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Zoonotic microorganisms in native and exotic invasive urban small mammals of bamako, Mali

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The increase in the incidence of zoonoses underlines the need for monitoring pathogens in wild animals. Recent studies have revealed the circulation of several microorganisms in rodents, in various geographic and environmental contexts, including African urban habitats. However, Mali, a landlocked country of West Africa, was not extensively studied for the circulation of the microorganisms in rodents. -borne zoonoses at a disadvantage. This is why we aimed through this study to improve knowledge of potentially zoonotic infectious agents carried by rodents in Mali (Bamako). Three hundred and seventy-one small mammal spleen samples taken from captures realized in 2021–2022 were analyzed by polymerase chain reaction. Eleven of them (i.e. 2.96%) were infected by microorganisms (*Bartonella* spp., *Coxiella burnetii* and *Trypanosoma otospermophili*). The most frequently detected microorganisms were *Bartonella* spp. (2.43%). W *B. elizabethae* (a species involved in some cases of infective endocarditis) and *B. mastomydis*. W *C. burnetii* MS type 12, thus showing active circulation of a human-pathogenic genotype of Q fever agent in wild rodents. For *Trypanosoma otospermophili* *Rattus norvegicus*.

For several decades now, humanity faces an increase in the emergence of infectious diseases whose negative impact on public health, global economy, and society in general has been growing steadily[1]. Numerous cases of hospitalization and death have been recorded because of these diseases. Indeed, according to the WHO, 39 million people living with HIV were registered at the end of 2022 and 630,000 people died during the same year due to HIV[2]. More than six million deaths followed the emergence of COVID-19 up to 2023 (https://data.who.int/dashboards/covid19/deaths?m49=001&n=c). Dengue is showing a worrying trend of progression in Africa. In 2023, more than 270,000 cases were reported across 18 African countries, resulting in 753 deaths (https://www.afro.who.int/countries/burkina-faso/publication/dengue-who-african-region-situation-report-01-19-december-2023). In the same year, 25 countries reported outbreaks of other arboviral diseases such as yellow fever, chikungunya, Rift Valley fever, and West Nile virus, with a total of 19,569 confirmed cases and 820 deaths[3].

Epidemics of Ebola, SARS, MERS, Zika, Lassa viruses, and many others have caused numerous deaths. They exemplify the ongoing emergence of large-scale, sometimes even pandemic, diseases and the significant threats they pose to public health. The world cannot remain indifferent to these threats; it is imperative to develop strategies to combat them. This begins with accurately identifying the reservoirs of microorganisms that cause infectious diseases[4]. Indeed, most of these microorganisms are known to have a zoonotic origin[5], and non-human

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primates, bats and rodents have been identified as major reservoirs for most of the zoonotic microorganisms that have emerged in recent decades[6].

Rodents are the world's most abundant mammals. They account for nearly 40% of all mammals[7]. They play a major role in the emergence and re-emergence of many infectious diseases. Hundreds of infectious microorganisms, including viruses, bacteria, parasites and fungi, have been discovered in rodents[8,9]. Their close proximity to humans, especially in dwellings and agricultural sites, makes them important links in the transmission of zoonoses to humans. This is why an increase in the incidence of zoonoses underlines the crucial need for real-time monitoring of pathogens in wild animals such as rodents, particularly in high-risk regional "hot spots" where new emerging infectious diseases have been reported[4,5].

In West Africa, in addition to the endemic Lassa hemorrhagic fever virus[10,11], many rodent-borne zoonotic pathogens have been identified. Recent studies carried out in Gabon[12] and Senegal[13–17] have revealed the circulation of several microorganisms in rodents, including *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *Anaplasma* spp., *Piroplasma* spp., *Hepatozoon* spp., *Trypanosoma* spp., *Leishmania* spp., etc. In addition, data obtained from screening of rodent-hosted parasitic arthropods may be biased by the detection of microorganisms genetically close to pathogens but associated with arthropods only. Mali, a landlocked country which borders Senegal and where Lassa haemorrhagic fever is endemic in the southern region[10], was not extensively studied for the circulation of microorganisms carried by rodents. Papers of Diarra et al.[18] and Schwan et al.[19] report different species of *Bartonella* detected in both small mammals and ectoparasites, whereas species of the *Anaplasmataceae* family and *C. burnetii* were detected exclusively in ectoparasites. A new genotype of the latter bacteria, causative agent of Q fever[20] was recently identified in wild rodents from Northern Senegal, relatively close to Mali borders, suggesting developing enzootic outbreak there[20].

Given the paucity of the information, the fight against zoonoses transmitted by rodents in Mali is disadvantaged. This is why we aimed through this study to improve knowledge of potentially zoonotic infectious agents carried by rodents in Mali, and more particularly in its capital, Bamako.

Results

Small mammal sampling

A selected set of three hundred and seventy-one (371) small mammals of six different species among the 1247 specimens that were captured in Bamako between October 2021 and October 2022 was subjected to analyses (S. Ag Atteynine et al., submitted). Four species (the house mouse *Mus musculus*, the brown rat *Rattus norvegicus*, the multimmmate mouse *Mastomys natalensis*, and the black rat *Rattus rattus*) are rodents of the family Muridae and one is from Nesomyidae (the giant rat *Cricetomys gambianus*). Shrews (*Crocidura olivieri*, Soricidae) were regularly captured at the same time with rodents.

