Original Article

Molecular Surveillance of *Yersinia pestis* from Stray Dogs and Cats and their Fleas in Algiers

Zaidi, S[1*], Bessas, A[2], Hezil, D[3], Benseghir, H[4], Bitam, I[5,6]

1. *Higher National Veterinary School, El Alia, Oued Smar, 1615, Algiers, Algeria.*
2. *Department of Biology, Faculty of Sciences, University of Algiers 1 (Benyoucef Benkhedda University), Algiers, Algeria.*
3. *Department of Biology, Faculty of Sciences, M'Hamed Bougara University, Boumerdes, Algeria.*
4. *Department of Microbiology, Faculty of Natural and Life Sciences, University of Batna2, Algeria.*
5. *Higher School of Food Sciences and Agri-Food Industries, Algiers, Algeria.*
6. *Aix Marseille Univ, IRD, VITROME, IHU Méditerranée Infection, Marseille, France.*

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ABSTRACT

In recent years, plague has re-emerged in several countries around the world and remains endemic in some regions. In a natural environment and in contact with rodents and their fleas, stray carnivores are most at risk of catching the disease and maintaining the spread of the bacillus. The present study aimed to demonstrate the presence or absence of *Yersinia pestis* in stray dogs and cats in the Algiers region by molecular methods and thus determine their role in epidemiology of this disease. Molecular research of *Yersinia pestis* has also been conducted on fleas from these carnivores. Preliminary identification of ectoparasites to genus and species level was performed. Real-time polymerase chain reaction targeting *Yersinia pestis pla* gene was used to survey the plague agent in fleas and carnivores captured as stray animals in Algiers (Algeria). Positive qPCR results were tested by PCR sequencing using glpD gene. Among 327 fleas captured from 107 dogs and 365 fleas from 140 cats, prevalence of *Ctenocephalides felis* was higher in cats (86,96%), whereas that of *Ctenocephalides canis* and *Xenopsylla cheopis* were higher in dogs (90,57% and 92,63%, respectively). While internal and external PCR positive controls were positive, none of the 107 dogs spleens and 140 cats spleens and none of the 256 analyzed fleas were positive for *Y. pestis*. These results suggested that stray cats and dogs are unlikely sources of plague in Algeria, contrary to what has been reported in other plague-endemic countries. This observation illustrates that the plague epidemiological chain varies from one region to another.

Keywords: Algeria, Cat, Dog, Fleas, Plague

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Corresponding Author's E-Mail:
zaidi.ensv@gmail.com

