphological characteristic, such as the width of the three thoracic segments being larger than the abdomen, the absence of pronotal or cephalic combs, and

an absence of the pleural rod within the mesothorax (Harimalala et al., 2021). Male fleas were identified by their smaller size, flatter abdomen that points upward, and the shape of the genitalia within the abdomen, when compared to female fleas (Harimalala et al., 2021). Fleas were further sorted based on feeding status by examining the amount of blood in their abdomen using a microscope. The feeding status was assigned from a range of 0-4, with 0 being unfed fleas, and 4 being fully fed fleas. Female fleas that had a feeding status of "4", "3" or "2" were selected for molecular biology analysis to maximize the chance of amplifying host DNA. Only male fleas with "4" feeding status were selected since their smaller abdomens held less blood compared to females. 125 individual fleas were selected from each village for a total of 500 samples for this study.

Multiplex PCR

DNA was extracted from *P. irritans* samples using Qiagen DNeasy® Blood & Tissue Kit. After extraction, multiplex PCRs were done using two separate protocols from the literature. Two different multiplex PCR protocols were used to amplify flea blood sources to identify greater host range. The first PCR protocol used a primer set targeting mitochondrial *cytochrome b* sequences detecting humans, non-human mammals, and avian species (Field et al., 2019) for 376 *P. irritans* samples. Mix composition for this avian protocol involved 14µl of GoTaq Master Mix, 5µl of nuclease free water, 0.5µl of the universal reverse primer, 0.5µl of each the forward primers (human, avian, non-human mammal) and 2µl of extracted DNA. PCR was performed with the Thermocycler conditions of 94 °C for 3 min; 36 cycles of 94 °C for 30 s, 60°C for 30 s, 72 °C for 30 s; and a final extension cycle at 72 °C for 5 min.

The second PCR protocol used a mammalian primer set amplifying mitochondrial *cytochrome b* sequences for human, dog, cow, goat, and pig (Kent et al., 2005) for 93 *P. irritans* samples. Mix composition for this mammalian protocol was 14µl of GoTaq Master Mix, 5µl of nuclease free water, 0.5µl of universal reverse primer, 0.5µl of each forward primers (human, dog, cow, pig, and goat) and 2µl of extracted DNA. PCR was performed with the thermocycler conditions of 95 °C for 5 min; 40 cycles of 95 °C for 60 s, 56°C for 60 s, 72 °C for 60 s; and a final extension cycle at 72 °C for 7 min.

The amplified PCR products were visualized using 1% ethidium bromide-stained, 190mL agarose gel electrophoresis. The samples ran under voltage conditions of 140V for 55 minutes using large gels that fit 30 samples at once. After completion, gels were assessed under a UV light with a BioRad Gel Imaging System. Band sizes for each product were compared with sizes from known positive controls of the primers. For the avian multiplex PCR protocol, the expected PCR sizes for human, nonhuman mammal, and avian hosts were 216bp, 386bp, and 518bp, respectively. For the mammlian multiplex PCR protocol, the expected PCR sizes for goat, human, pig, cow, and dog were 132bp, 334bp, 453bp, 561bp, and 680bp, respectively. If the PCR products matched with these expected sizes, then the flea specimen pertaining to the PCR product was indicated to have fed on the matched hosts (Figure 1). For fleas that have fed on multiple hosts, multiple bands should appear on the gel electrophoresis corresponding to the expected sizes of the five mammals (Figure 1).

Results

Singleplex PCR

Singleplex PCR of fleas fed on one blood source had highest accuracy for rat blood-fed fleas, correctly identifying all flea samples that fed on rat blood with an average percent

identification of 99.96% (Table 1). For fleas that fed on mouse blood, there was a 100% identification for three out the five flea samples (Table 1). However, two of these mouse blood samples yielded incorrect species results: *Homo sapiens* and *Xenopsylla cheopis* (Table 1). Singleplex PCR had the lowest accuracy for chicken blood-fed fleas, with an average of 76.84% for four out of the six samples (Table 1). Two of these samples had incorrect species results for *Xenopsylla cheopis* (Table 1).