Mus musculus, the most abundant species, represents 51.8% of the sample (192/371), followed by *R. norvegicus* (17.8%; 66/371), *C. olivieri* (11.6%; 43/371), *M. natalensis* (11.1%; 41/371), *R. rattus* (7.0%; 26/371) and *C. gambianus* (0.8%; 3/371). These proportions are similar to those observed in this small mammal community at the scale of the whole city. It is worth noting here that quantitatively, exotic invasive rodents (*M. musculus* and *Rattus* spp.) represent the majority (i.e. more the 75%) of the specimens in this sample, as well as in the urban community of small mammals of Bamako.

Molecular detection of microorganisms (Bacteria and Protozoa)

A total of 371 individual small mammal spleen samples were analysed in this study.

Eleven of them (i.e. 2.96%), all from rodents, were infected by microorganisms (*Bartonella* spp., *C. burnetii* and protozoa of the Kinetoplastida class). The most frequently detected microorganisms were *Bartonella* spp. (9/371; 2.43%). The frequency of detection of *C. burnetii* and Kinetoplastida was less than 1% (1/371 each) (Table 1).

Only one rodent of the species *R. norvegicus* showed a mixed infection with *Bartonella* spp. and Kinetoplastida. *Bartonella* spp. were mainly detected in *R. norvegicus* (6/66; 9.09%) but also in *C. gambianus* where 2 of the 3

			Mammal species						
	Microorganism taxa	Target gene	***Cricetomys gambianus*** *N* = 3*	***Crocidura olivieri*** *N* = 43*	***Mastomys natalensis*** *N* = 41*	***Mus musculus*** *N* = 192*	***Rattus norvegicus*** *N* = 66*	***Rattus rattus*** *N* = 26*	**Total** *N* = 371*
qPCR/PCR	***Coxiella burnetii***	IS1111	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3,85%)	1 (0,27%)
		IS30A							
	Borrelia spp.	16 S	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Bartonella spp.	ITS	**2** (66,67%)	**1** (2,33%)	0 (0%)	0 (0%)	**6** (9,09%)	0 (0%)	9 (2,43%)
	Anaplasmatacea	23 S	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Piroplasma	5.8 S	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Pan-Kinetoplastida	**28 S**	**0 (0%)**	**0 (0%)**	**0 (0%)**	**0 (0%)**	**1 (1,52%)**	0 (0%)	**1 (0,27%)**
PCR	*Hepatozoon*	18 S (620-bp)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Total		2 (66,67%)	1 (2,33%)	0 (0%)	0 (0%)	**7 (10,61%)**	1 (3,85%)	**11 (2,96%)**

Table 1. Different infectious agents identified in this study and their host rodent species. * - the total number of animals collected in this study.

individuals tested were found positive, and in *C. olivieri* (1/43; 2.33%). *C. burnetii* and Kinetoplastida were exclusively detected in *R. rattus* and *R. norvegicus*, respectively. The highest infection rate was observed in *R. norvegicus* where 7/66 (10.61%) of the individuals tested were found positive for microorganisms. Regarding *Bartonella*, PCRs targeting the ITS, *rpoB* and *ftsZ* genes provided nine, six and two informative partial sequences, respectively, at the end of the sequencing. The result of the BLAST analysis of the sequences of the ITS, *rpoB* and *ftsZ* genes allowed us to obtain a total of three groups of different sequences of these three genes. In view of the low inter-species similarity value of the *rpoB* gene compared to other genes (ITS, *ftsZ*) as described by La Scola (21), the *rpoB* gene sequences has been selected to identify the new genotypes (PP264222-PP264225 and PP264227). Indeed, three new genotypes of *B. elizabethae* (PP264223-PP264225), a known genotype of *B. elizabethae* (PP264226) and two new genotypes of *B. mastomydis* (PP264222 and PP264227) were found (Table 2).

The phylogenetic position of the *Bartonella* species identified by the *rpoB* gene in our study is presented in Fig. 1.

We were unable to amplify the *rpoB* and *ftsZ* genes on one spleen sample of the *C. olivieri* specimen previously tested positive for ITS. Furthermore, it should be noted that the sequencing of spleen samples of two *R. norvegicus* previously tested positive for ITS and *rpoB* has failed. Analyses of these three DNA sequences obtained with the 16–23 S intergenic spacer (ITS) revealed genotypes with homologies of 87.01%, 90.20% and 99.34% respectively with *Bartonella florencae* strain R4 from France 16–23 S ribosomal RNA intergenic spacer, partial sequence, *Bartonella tribocorum* main chromosome complete genome, strain BM1374166 and *B. elizabethae* strain NCTC12898 genome assembly, chromosome: 1, respectively. We failed to amplify the *gltA* gene in all nine samples previously tested positive for ITS.

C. burnetii DNA was detected in one black rat spleen sample by qPCR with primers and probes targeting IS1111 and IS30A spacers. To identify the genotype, we amplified six pairs of intergenic spacer primers, Cox2F/R, Cox5F/R, Cox18F/R, Cox56F/R, Cox57F/R, and Cox61F/R. Multispacer sequence typing (MST) genotyping of *C. burnetii* strains[22] (https://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/blast.html) using sequences from the amplification of these six primer pairs revealed *C. burnetii* MST group 12.