1. Introduction

Plague is a zoonotic disease affecting humans, animals, and their fleas (1). In humans, plague is a deadly disease with a reported case fatality rate of 30% to 100% in the absence of treatment. This disease has resulted in major historical epidemics, sometimes re-emerging from infected soil known as plague foci, as described in Maghreb countries, including Algeria (2,3). During the 18 years from 1985 to 2003, more than 43,000 human cases of plague were reported to the World Health Organization (WHO) by 25 countries. In Algeria, archives reported epidemics compatible with plague as far back as the 14th century. These epidemics mainly affected ports, particularly Oran between 1556 and 1678 with 3,000 deaths. In 1899, after nearly a 100-year, plague reappeared in the port of Philippeville (now Skikda). Three large epidemics were subsequently reported in 1921 (185 cases), 1931 (76 cases), and 1944 (95 cases) as well as 158 sporadic cases. These cases occurred in ports, but no natural plague foci had ever been described (4). In Algeria, plague reemerged in 2003 in Oran after a period of inter-epidemic silence of 50 years (2,4). The epidemic began in Kehailia, a village of 1,300 inhabitants 25 km south of Oran, in a rural region with inadequate sanitation. Plague cases were subsequently reported in neighboring regions, such as Mascara and Ain Temouchent (4). All cases had bubonic plague; septicemia and coma later developed in two patients (2). In this outbreak, it was unclear if reemergence resulted either from importation through the international port of Oran or from a previously unknown rural focus (2). In July 2008, a new episode took place in the Laghouat area in a nomad camp in Thait El Maa with four cases (3). This incidence is the last episode of plague in Algeria to the present day. To find the reservoirs responsible for this epidemic, in January 2009, eight individuals of the rodent species *Meriones shawii* and two *Psamommys obesus* were trapped inside nomads' tents. The PCR amplification of *pla* gene revealed positivity of 16%. Identification was further confirmed by culturing and sequencing *pla*, *caf*, and *glpD* genes. The Laghouat area was not previously known as a plague focus, and plague, therefore, must be regarded as an emerging disease in this region (3). To better understand the diversity of small mammals, including rodents maintaining *Y. pestis* in zoonotic foci throughout the country, an investigation was conducted in 2009 to 2012 in twelve regions of Algeria (Tlemcen, Aïn Témouchent, Mascara, Laghouat, Djelfa, M'Sila, Biskra, Batna, Algiers, Boumerdès, Cap Djinet). The PCR sequencing of *pla*, *glpD*, and *rpoB* genes revealed a positivity of 8% (18/237) in rodents of five species, including *Apodemus sylvaticus*, previously undescribed as pestiferous, and disclosed three new plague foci (Figure 1) (5). In Algerian rodents, only the Orientalis biovar has been observed. This finding is in agreement with the previous observations according to which the patients infected by *Y. pestis* in Algeria belonged to the biovar Orientalis (2). Previous studies have shown that contacts with domestic animals such as dogs and cats was associated with the risk for plague infection (6-10). In a natural and untreated environment and in contact with rodents, the wandering population of these carnivores is most at risk of catching the disease and subsequently maintaining the spread of the bacteria (11,12). Here, we investigated stray cats and dogs and their fleas in Algiers by means of PCR-based assays for the presence of *Y. pestis*.

2. Materials and Methods

2.1. Ethic statement

The study was submitted to and approved by the Ethics Committee and Decision Board of EPIC- H.U.P.E (EPIC: Entreprise publique à caractère industriel et commercial; H.U.P.E: Hygiène Urbaine et Protection de l'environnement) of Wilaya of Algiers (Ex-HURBAL). The HURBAL was created in 1994 with a new status: EPIC-H.U.P.E under the register number: 16/00-0013132B00. EPIC- H.U.P.E is an institution affiliated with the Algerian Ministry of the Interior, the Local Government, and the Algerian Ministry of Water Resources and Environment. In the context of the National Program for Rabies Control, EPIC- H.U.P.E captures stray dogs and cats in Algiers. Once captured, stray animals were housed in cages, being euthanized after expiration of the seven-day legal waiting time (in order to allow owners to claim their pets). As complying with the Algerian legislation for the protection of animals (Law 01/04/1994), to which our protocol was adhered.

2.2. Sample collection

From February 2015 to September 2016, 107 stray dogs and 140 stray cats were randomly captured in Algiers, Algeria. A necropsy was performed immediately following euthanasia of animals. Spleen fragments were collected aseptically into sterile tubes. Additionally, fleas were collected from these stray dogs and cats. The randomly selected animals were examined in general. Information (species, breed, sex, age of the animal, and the clinical signs) were noted for each animal. The assessment of the age of the animals was based on tooth wear and the animals were classified in age groups. All specimens (spleens and fleas) were stored in 70% ethanol at room temperature (13). A total of 140 spleens of cats, 107 spleens of dogs, and 256 randomly selected fleas collected from these carnivores were analyzed by molecular biology targeting the *Y. pestis pla*. Positive

qPCR results were tested by PCR sequencing using glpD gene.

2.3. Identification of ectoparasites

Identification of ectoparasites to the genus and species level was performed using a stereomicroscope based on morphological criteria and standardized taxonomic keys. In the entomology laboratory, the handling of these ectoparasites was carried out in a Petri dish using fine forceps. During the observation, the arthropod was moistened with alcohol at 70°C to avoid its desiccation and remove debris surrounding it.