For samples with two fleas fed with two different known blood sources, BLAST was not able to pick up more than one host for any of the samples (Table 2). For samples with fleas fed from chicken and rat blood, all the samples were positive for rats (*Rattus sp.*), with an average percent identification of 99.13% (Table 2). All the samples with fleas fed with chicken and mouse yielded results of *Mus musculus* (mouse), with a 100% percent identification rate (Table 2). The samples fed with mouse and rat were the only one where there were different BLAST results for each of the three samples, with one of the samples being positive for *Rattus sp*., one of the samples being positive for *Mus musculus,* and a third sample that had an incorrect species result of *Xenopsylla cheopis* (Table 2).

Multiplex PCR

From 376 *P. irritans* collected from rural villages in Madagascar, the hosts of 81.12% of the samples could be identified by multiplex PCR protocols using the avian primer set, with 81.12% positive for humans, 5.59% for avian, and 0.50% for non-human mammals DNA (Figure 2). All avian DNA-positive samples were positive with human DNA, and all non-human mammal positive samples were positive with human and avian DNA.

The mammalian multiplex primer set was applied to 93 *P. irritans* samples with 78.49% positive for humans, 12.90% positive for pigs, 6.45% positive for cows, and none were positive for dogs or goats (Figure 3). In addition, 19.35% of the samples did not test positive for any of the hosts from the mammalian primer set (Figure 3). The mammalian primer set was also validated using the same technique with known positive controls from seven different blood sources to test for contamination. Two of these samples were contaminated: a mosquito sample with human blood tested positive for cow host, and a flea sample with sheep blood tested positive for human host.

The same *P. irritans* samples that were analyzed using the mammalian primer set were compared with those from the avian primer set to have a more direct comparison between the two. Statistical comparisons using a two-proportion z test indicate that the differences between the primer sets for human DNA are not significant (p-value = 0.60) but is significant for non-human mammals (p-value = 0.00094). Compared to the avian primer set, the mammalian primer set was able to identify 14.19% more samples with non-human mammal DNA (Figure 4). Additionally, the avian primer set yielded 5.38% more samples that did not test positive for any blood source (Figure 4). The two primer sets did not differ significantly for amount of samples not testing positive for any mammals (p-value = 0.38).

Discussion

This study compared the suitability of singleplex and multiplex PCR to accurately detect flea blood-meal sources. Multiplex PCR demonstrated that although the primary host preference for *P. irritans* was humans, they also fed on pigs, cows, and birds. Although previous studies have confirmed *P. irritans* infesting dogs and goats (Garcia et al., 2008, Christodoulopoulos et al., 2006), I did not detect either using multiplex PCR with the mammalian primer set. There are

several possible explanations for this divergence. First my sampling protocol using candle traps was more passive than direct flea removal from hosts as done in the previous studies (Garcia et al., 2008, Christodoulopoulos et al., 2006). In addition, my sampling period may have been too short to reflect all *P. irritans* blood-meal sources. Lastly, fleas digest blood from animal sources at differential rates in the gut (Eisen et al., 2008), thus dog or goat blood may have been digested faster. Future studies should target other species-specific hosts, such as rodents, by changing the primer and PCR design. Rodents are a particularly important mammal to investigate as a potential flea blood-meal source, as some rodent species are reservoirs of plague in Madagascar (Dennis and Mead, 2006).