To identify the Kinetoplastida species found in one brown rat sample, we used a broad species PCR tool targeting the 28 S gene. This *R. norvegicus* DNA sample was successfully amplified and sequenced. The blast analysis of reverse strand of 392-bps-long portion were 100% identical to *Trypanosoma otospermophili* clone 14RD1608 large subunit ribosomal RNA gene, partial sequence (MT271797.1). It should be noted that this same sample showed a *B. elizabethae* / *T. otospermophili* mixed infection.

Discussion

Through this study, we wanted to contribute to improving knowledge of blood-borne microorganisms circulation in small mammals in Mali, and more precisely in its capital city, Bamako. We recruited 371 rodents from which 371 spleen samples were taken. Of the seven pathogens tested, three (*Bartonella* spp., *C. burnetii* and Kinetoplastida sp.) were detected by qPCR. They were then amplified by conventional PCR and sequenced for identification. Less than 3% (11/371) of the small mammals analyzed were infected by at least one of the three microorganisms detected. This result is difficult to compare with the results of other studies in the region, given that several studies based on epidemiological investigation of communities of microorganisms (bacteria, parasites, viruses, etc.) circulating in small mammals have not mentioned overall infection rates. Examples include the studies carried out in Mali in 2020[18], in Senegal in 2020[14], and Tunisia in 2021[23]. However, compared with one of the very few studies to have these data in the region, in Senegal in 2023[13], the infection rate obtained in our study is much lower. It is also much lower than the rate obtained in Germany in 2019[24]. The difference in rates observed between this study and ours could be due to the fact that the rodent species included in this study were different from those in our study. We could speculate that the species included in our study, and especially *M. musculus* which dominates in our sample, would have a less diverse and smaller pool of microorganisms than the other species. However, due to lack of evidence through exhaustive studies, we cannot confirm this. Furthermore, this difference could be because most of the specimens of small mammals on which the microorganisms were detected in our study belong to exotic invasive species. As such, the latter would have a lower reserve of microorganisms than the native species living in Europe: They may have lost some of their native microorganisms during introduction / invasion processes, and at the same time the colonization of these species by microorganisms from the regions where they are introduced would not compensate for the loss of indigenous microorganisms[25].

In this study, *Bartonella* spp. was the most frequently detected microorganism, with a prevalence rate of 2.43% (9/371). This prevalence is similar to the ones recorded in studies carried out in the Ferlo region of Senegal in 2020[14] and in 2023[13], and in Franceville, Gabon in 2021[12], where conventional PCR results suggested rates of 5.85% (10/171), 4% (5/125) and 2.53% (5/198), respectively. In contrast, our results differ from those obtained in Germany[24], Germany and the Czech Republic[26], which describe prevalence rates of 78.2% (129/165) and 67.3% (216/321), respectively. In view of the argument presented above, it would be logical that the prevalence of *Bartonella* among invasive exotic species in Africa is much lower than that described among native species in Europe. However, an exhaustive comparative study should be carried out in order to provide more information and confirm this observation.

Analysis of the six DNA sequences obtained with the *rpoB* genes revealed sequences closely related to either *B. elizabethae* or *B. mastomydis*. Three new *B. elizabethae* genotypes detected in three rodents (two *R. norvegicus* and one *C. gambianus*) each showed homologies of 99.47%, 99.14% and 96.13% with *B. elizabethae* strain NCTC12898 genome assembly. In a fourth rodent (*R. norvegicus*), the *Bartonella* sequence detected showed 100% homology with *B. elizabethae* strain THSKR-008. These results are supported by the fact that, like the identification of *B. elizabethae* as the cause of human endocarditis[27], this species had previously been identified

Sample number	Small mammal species	Target gene	bps	Quality	Closest sequence	Accession	Geographic origin	Per. Ident	Isolation source	Closest officially recognized species, geographic origin	Per. Ident	Conclusion *rpoB*
LG1645 (300)	*Rattus norvegicus*	*rpoB*	885	partial	*UnculturedBartonellasp. clone F RNA polymerase beta subunit (rpoB) gene, partial cds*	JQ425630.1	Ethiopia	100%	*Mastomys awashensis*	*Bartonella mastomydis strain 008, Senegal*	99.41%	**New genotype ofB. *mastomydis***
LG1991 (320)	*Rattus norvegicus*	*rpoB*	567	partial	*UnculturedBartonellasp. clone TNF20-01 RpoB (rpoB) gene, partial cds*	*MZ835681.1*	Taiwan	100%	*Xenopsylla cheopis*	*Bartonella elizabethae strain NCTC12898, USA*	99.47%	**New genotype ofB. *elizabethae***
LG2097 (327)	*Rattus norvegicus*	*rpoB*	856	partial	*UnculturedBartonellasp. clone TNF20-01 RpoB (rpoB) gene, partial cds*	*MZ835681.1*	Taiwan	100%	*Xenopsylla cheopis*	*Bartonella elizabethae strain THSKR-008, Thailand*	100%	***B. elizabethae***
LG1741 (363)	*Cricetomys gambianus*	*rpoB*	899	partial	*UnculturedBartonellasp. clone G RNA polymerase beta subunit (rpoB) gene, partial cds*	*JQ425631.1*	Ethiopia	97.39%	*Stenocephalemys albipes*	*Bartonella mastomydis strain 008, Senegal*	96.87%	**New genotype ofB. *mastomydis***
LG1651 (301)	*Rattus norvegicus*	*rpoB*	408	partial	*UnculturedBartonellasp. clone TNF20-01 RpoB (rpoB) gene, partial cds*	*MZ835681.1*	Taiwan	99.71%	*Xenopsylla cheopis*	*Bartonella elizabethae strain NCTC12898, USA*	99.14%	**New genotype ofB. *elizabethae***
LG2022 (364)	*Cricetomys gambianus*	*rpoB*	853	partial	*Bartonellasp. KS4-1 rpoB gene for RNA polymerase beta subunit, partial cds*	*AB529943.1*	Japon	97.45%	*Acomys russatus*	*Bartonella elizabethae strain NCTC12898, USA*	96.13%	**New genotype ofB. *elizabethae***

Table 2. Identification of *Bartonella* species in small mammal samples based on *rpoB* gene sequencing.

