16S rRNA amplicon sequencing for epidemiological surveys of bacteria in wildlife: the importance of cleaning post-sequencing data before estimating positivity, prevalence and co-infection

Maxime Galan[1], Maria Razzauti[1], Emilie Bard[2], Maria Bernard[3,4], Carine Brouat[5], Nathalie Charbonnel[1], Alexandre Dehne-Garcia[1], Anne Loiseau[1], Caroline Tatard[1], Lucie Tamisier[1], Muriel Vayssier-Taussat[6], Hélène Vignes[7], Jean-François Cosson[1,6]

1: INRA, CBGP, Montferrier sur Lez, France

2: INRA, EpiA, Clermont-Ferrand, France

3: INRA, Sigenae, France

4 : INRA, GABI, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France

5: Ird, CBGP, Montferrier sur Lez, France

6: INRA, Bipar, Maisons-Alfort, France

7: CIRAD, AGAP, Montpellier, France

Corresponding authors: galan@supagro.inra.fr; cosson@supagro.ira.fr

Importance

Several recent public health crises have shown that the surveillance of zoonotic agents in wildlife is important to prevent pandemic risks. Rodents are intermediate hosts for numerous zoonotic bacteria. High-throughput sequencing (HTS) technologies are very useful for the detection and surveillance of zoonotic bacteria, but rigorous experimental processes are required for the use of these cheap and effective tools in such epidemiological contexts. In particular, HTS introduces biases into the raw dataset that might lead to incorrect interpretations. We describe here a procedure for cleaning data before estimating reliable biological parameters, such as bacterial positivity, prevalence and coinfection, by 16S rRNA amplicon sequencing on the MiSeq platform. This procedure, applied to 711 commensal rodents collected from 24 villages in Senegal, Africa, detected several emerging bacterial genera, some in high prevalence, while never before reported for West Africa. This study constitutes a step towards the use of HTS to improve our understanding of the risk of zoonotic disease transmission posed by wildlife, by providing a new strategy for the

use of HTS platforms to monitor both bacterial diversity and infection dynamics in wildlife. In the future, this approach could be adapted for the monitoring of other microbes such as protists, fungi, and even viruses.

Summary

Human impact on natural habitats is increasing the complexity of human-wildlife interfaces and leading to the emergence of infectious diseases worldwide. Highly successful synanthropic wildlife species, such as rodents, will undoubtedly play an increasingly important role in transmitting zoonotic diseases. We investigated the potential of recent developments in 16S rRNA amplicon sequencing to facilitate the multiplexing of large numbers of samples, to improve our understanding of the risk of zoonotic disease transmission posed by urban rodents in West Africa. In addition to listing pathogenic bacteria in wild populations, as in other high-throughput sequencing (HTS) studies, our approach can estimate essential parameters for studies of zoonotic risk, such as prevalence and patterns of coinfection within individual hosts. However, the estimation of these parameters requires cleaning of the raw data to eliminate the biases generated by HTS methods. We present here an extensive review of these biases and of their consequences, and we propose a trimming strategy for managing them and cleaning the dataset. We also analyzed 711 commensal rodents collected from 24 villages in Senegal, including 208 *Mus musculus domesticus,* 189 *Rattus rattus*, 93 *Mastomys natalensis* and 221 *Mastomys erythroleucus*. Seven major genera of pathogenic bacteria were detected: *Borrelia*, *Bartonella*, *Mycoplasma, Ehrlichia*, *Rickettsia*, *Streptobacillus* and *Orientia*. The last five of these genera have never before been detected in West African rodents. Bacterial prevalence ranged from 0% to 90%, depending on the bacterial taxon, rodent species and site considered, and a mean of 26% of rodents displayed coinfection. The 16S rRNA amplicon sequencing strategy presented here has the advantage over other molecular surveillance tools of dealing with a large spectrum of bacterial pathogens without requiring assumptions about their presence in the samples. This approach is, thus, particularly suitable for continuous pathogen surveillance in the framework of disease monitoring programs