Both the mammalian and avian primer sets were compared to validate the human and non-human mammal positive results. The detection rate for human DNA between the two primer sets did not differ; however, the detection rate for non-human mammals was higher with the mammalian primer set. One possible explanation for this difference may be the degenerate primer sequences in the avian primer set, which can result in a mismatch between the primer and template DNA sequences, leading to insufficient amplification of target DNA during amplification (Linhart and Shamir, 2005; Green et al., 2015). Since the avian primer set contained several degenerate sequence positions, it could have amplified less of the non-human mammal blood due to such a mismatch. In addition, since the mammalian primer set targets specific mammal species instead of a broad group of mammals, there could be greater specificity between the primer and template DNA leading to more accurate amplification. Therefore, using this avian primer set to determine if fleas contain non-human mammal blood sources is not reliable. However, the avian primer set is still a reliable method for detecting avian DNA as a

potential blood source. There is greater confidence for detecting humans with both the avian and mammalian primer sets as they both had similar accuracy for detecting positive results.

Singleplex PCR paired with sequencing was problematic, with 20% of samples not matching positive controls. This method was also prone to contamination with human DNA for known blood sources without humans. Surprisingly, even though a vertebrate primer was used for amplification, three samples were positive for *Xenospylla cheopis*, the rat flea. In addition, I was able to confirm that singleplex PCR was not capable of identifying multiple blood sources from the same sample when using BLAST. Although singleplex PCR was clearly prone to contamination issues with a generalist vertebrate primer, this method could still be useful when using a primer that is more specific to a single species. Other studies have successfully used this approach to identify blood sources among various blood-feeding taxa, so controlling for contamination could yield more accurate results (Towzen et al., 2008, Molaei and Andreadis, 2006).

As evidenced by the singleplex PCR results, contamination could lead to inaccurate identification of blood sources. Even with the multiplex PCR, there were two samples that did not match positive controls of known blood sources. Practicing best practices to minimize cross contamination risk is critical when conducting PCR blood source identification studies. Some practices I recommend for future studies are to always vortex DNA extracts before transferring to PCR reaction, to use pipette tips with aerosol filters for DNA extraction and PCR, and to keep equipment for PCR and non-PCR stations separate. I would also recommend validating with more positive control samples to ensure greater confidence that results are contamination free. In addition, having more negative controls, such as through unfed fleas and sterile water, at the core

steps of a PCR protocol (DNA extraction, preparation of PCR mixes) can help track down potential contamination.

There are other PCR methods in the literature that could be used to address the limitations from the conventional PCR used in this study. One study used a conventional singleplex PCR for identifying blood sources from cat fleas using a single primer to amplify vertebrate DNA and then identifying species by sequencing (Graham et al., 2013). However, instead of using conventional PCR, the researchers used real-time PCR (Graham et al., 2013). Real-time PCR allows for quantitative monitoring of the amplification process through fluorescent technology and melting points to identify how many unique DNA sequences are amplified (Valasek, 2005). Using this method, the researchers were able to identify if fleas had enough vertebrate DNA to amplify and whether there were multiple blood sources (Graham et al., 2013). They were then able to infer haplotypes using a DNA polymorphism algorithm and BLAST to identify each of the mixed samples (Graham et al., 2013). Even with the conventional multiplex PCR approach used in this study, amplification for the primer set chosen was limiting since the blood source could have other vertebrate hosts that the primers were not designed for. The real-time PCR approach could allow for a more accurate approximation of how many vertebrate blood sources *P. irritans* fed on and to identify the specific species for each source. However, future studies should account for the time it might take to adopt this more complex procedure and conduct sequencing analyses for multiple blood sources of a single flea.

Another potential alternative to PCR-based Sanger sequencing is using amplicon deep sequencing for bloodmeal analyses. This approach utilizes next-generation sequencing to barcode and sequence amplicons from conserved loci (Balasubramanian et al., 2021). This is advantageous to the Sanger sequencing method as it allows for hundreds of thousands of

sequences reads for one vector as opposed to a single read with Sanger sequencing (Balasubramanian et al., 2021). One study successfully used this technique for seven different triatomine species and found that amplicon deep sequencing detected multiple blood sources in a single vector and yielded positive results for additional blood sources not detected by Sanger sequencing (Balasubramanian et al., 2021). This powerful tool could be applied for blood source identification of *P. irritans* to overcome the limitations identified with singleplex PCR and Sanger sequencing. However, cost per sample is a major limiting factor with this approach since more expensive technology is required to perform next generation sequencing (Balasubramanian et al., 2021).