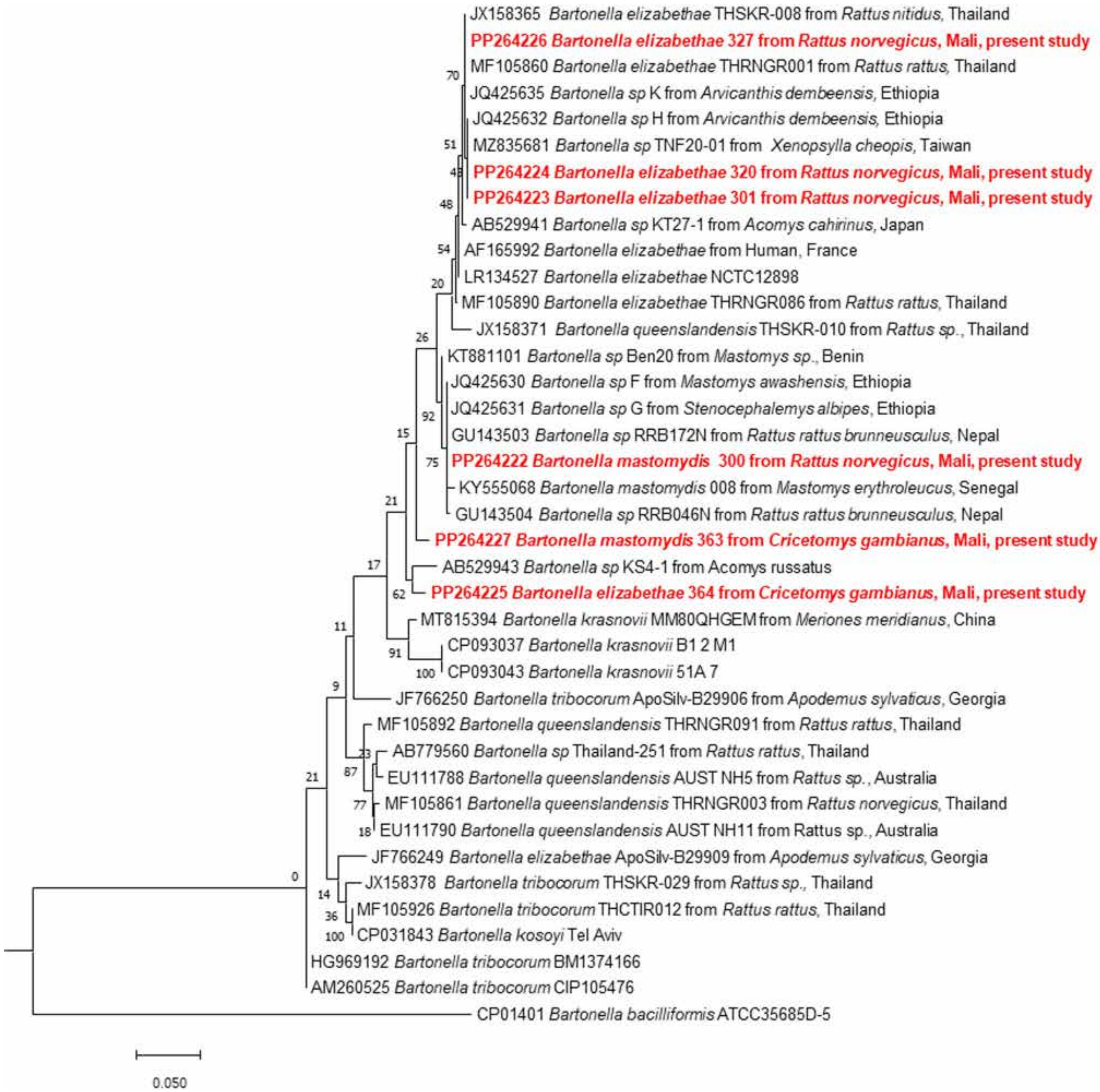

Fig. 1. Maximum-likelihood phylogenetic tree of the *rpoB* gene portion of *Bartonella* genus bacteria showing the position of the identified *Bartonella* sequences obtained from different rodents. The percentage of trees in which the associated taxa clustered together is shown next to the branches. GenBank accession numbers are provided. Scale bar indicates nucleotide sequence divergence.

in small mammals[27]. It is a species that circulates in rodents, mostly in rats. Species similar to *B. elizabethae* also infect other genera of rats, shrews and gerbils[28]. *B. elizabethae* has a worldwide distribution, reflecting its close host–pathogen relationship with rats. As rats are invasive species found across the globe and tend to follow human populations, it is not surprising that associated *Bartonella* strains from different regions exhibit close genetic similarities. This appears to be the case for *B. elizabethae* and its rat hosts: as shown in the phylogenetic tree, the *B. elizabethae* strains identified in our study are genetically similar to strains from various parts of the world, including Asia, Europe, and the Americas.