2.4. DNA extraction

2.4.1. Extraction

The specimens (spleens and fleas) were rinsed with distilled water, and each spleen sample was incised using an individual scalpel, and fleas were crushed into sterile tubes (Eppendorf, Hamburg, Germany). The samples were subsequently immersed in 180 µL of buffer G2 (30 mMTris-Cl, 30 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 800 mMGuHCl) 20 µL of proteinase K (activity = 600 mAU / mL of solution or 40 mAU / mg of protein), and incubated overnight at 56°C. After this pre-lysis step, a total of 100 µL of the DNA was extracted from half of each flea and a small fragment of each spleen using the QIAamp Tissue kit (Qiagen, Hilden, Germany). This kit contains reagents that allow the purification of DNA from tissues using an EZ1 automated robot (QIAGEN-BioRobot® EZ1, Tokyo, Japan). Extracted DNA was stored at -20°C under sterile conditions until used in PCR assays.

2.5. Real-time PCR

After extraction, all DNA samples were amplified by qPCR.

2.5.1. Mix preparation

This operation was performed under aseptic conditions in an isolated room and under a UV hood to prevent contamination of the mix. We prepared the mix with the addition of Master mix containing polymerase, primers, and probe (TakyonqPCR kit, Qiagen, Hilden, Germany). A quantity of 15 µL Mix was deposited in 96-well PCR plates.

2.5.2. Systems used during the qPCR

2.5.2.1. System for the detection of *Y. pestis*

Using qPCR, the DNA samples were tested for *Y. pestis* by targeting the plasminogen activator gene (*pla gene*) located in plasmid *pPCP1*. These primers amplify a fragment of approximately 98 bp (Table 1).

2.5.2.2. Control systems

As the positive control of *Y. pestis* was prohibited, parallel control qPCRs were performed on the extracted DNAs to confirm the efficient running of the PCR. Beta-actin was used as an internal control indicating the presence of host DNA, and thus the efficient progress of extraction and amplification. We used human beta-actin, as it is homologous to that of the dog and the cat, primers amplified a 172 bp fragment.

2.5.2.3. External control: TISS

TISS is a synthetic DNA sequence added before extraction as an external control. Its presence at the end indicated the absence of PCR inhibitors and, thus, an efficient progress of the amplification (Table 2). These primers amplify a fragment of approximately 135 bp.

2.5.2.4. The addition of extracted DNAs to the mix

In another room, the DNAs from our samples were added to the mix with an amount of 5 µL of DNA from each sample. The negative controls used in all qPCR reactions were sterile distilled water. Positive control was not used to avoid contamination (5). The addition of the positive controls was carried out in a separate box. A total of 20 µl of the reaction mixture is contained in each microtube of the PCR plate.

2.5.2.5. qPCR Cycling Parameters

The PCR plate is first covered with an adhesive film, centrifuged at 1300 rpm for 3 min, and then placed in a real-time quantitative PCR apparatus (qPCR CFX96 ™, Bio-Rad, California, USA). The latter is controlled by a computer that allows the acquisition and monitoring of real-time data and their processing. The qPCR program (Takyon protocol) was as follows: the reaction mixtures were maintained at 50°C for 2 min, then 95°C for 3 min and then passed through 39 cycles 95°C for 10 sec and 60°C for 1 min. The results are considered positive when the cycle threshold (Ct) was less than 36.

3. Results

3.1. Identification of ectoparasites

A total of 692 fleas were collected from stray dogs and cats in Algiers, Algeria, comprised of *Ctenocephalides felis, Ctenocephalides canis,* and *Xenopsylla cheopis*. The number of fleas identified for each species was 391 (56.50%) of *Ctenocephalides felis*, 163 (23.55%) of *Xenopsylla cheopis*, and 138 (19.94%) of *Ctenocephalides canis*. The identification of fleas by animal species is shown in Table 3. From 1 to 5 fleas were sampled from each host, depending on the rate of infestation. Some animals had mixed flea infestations. *Ctenocephalides felis* was the most frequently recovered flea species in cats, and the rat flea *Xenopsylla cheopis* was the most abundant flea in dogs. A total of 149 fleas collected from 107 infested dogs and 107 fleas sampled from 140 cats were randomly selected for further analysis. (Table 3).