In conclusion, this study showed that multiplex PCR can be used to identify blood-meal sources from *P. irritans* for multiple species, while singleplex PCR might be more appropriate using species-specific primers instead of a generalist primer. These results are an important first step for understanding host specificity and preference of *P. irritans* that affect their behavior within the plague transmission cycle. Future research should include testing for other species using similar PCR methods to better understand *P. irritans* host range.

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Tables and Figures

Table 1. Singleplex PCR BLAST Results from Known Flea Blood Sources of Single Organisms. Although fleas that fed on mouse blood had no inaccurate BLAST results, those that fed on mice blood had incorrect BLAST identification as *Homo sapiens* and *Xenospylla cheopi*s, and samples that fed on chicken blood had incorrect results as *Xenospylla cheopis*. Samples that fed on chicken had the lowest percent identification for correct results among the different hosts, with an average of 76.84%.

Organism	Total Samples	Samples with Correct BLAST Results	Average % Identification for Correct Results	Samples with Incorrect BLAST Results	Incorrect Species Results
Mouse	5	3	100%	2	*Homo sapiens, Xenopsylla cheopis*
Rat	6	6	99.96%	0	n/a
Chicken	6	4	76.84%	2	*Xenopsylla cheopis*

Table 2. Singleplex PCR BLAST Results from Known Flea Blood Sources of Multiple Organisms. None of the known blood flea sources of multiple organisms had more than one organism identified through BLAST. Samples with combined blood sources from mouse and rat had an inaccurate BLAST result of *Xenopsylla cheopis*. Chickens were never identified for any of the combined blood sources.

Organism	Total Samples	BLAST Result	Average % Identification	Samples with Incorrect BLAST Results	Incorrect Species Results
Rat and Chicken	3	*Rattus sp.*	99.13%	0	n/a
Chicken and Mouse	2	*Mus musculus*	100%	0	n/a
Mouse and Rat	3	*Rattus sp.* (1) *Mus musculus* (1)	98.02%	1	*Xenopsylla cheopis*