65 # Introduction

66 Pathogen monitoring in wildlife is a key method for preventing the emergence of
67 infectious diseases in humans and domestic animals. More than half the pathogens
68 causing disease in humans originate from animal species [1]. The early identification
69 of zoonotic agents in animal populations is therefore of considerable human health
70 interest. Wildlife species may also act as a reservoir for pathogens capable of
71 infecting livestock, with significant economic consequences [2]. The monitoring of
72 emerging diseases in natural populations is also important for preserving biodiversity,
73 because pathogens carried by invasive species may cause the decline of endemic
74 species [3]. There is, therefore, a need to develop screening tools for identifying a
75 broad range of pathogens in samples consisting of large numbers of individual hosts
76 or vectors.

77 Modern high-throughput sequencing (HTS) approaches require no prior assumptions
78 about the bacterial communities present in samples of diverse natures, including
79 non-cultivable bacteria. Such metagenomics approaches are based on the
80 sequencing of all (WGS: whole-genome sequencing) or some (RNAseq or 16S rRNA
81 amplicon sequencing) of the bacterial DNA or RNA in a sample, with the sequences
82 obtained then compared with those in a reference sequence database [4].
83 Metagenomics has made a major contribution to the generation of comprehensive
84 inventories of the bacteria, including pathogens, present in humans [5]. Such
85 approaches are now being extended to the characterization of bacteria in wildlife [6-
86 13, 90]. However, improvements in the estimation of infectious risks will require more
87 than just the detection of bacterial pathogens. Indeed, we will also need to estimate
88 the prevalence of these pathogens by host taxon and/or environmental features,
89 together with coinfection rates [14,15] and pathogen interactions [16,17].

90 Razzauti *et al*. [8] recently used 16S rRNA amplicon sequencing with the dual-index
91 sequencing strategy of Kozich *et al*. [18] to detect bacterial pathogens in very large
92 numbers of rodent samples (up to several hundred samples in a single run) on the
93 MiSeq Illumina sequencing platform. The 16S rRNA amplicon sequencing technique
94 is based on the amplification of small fragments of the hypervariable region of the
95 16S rRNA gene. The sequences of these fragments are then obtained and compared
96 with those in a dedicated database, for taxonomic identification [4,19]. Multiplexed

97 approaches of this kind include short indices (or tags) specific to a PCR product. This
98 makes it possible to assign the sequences generated by the HTS run to a particular
99 sample following bioinformatic analysis of the dataset [18]. Razzauti *et al.* [8]
100 demonstrated the considerable potential of this approach for determining the
101 prevalence of bacteria within populations and for analyzing bacterial interactions
102 within hosts and vectors, based on the good characterization of bacterial diversity
103 within each individual samples it provides. However, the various sources of error
104 during the generation and processing of HTS data [20] may make it difficult to
105 determine which samples are really positive or negative for a given bacterium. The
106 detection of one or a few sequences assigned to a given taxon in a sample does not
107 necessary mean that the bacterium is effectively present in that sample. We carried
108 out an extensive literature review, from which we identified several potential sources
109 of error involving all stages of a 16S rRNA amplicon sequencing experiment — from
110 the collection of samples to the bioinformatic analysis — that might lead to false-
111 negative or false-positive screening results (Table 1, [18,19,21-40]). These error
112 sources have now been documented, and recent initiatives have called for the
113 promotion of open sharing of standard operating procedures and best practices in
114 microbiome research [41]. However, no experimental designs minimizing the impact
115 of these sources of error on HTS data interpretation have yet been reported.