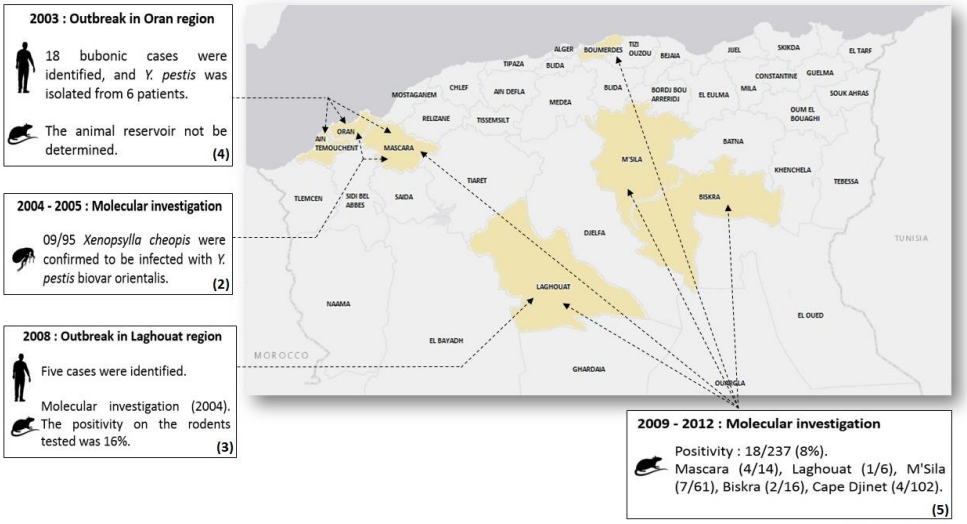

Figure 1. Plague studies found in Algeria

Table 1. Primers and probe used for the qPCR identification of *Y. pestis*

Bacteria	Target gene	Primers and probe	Sequences
Yesinia pestis	*pla gene*	Yper_Pla_F	5′- ATGGAGCTTATACCGGAAAC -3′
		Yper_Pla _R	5′-GCGATACTGGCCTGCAAG-3′
		Yper_Pla _P	5'-6FAM-TCCCGAAAGGAGTGCGGGTAATAGG-TAMRA-3′

Table 2. Primers and Probe used for the identification of the TISS at the qPCR

Target organismes	Name	Sequences	Dilution	Amplicon
TISS : Synthetic inhibition control	Tiss_F	CTGAGTCGTACCTAATGCATGACC	1nmol/uL followed by 1/50	135 pb
	Tiss_R	GTATCGCGATTCGCTAAAGTTC	1nmol/uL followed by 1/50	
	Tiss_P	6FAM- TCGAGACTCGACGCATGCACG-Tamra	0,1nmol/uL followed by 1/40	

Table 3. Number of fleas identified on sampled cats and dogs and number of fleas destined for molecular analysis

Flea species	Fleas identified					Fleas destined for molecular analysis					
	Dogs		Cats		Total	Dogs		Cats		Total	
	Nbr	%	Nbr	%		Nbr	%	Nbr	%	Nbr	%
Ctenocephalides felis	51	13.04	340	86.96	391	32	62.75	92	27.06	124	31.71
Xenopsylla cheopis	151	92.63	12	7.36	163	65	43.05	7	58.33	72	44.17
Ctenocephalides canis	125	90.57	13	9.42	138	52	41.60	8	61.54	60	43.48
Total fleas	327	47.25	365	52.74	692	149	45.57	107	29.32	256	37

3.2. Molecular results

The results of the real-time PCR targeting the *pla* gene on spleen samples and fleas collected from animals were all negative. No signal was obtained from the negative controls. Tiss and Actin controls were positive for all samples, indicating good DNA extraction and good PCR.