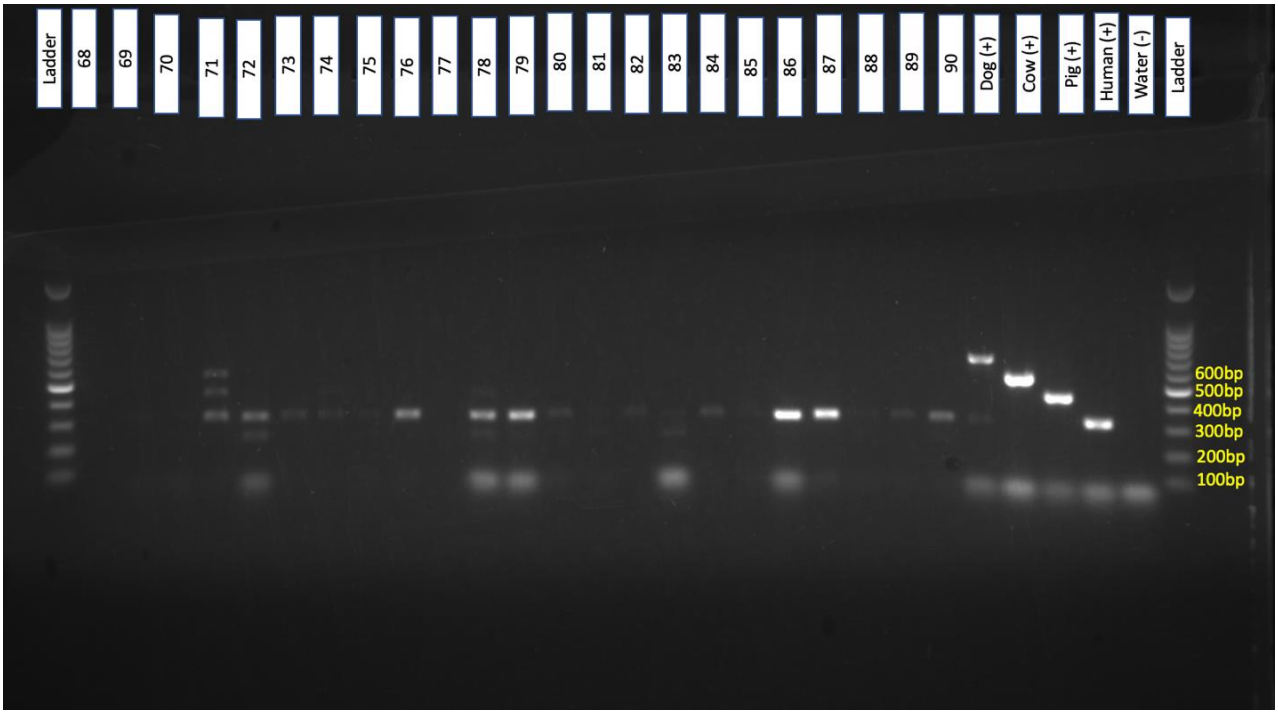

Figure 1. Example of Gel Electrophoresis Visualization with *P. irritans* Blood-Meal Samples 68-90 Using the Mammalian Primer Set. Samples were indicated as positive for a host type if the band sizes matched the positive control samples on the right side of the gel, with a DNA ladder as reference. In the gel electrophoresis pictured, 16 of the samples tested positive for humans. Samples 71 and 78 also tested positive for pigs, and sample 71 also tested positive for cows.