B. elizabethae is a relatively rare species in humans but is considered potentially pathogenic. It has been isolated from a patient with infectious endocarditis, confirming its ability to cause cardiovascular infections[29].

Two new genotypes of *B. mastomydis* with homologies of 99.41% and 96.87% with *B. mastomydis* strain 008 were detected in *R. norvegicus* and *C. gambianus* respectively. This result reveals the participation of *R. norvegicus* and *C. gambianus* in the circulation of new *Bartonella* genotypes. Indeed, the role of *C. gambianus* as a reservoir of *Bartonella* was recently mentioned in a study carried out in Dakar, Senegal in 2022[30].

B. mastomydis has not been associated with any human cases to date. It was recently described in West Africa from the rodent *Mastomys erythroleucus*, and no link to human disease has been established[31].

Analyses of three DNA sequences obtained with the 16–23 S intergenic spacer (ITS) revealed genotypes with homologies of 87.01%, 90.20% and 99.34% respectively with *B. florencae* strain R4 from shrew in France, *B. tribocorum* strain BM1374166 and *B. elizabethae* strain NCTC12898 respectively. It should be noted, however, that these results are not sufficient to identify these genotypes as new species in accordance with the criteria for describing new *Bartonella* species described by La Scola et al.[21].

The detection of different types of *Bartonella* genotype in this study concur with the fact that species of the *Bartonella* genus are characterized by a diversity of genotypes carried by small mammals[28,32].

In this study, we found only one rodent (*R. rattus*) out of 371 small mammals tested (0.27%) infected with *C. burnetii*. This result is similar to that of a study carried out in the Ferlo region of Senegal in 2020[14], which was unable to detect *C. burnetii* in the following species of the Muridae family: *Arvicanthis niloticus*, *Gerbillus nigeriae*, *Taterillus* sp., *Mastomys erythroleucus*, *Mus musculus*. The prevalence obtained in our study is also close to that obtained previously in Mali (prevalence rate 2.3%)[18]. Conversely, it should be noted that in 2023 in the Ferlo region in Senegal (neighboring of the Kayes region in Mali)[20] a prevalence rate of 19.2% in a rodent community made up of the following species: *A. niloticus*, *Desmodilliscus braueri*, *Gerbillus nancillus*, *G. nigeriae*, *Jaculus jaculus*, *Taterillus* sp., and *Xerus erythropus*. All these rodents were infected by the same new genotype of *C. burnetii*. We did not identify this new genotype in small mammals from Bamako, the only genotype identified in *R. rattus* corresponding to the previously described genotype 12. Thus, we believe that the epizootic outbreak of *C. burnetii* infection in Ferlo, Senegal in 2023[20] is probably local and has not (yet?) extended in Mali. Such discordance in prevalence corresponds to previous observations that *C. burnetii* infection rates in domestic and wild animals vary from place to place[33].

It should be noted that genotype 12 of *C. burnetii* had previously been detected in Algeria in cow's milk[34,35] and in Italy in cheese produced from ewe's, goat's and bovine milk[35–37]. It had also been identified in human clinical samples (in Switzerland, France and Senegal)[34,35,37,38]. However, to our knowledge, this is the first time that the MST12 genotype has been identified in rodents.

Since the beginning of studies on Q fever, various rodent models have proved highly susceptible to infection[39]. Despite this fact, wild rodents have not often been considered important reservoirs of Q fever. Several studies suggest, however, that rodents are potential sources of human infection such as capybaras in French Guiana[40]. In Egypt, rats seem to be an important excreting reservoir of Q fever harboring *C. burnetii* genotypes almost identical to those isolated from humans[41]. This would not exclude the hypothesis that the *R. rattus* tested positive for *C. burnetii* MST12 in our study could be a reservoir of human Q fever in Mali.

Here, *T. otospermophili* was also identified in a rodent of the species *R. norvegicus*. In fact, *T. otospermohili* is a species of the *Trypanosoma* genus[42]. This result matches those of a study carried out in Gabon[12], that identified *T. otospermophili* in *R. rattus*. In Mali, *Trypanosoma lewisi* was detected in *R. rattus* as well as in *Mastomys natalensis*[43], . Our study has the merit of having identified *T. otospermophili* in a rodent in Mali for the first time. This microorganism also parasitizes ground squirrels (*Spermophilus* spp.) in North America, Europe and Asia[44]. To date, no evidence of its pathogenicity in humans has been demonstrated.

In conclusion, identification of a MS type 12 of *C. burnetii* in *R. rattus* is an important finding showing active circulation of human-pathogenic genotype of Q fever agent in wild rodents. Permanent presence of Q fever reservoir in such a big city as Bamako may present an important sanitary threat. Therefore, monitoring should be organized to determine whether this genotype is circulating in Bamako.

We identified new genotypes of *B. elizabethae and B. mastomydis* in rodents. It is important to note that in the absence of clear evidence, we cannot say whether these new genotypes detected in rodents in Bamako are pathogenic for humans. However, in view of the role of *B. elizabethae* in the occurrence of certain cases of infective endocarditis in humans[45,27], epidemiological surveillance of this bacterium is important.

We also note that for the first time in Mali, *T. otospermophili* was identified in a specimen of brown rat *R. norvegicus*. *T. otospermophili* is neither common nor currently recognized as pathogenic in humans. To date, no human infections have been documented in the scientific literature, and its role in human and animal pathology remains to be elucidated.