4. Discussion

Although Algeria is declared among the plague-infected countries by WHO, we do not have any epidemiological data on this pathology on animal species likely to be a source of contamination for humans. Few studies have been carried out on rodents and during declared outbreaks of plague. Feline plague is the source of human infection in many parts of the world, and several cases of cats with this infection have been reported (11). The role of the dogs as a source of plague infection has not been well-elucidated by scientists, despite the fact that this animal species was incriminated as a source of contagion several times (10). In our country, the population of stray dogs and cats is high. These animals live in unsanitary conditions and in contact with rodents, the main reservoirs of the plague. To know if stray dogs and cats could carry this dangerous pathogen in Algiers, a region that has experienced outbreaks of plague in the past, a total of 267 spleens and 256 fleas collected from these animals were analyzed for bacterial DNA of *Y. pestis*. Our investigation was based on molecular methods targeting *pla* gene. Our negative results on cats suggest that they are not dangerous as a source of plague in Algiers during our period of study, as was the case during studies in some other parts of the world. However, we can think of the possibility that some cats died of plague before being captured or sampled. Exposure to infected cats with *Y. pestis* has been known for a long time, and can be a source of infection for humans through scratches, bites, or even contact (14). Primary pulmonary plague contracted in cats has become increasingly common. The first risk factor for feline plague appears to be the hunting and ingestion of dead rodents in endemic areas. The increase of stray cats is a risk factor for their acquisition of plague agent (6). Feline plague is the source of human infection in many parts of the world. During the outbreaks reported in 1982 in South Africa, it appears that at least one human case has contracted the plague of an infected domestic cat (15). Bubonic plague was transmitted to a 10-year-old girl in the United States by a scratch from a domestic cat. A total of 297 cases of feline plague were reported in the western United States between 1977 and 1998, of which 23 (7.7%) were associated with human cases (11). A plague epidemic has been reported in hunters in India, Joshi et al. (2009) reported that dead rodents and their fleas have not been reported or identified, suggesting that the cause was likely contact with domestic and feral cats (16). in six cats that had prior contact with wild rodents or rabbits (17).. Cats are the only known carnivores which can present the three forms of disease after exposure to *Y. pestis*. Orally infected cats exhibit the same symptoms as naturally infected cats (ingestion of infected rodents), with the appearance of numerous lesions in the head and neck ganglia. Cats can act as sentinels and develop antibodies that can be detected for several months. Serological screening for such an infection is particularly useful for epidemiological surveys or in the case of animal or human clinical suspicion (14). Serologic studies have been conducted in several regions of the world with different prevalence rates from one region to another, and they are summarized in Table 4. The close relationship between dogs and humans and the possibility of pathogens agents' transmission from dogs to humans calls for frequent assessment of potential zoonotic microbes in dogs (17-19). Despite the unsanitary conditions of the stray dog population and their close contact with rodents, the results on the dogs were all negative. During molecular research by Kassem et al. (2016) in the United States, on spleen and blood samples from five suspect dogs, the results were negative, suggesting that the dog is a plague resistant species (20). The role of the dog as a source of plague infection has not been well-understood. In endemic areas, the dog can play an important role in the epidemiology of this pathology. This role was noted during the epidemic reported in China in 2009 when a dead dog appeared to be the source of the infection (10). This epidemic was remarkable because it was the first outbreak of pulmonary plague in a canine species because dogs are considered to be naturally resistant to the disease despite the ingestion of infected rodents in endemic areas (21). Domestic dogs develop a relatively benign or asymptomatic form of plague infection but may be a source of human infection for their owners when exposed to infected wild animals. They can facilitate the transfer of infected fleas from plague-infected rats into homes (22). A study carried out in the USA between 2003 and 2011 on 62 dogs showed that dogs can develop moderate to severe illness and mortality. Contamination of dogs with *Y. pestis* occurred following hunting, exposure to rodents or rabbits and residence in rural areas (23). Domestic dogs appear to be very resistant to plague; however, they can develop high detectable antibody levels after several months without symptoms. Therefore, the dog is considered an indicator animal for plague surveillance, and the serological study can indicate the areas where the infection is active (10, 24). Several serological studies have been conducted in

different parts of the world (Table 5). The carnivore flea infestation studied in our survey indicated a high prevalence. It was 327 (47.25%) and 365 (52.74%) fleas in dogs and cats, respectively. This result suggests that ecological and climatic conditions, outdoor habitat, contact with other animals and the non-use of preventive or therapeutic antiparasitic are factors associated with a large exposure of stray dogs and cats from Algiers to arthropods vectors. However, the PCR results for *Y. pestis* in fleas were all negative. These results are consistent with the results obtained by Nyirenda et al. (2017), where 82 fleas were collected from dogs (*C. canis*) and rodents (*X. cheopis*) in Zambia and which were all negative (25).