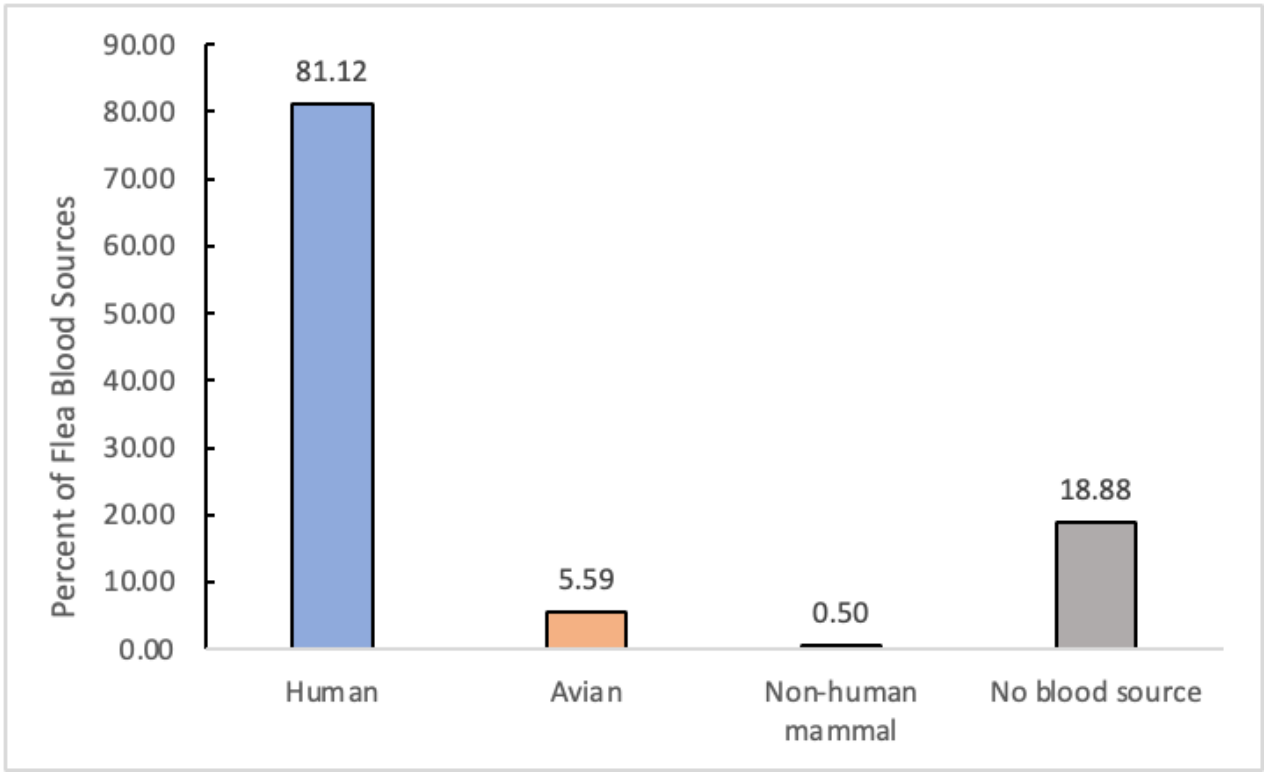

Figure 2. Percent of Flea Blood Sources Using the Avian Primer Set for *P. irritans* samples (n=376) from rural Madagascar.

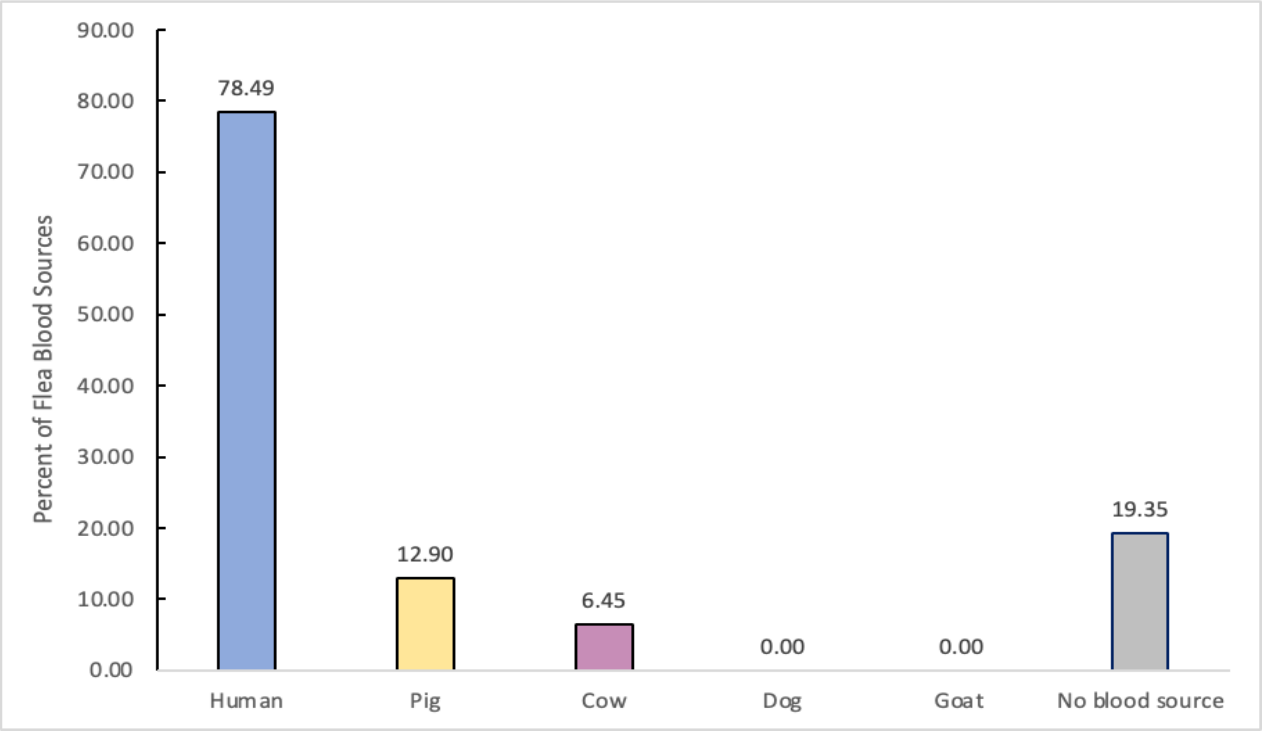

Figure 3. Percent of Flea Blood Sources Using the Mammalian Primer Set for *P. irritans* Samples (n=93) From Rural Madagascar.