Understanding some limitations of the present study we encourage performing a large-scale continuous screening of rodents in African cities in order to clarify their potential roles as reservoir of emerging human infections. Interactions between exotic invasive and native species should also be followed, to check for their potential effects on zoonotic agents exchange and circulation.

Materials and methods

Study area and samples collection

The "Small mammals of Bamako: inventory, distribution determinants, hosted parasites" project (including the trapping of small mammals, their sacrifice and autopsy for biological samples intended for any genetic or epidemiological study) was approved by the ethics committee of the National Institute of Public Health of Mali (decision no. 18/2021 / CE-INSP). It also received prior informed consent, in accordance with the provisions of the Nagoya protocol signed by Mali, from the National Director of Water and Forests of Mali (letter 0976/MEADD-DNEF of November 25, 2021).

The study area corresponds to the district of Bamako in southwest Mali. The samples analysed here were taken from captures realized between October 2021 and October 2022 in various quarters of the six communes of the city. Elements of the trapping procedures followed here have already been described elsewhere[46]. Twelve quarters were sampled as representative of the cityscape diversity in terms of urbanization and socio-economic characteristics, without being too close from each other in order to ensure good spatial coverage of the city (Fig. 2). Captured small mammals were identified following keys provided in Granjon & Duplantier (2009)[47], euthanized

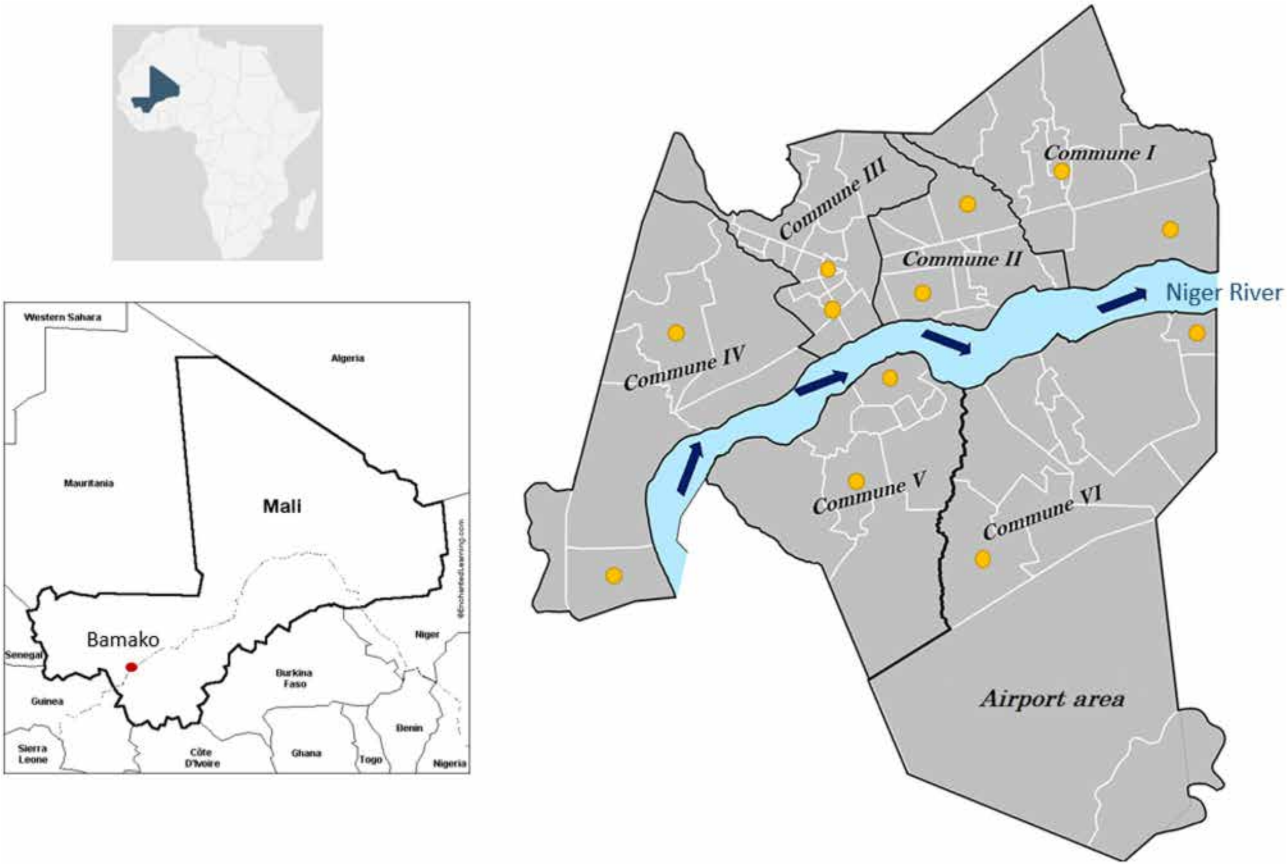

Fig. 2. Map of Mali and the district of Bamako. Yellow dots point to the quarters of Bamako district sampled for their small mammals.

by cervical dislocation and handled in accordance with the relevant requirements of Malian legislation and the live animal capture guidelines of the American Society of Mammalogists[48]. Body measurements were taken before autopsy and reproductive status was noted. Tissues, including spleen, were preserved in 95% ethanol for further analyses. We chose to use spleen samples because the spleen of small mammals, particularly rodents, is a key organ for detecting zoonotic pathogens, due to its central role in immune function and its capacity to accumulate blood-borne microorganisms. Its accessibility and usefulness in large-scale field studies make it a valuable tool for epidemiological surveillance and understanding host-pathogen dynamics.