Table 4. Seroprevalence surveys of feline *Yersinia pestis* infection

Region (Country)	Period of study	Number of sera collected	Positive		References
			Prevalence	%	
South Africa	1982	6	2	33,3%	(15)
Canada	1995	242	13	5,4%	(8)
USA	1979-1991	466	15	3,22%	(7)
China	2006	151	40	26,5%	(22)
Brazil	1997-2006	50 849	395	0,8%	(9)
Brazil	1990-2014	61 135	426	0.7%	(30)

Table 5. Seroprevalence surveys of canine *Yersinia pestis* infection

Geographic area	Year of study	Number of sera collected	Positive		References
			Prevalence	%	
South Africa	1982	5 938	21	0,35%	(15)
Tanzania	1980-1990	176	11	6,3%	(31)
USA	1979-1991	4 115	86	2,09%	(7)
Canada	1995	240	24	10%	(8)
Saudi Arabia	1997	17	3	17,6%	(32)
USA	2004-2005	183	5	2,7%	(33)
China	2006	689	162	23,5%	(22)
Brazil	1997-2006	95 883	2 234	2,3%	(9)
Madagascar	1999	63	15	23,8%	(24)
Brazil	2009	480	0	0%	(34)
Iran	2011-2012	117	4	3,42%	(35)
USA	2003-2011	62 sick	62	100%	(23)
China	__	226	49	21,6%	(10)
Zambia	2012-2013	165	5	3%	(12)
Brazil	1990-2014	203 311	3 023	1.5%	(30)

Although at least one flea was tested from each dog or cat in our study, Y. pestis can be missed when only subsets of fleas are tested because larger flea pools may harbor more plague bacilli than individual fleas alone. During the reappearance of human plague in Algeria in 2003, 95 *Xenopsylla cheopis* were collected from four species of rodents (*R. rattus*, *R. norvegicus*, *Mus musculus*, and *M. spretus*) trapped inside human residences and peridomestic in the area of Oran and Mascara. The *Y. pestis* DNA was detected by real-time PCR with primers against *Pla* gene. The positive results were confirmed and sequenced by the spacers YP8 and YP9. Nine fleas were infected with *Y. pestis* biovar orientalis (2). A molecular study carried out on populations of domestic fleas taken from animals in Benin, Tanzania and the Congo revealed a positivity of 3.25% (4/126) (26). Although many species of fleas can transmit *Y. pestis*, the most common and effective plague vector is the eastern rat flea Xenopsylla cheopis. The latter is more likely to spread the disease than other fleas because of its ability to block a blood meal containing Y. pestis in its proventriculus, permitting regurgitation of blood, carrying bacteria from the midgut or the proventriculus along with it, back into the bite site. (27). The cat flea (*Ctenocephalides felis*) and the dog flea (*Ctenocephalides canis*) can transmit the plague to humans (1, 28). However, they are considered poor vectors and rarely cause human disease. In the past, *C. felis* has been viewed as only a nuisance biting insect because limited laboratory studies have suggested that it is incapable of transmitting *Y. pestis* or is an inefficient vector (29). The *C. felis* is a common household and peri-domestic flea in parts of Africa and China where plague is endemic. In northwestern Uganda, China, and the United States, this flea has been reported as the most common flea in the human family environment (29). The *C. felis* feeds on various hosts, including humans; it also feeds on wild rodents, main reservoirs of *Y. pestis* and, therefore, may play a role in the transmission of plague to humans (28). The recent reappearance of several outbreaks of the plague remained silent for several decades, indicating that the eradication of an established plague outbreak can never be taken for granted. In the present study, we did not confirm the reservoir role of stray cats and dogs in the Algiers region in relation to the plague. This report showed that although the epidemiology of the plague is not unique, it follows different routes in different regions.

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Authors' Contribution

Study concept and design: Z.S., B.A. and H.D.
Acquisition of data: Z.S. and B.A.
Analysis and interpretation of data: Z.S. B.H.
Drafting of the manuscript: Z.S. and B.A.
Critical revision of the manuscript: B.A. and B.I.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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