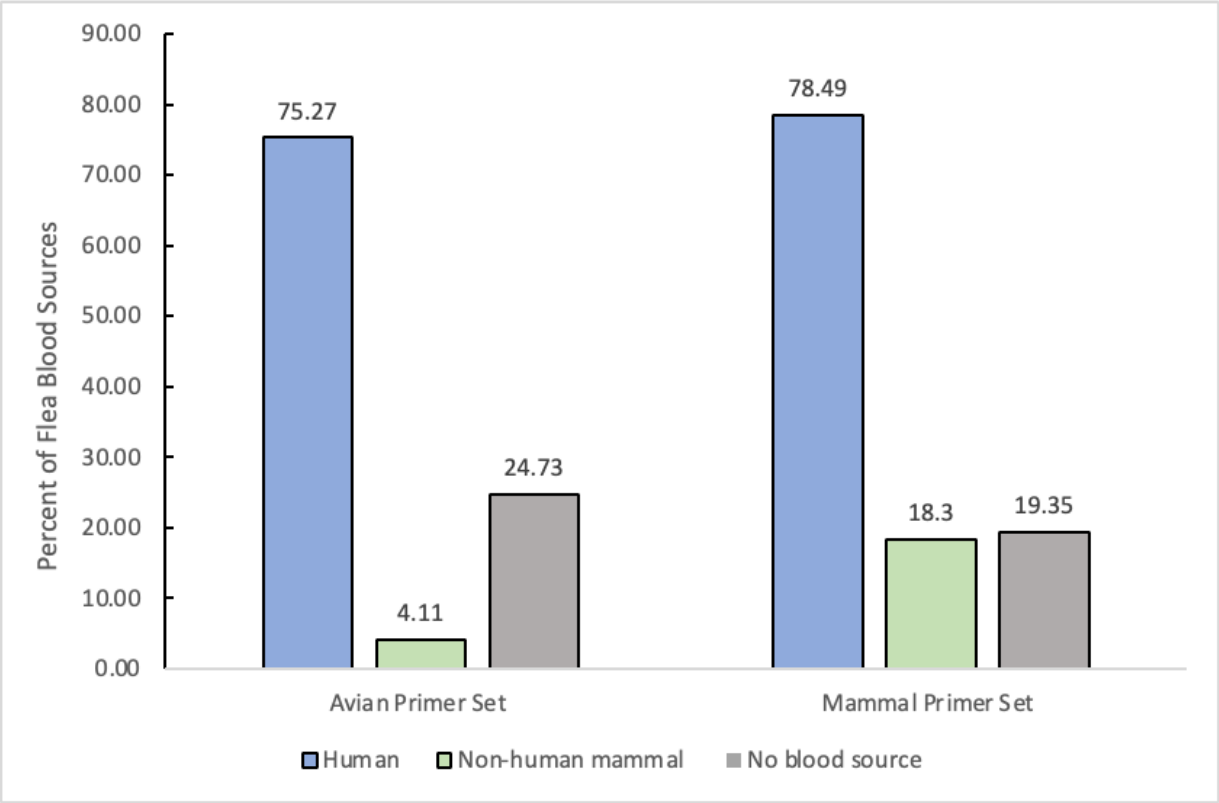

Figure 4. Percent of Flea Blood Sources Using Avian vs Mammalian Primer Sets for *P. irritans* Samples (n=93) from Rural Madagascar.