Molecular detection

DNA extraction

For each spleen, a small piece (10 mg) was crushed and incubated for at least four hours with proteinase K. DNA extraction was performed using NucleoMag Tissue kit for DNA purification from cells and tissue (Macherey-Nagel, France), according to the manufacturer's protocol. DNA extracts were then stored at -20 °C until PCR analysis.

PCR amplification

Seven groups of blood-borne pathogens, most of them zoonotic, have been screened: *Bartonella* spp., *C. burnetii*, *Borrelia* spp., *Anaplasmataceae* spp., *Piroplasma* spp., *Hepatozoon* spp., and *Kinetoplastida*.

The initial screening of samples was performed using qPCR systems with wide specificity (genus or family-specific) (Table 3). For Real-Time PCR, the reaction mix contained 5 µL of the DNA template, 10 µL of the Roche master mix (Roche Applied Science, Mannheim, Germany), 0.5 µL (20 µM) of each reverse and forward primers, 0.5 µL (5 µM) of the FAM-labeled probe), 0.5 µL of uracil DNA glycosylase (UDG), and 3 µL of distilled water DNAse and RNAse free, for a final volume of 20 µL. The real-time qPCR amplification was carried out in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA) using the following thermal profile: incubation at 50 °C for two minutes for UDG action (eliminating PCR amplicons' contaminant), then an activation step at 95 °C for three minutes followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing-extension at 60 °C for 30 s. With the exception of *C. burnetii* for which confirmation of a positive sample was obtained after two qPCRs (IS1111 and IS30A), for all systems, any sample presenting a cycle threshold (Ct) value less than or equal to 33 Ct was analyzed by conventional PCR for confirmation of positivity.

Target Organism	Target sequence	Technique	Name	Primer and probe sequences (5'-3')	Annealing Temperature	Reference
Bartonella sp.	ITS (Intergenic 16–23 S)	Broad-range qPCR	Barto_ITS3_F Barto_ITS3_R Barto_ITS3_P	GATGCCGGGGAAGGTTTTCGCCTGGGAGGACTTGAAC CT FAM-GCGCGCGCTTGATAAGCGTG	60 °C[49]	
	ITS (733 bp) (Intergenic 16–23 S)	Broad-range conventional PCR	Urbarto1 Urbarto2	CTTCGTTTCTCTTTCTTCACTTCTCTTCACAATTTCAAT	50 °C[50]	
	rpoB (900 bp)	Broad-range conventional PCR Heminested PCR	rpoB1400F rpoB2300R rpoB1596R rpoB2028F rpoB1873R	CGCATTGGCTTACTTCGTATGGTAGACTGATTAGAAC GCTGGGACAAATACGACCATAATGCG (for sequencing) GGAAAATGATGATGCGAATCGTGC (for sequencing) TCYTCCATMGCWGAMAGATAAA (for sequencing)	48 °C[51]	xx
	gltA (1017 bp)	Broad-range conventional PCR	BgltA1F BgltA2R	TTACTTATGATCCKGGYTTTA AATGCAAAAAGAACAG TAAACA	42 °C[52]	xx
	ftsZ (915 bp)	Broad-range conventional PCR	BftsZ1F BftsZ2R BftsZ3F BftsZ4R	ATTAATCTGCAYCGGCCAGA ACVGADACACGAATAACACC TTACAAAAATCYGTTGATAC (for sequencing) GTATCAACRGATTTTTGTAA (for sequencing)	55 °C	
Coxiella burnetii	IS1111	Broad-range qPCR	CB_IS1111_0706F CB_IS1111_0706R CB_IS1111_0706P	CAAGAAACGTATCGCTGTGGCCACAGAGCCACCGTATG AATC FAM-CCGAGTTCGAAACAATGAGGGCTG	60 °C[53]	(XX)
	IS30A	Broad-range qPCR	CB_IS1111_0706P CB_IS30A_3R CB_IS30A_3P	CGCTGACCTACAGAAATATGTCCGGGGTAAGTAAATA ATACCTTCTGG FAM-CATGAAGCGATTTATCAATACGT GTATGC	60 °C[53]	(XX)
	Cox2 (397 bp) (intergenic spacer)	Broad-range conventional PCR	COX2_20766 COX2_21004	CAACCCTGAATACCCAAGGAGAAGCTTCTGATAGGC GGGA	57 °C[54]	xx
	Cox5 (395 bp) (intergenic spacer)	Broad-range conventional PCR	COX5_77554 COX5_77808	CAGGAGCAAGCTTGAATGCGTGGTATGACAACCCGT CATG	57 °C[54]	
	Cox18 (557 bp) (intergenic spacer)	Broad-range conventional PCR	COX18_283060 COX18_283490	CGCAGACGAATTAGCCAATCTTCGATGATCCGATGG CCTT	57 °C[54]	
	Cox56 (479 bp) (intergenic spacer)	Broad-range conventional PCR	COX56_886418 COX56_886784	CCAAGCTCTCTGTGCCCAATATGCGCCAGAAACGCA TAGG	57 °C[54]	
	Cox57 (617 bp) (intergenic spacer)	Broad-range conventional PCR	COX57_892828 COX57_893316	TGGAAATGGAAGGCGGATTCGGTGGAAGGCGTAAGC CTTT	57 °C[54]	
	Cox61 (611 bp) (intergenic spacer)	Broad-range conventional PCR	COX61_956825 COX61_957249	GAAGATAGAGCGGCAAGGATGGGATTTCAACTTCCG ATAGA	57 °C[54]	xx
Borrelia sp.	16 S	Broad-range qPCR	Bor_16S_3F Bor_16S_3R Bor_16S_3P	AGCCTTTAAAGCTTCGCTTGTAGGCCTCCCGTAGGAGT CTGG FAM-CCGGCCTGAGAGGGTGAACGG	60 °C[55]	(XX)
Anaplasmataceae	23 S	Broad-range qPCR	TtAna_F TtAna_R TtAna_P	TGACAGCGTACCTTTTGCATGTAACAGGTTCGGTCCTC CA FAM-GGATTAGACCCGAAACCAAG	55 °C[56]	xx
	23 S (960 bp)	Broad-range conventional PCR Hemisted PCR	Ana23S-212 F Ana23S-753 R Ana23S-908R	ATAAGCTGCGGGGAATTGTCTGCAAAAGGTACGCTGTC ACGTAACAGGTTCGGTCCTCCA	58 °C[56]	xx
Piroplasma sp.	5.8 S	Broad-range qPCR	5.8 S-F5 5.8 S-R1 5.8 S-P	AYYKTYAGCGRTGGATGTC TCGCAGRAGTCTKCAAGTC FAM-TTYGCTGCGTCCTTCATCGTTGT	60 °C[57]	(XX)
Pan-Kinetoplastida	28 S LSU (24 alpha)	Broad-range qPCR	F. 24a; 5198 R. 24a; 5412 P. 24a; 5345	AGTATTGAGCCAAAGAAGGTTGTCACGACTTCAGGTTC TAT FAM-TAGGAAGACCGATAGCGAACAAGTAG	60 °C[58]	(XX)
	28 S LSU (550 bp) (24 alpha)	Broad-range conventional PCR	F2 28 S R1 28 S	ACCAAGGAGTCAAACAGACGGACGCCACATATCCCT AAG	53 °C[58]	(XX)
Hepatozoon	18 S (620 bp)	Broad-range conventional PCR	H14Hepa18SFw H14Hepa18SRv	GAAATAACAATACAAGGCAGTTAAAATGCTGTGCTGAA GGAGTCGTTTATAAAGA	58 °C[59]	(XX)

Table 3. Oligonucleotide sequences of primers and probes used for real-time PCR and conventional PCR to detect and identify bacteria and protozoa in this study.

Amplification of these samples by conventional PCR was carried out using an automated DNA thermal cycler (GeneAmp PCR Systems Applied Biosystems, Courtaboeuf, France). PCR reactions contained 5 µL of the DNA template, 12.5 µL of Ampli Taq Gold master mix, 0.75 µL of each primer (20 µM) and 6 µL of distilled water DNAse and RNAse free. The conditions for conventional PCR were as follows: one incubation step at 95 °C for 15 min, 35 cycles of 30 s at 95 °C, 30 s annealing at a different hybridization temperature for each PCR assay, and one minute at 72 °C, followed by a final extension for five minutes at 72 °C (Table 3). Negative and positive controls were included in each molecular assay. The success of amplification was confirmed by electrophoresis on a 1.5% agarose gel. We considered positive PCR product when the specific DNA band of interest was observed in the transilluminator. The positive PCR product was purified using NucleoFast 96 PCR plates (Macherey–Nagel, Hoerdt, France) according to the manufacturer's instructions.

We selected two genetic markers for improved characterization of *Bartonella* spp. identified by qPCR: the *rpoB* gene and the ITS region, both of which are routinely used for *Bartonella* identification and characterization[21,60].

Sequencing and phylogenetic analysis

The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The obtained sequences were assembled and edited using ChromasPro software (ChromasPro 1.7.7, Technelysium Pty Ltd., Tewantin, Australia). Then, the sequences were compared with those available in the GenBank database by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Evolutionary analyses were conducted using TOPALi version 2.5 (http://www.topali.org). The sequences of the *rpoB* genes amplified in this study with other *Bartonella rpoB* sequences available on GenBank (852 positions in the final dataset) were aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw) implemented on BioEdit version 3 (https://bioedit.software.informer.com). The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa–Kishino–Yano model plus invariant sites plus gamma distribution.

Data availability

The datasets generated and analysed during the current study are available in the Genbank repository with following accession codes: PP264222, PP264223, PP264224, PP264225, PP264226, PP264227Links : https://www.ncbi.nlm.nih.gov/nuccore/PP264222.1?report=GenBank https://www.ncbi.nlm.nih.gov/nuccore/PP264223.1?report=GenBank https://www.ncbi.nlm.nih.gov/nuccore/PP264224.1?report=GenBank https://www.ncbi.nlm.nih.gov/nuccore/PP264225.1?report=GenBank https://www.ncbi.nlm.nih.gov/nuccore/PP264226.1?report=GenBank https://www.ncbi.nlm.nih.gov/nuccore/PP264227.1?report=GenBank. This study was conducted and reported in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) available at https://arriveguidelines.org, and in compliance with applicable ethical standards for research involving animals.

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Author contributions
CK wrote the main manuscript. CK, LG and JM contributed to the drafting of the methodology. All authors reviewed the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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