116 We describe here a rigorous experimental design for the direct estimation of biases
117 from the data produced by 16S rRNA amplicon sequencing. We used these bias
118 estimates to control and filter out potential false-positive and false-negative samples
119 during screening for bacterial pathogens. We applied this strategy to 711 commensal
120 rodents collected from 24 villages in Senegal, Western Africa: 208 *Mus musculus*
121 *domesticus,* 189 *Rattus rattus*, 93 *Mastomys natalensis* and 221 *Mastomys*
122 *erythroleucus*. Rodents were screened for bacteria as described by Kozich *et al.* [18],
123 in a protocol based on MiSeq sequencing (Illumina) of the V4 hypervariable region of
124 the 16SrRNA gene. We considered the common pitfalls listed in Table 1 during the
125 various stages of the experiment (see details in the workflow procedure, Figure 1).
126 Biases in assessments of the presence or absence of bacteria in rodents were
127 estimated directly from the dataset, by including and analyzing negative controls
128 (NC) and positive controls (PC) at the various stages of the experiment, and
129 systematically using sample replicates. This strategy delivers realistic and reliable

130 **Table 1.** Sources of bias during the experimental and bioinformatic steps of 16S rRNA
131 amplicon sequencing, consequences for data interpretation and solutions for
132 decreasing these biases.

Experimental steps	Sources of errors	Consequences	Solutions
Sample collection	Cross-contamination between individuals [21]	False-positive samples	Rigorous processing (decontamination of the instruments, cleaning of the autopsy table, use of sterile bacterial-free consumables, gloves, masks) Negative controls during sampling (e.g., organs of healthy mice during dissection)
	Collection and storage conditions [21]	False-positive & negative samples	Use of appropriate storage conditions/buffers. Use of unambiguously identified samples. Double checking of tube labeling during sample collection.
DNA extraction	Cross-contamination between samples [22]	False-positive samples	Rigorous processing (separation of pre- and post-PCR steps, use of a sterile hood, filter tips and sterile bacterial-free consumables)
	Reagent contamination with bacterial DNA [21,23]	False-positive samples	Negative controls for extraction (extraction without sample)
	Small amounts of DNA [21, 24]	False-negative samples	Use of an appropriate DNA extraction protocol. Discarding of samples with a low DNA concentration
Target DNA region and primer design	Target DNA region efficacy [19,25]	False-negative due to poor taxonomic identification	Selection of an appropriate target region and design of effective primers for the desired taxonomic resolution
	Primer design [21,26]	False-negative samples due to biases in PCR amplification for some taxa	Checking of the universality of the primers with reference sequences
Tag/Index design and preparation	False-assignments of sequences due to cross-contamination between tags/indices [27,28]	False-positive samples	Rigorous processing (use of sterile hood, filter tips and sterile bacterial-free consumables, brief centrifugation before the opening of index storage tubes, separation of pre- and post-PCR steps) Negative controls for tags/indices (empty wells without PCR reagents for particular tags or index combinations) Positive controls for alien DNA, i.e. a bacteria strain highly unlikely to infect the samples studied (e.g., a host-specific bacterium unable to persist in the environment) to estimate false assignment rate
	False-assignments of sequences due to inappropriate tag/index design [29]	False-positive samples	Fixing of a minimum number of substitutions between tags or indices. Each nucleotide position in the sets of tags or indices should display about 25% occupation by each base for Illumina sequencing
PCR amplification	Cross-contamination between PCRs [28]	False-positive samples	Rigorous processing (brief centrifugation before opening the index storage tubes, separation of pre- and post-PCR steps) Negative controls for PCR (PCR without template) with microtubes left open during sample processing
	Reagent contamination with bacterial DNA [21,23]	False-positive samples	Rigorous processing (use of sterile hood, filter tips and sterile bacterial-free consumables) Negative controls for PCRs (PCR without template), with microtubes closed during sample processing
	Chimeric recombinations by jumping PCR [27,30,31,32,33]	False-positive samples due to artifactual chimeric sequences	Increasing the elongation time. Use of a bioinformatic strategy to remove the chimeric sequences (e.g., Uchime program)
	Poor or biased amplification [45]	False-negative samples	Increasing the amount of template DNA; Optimizing the PCR conditions (reagents and program) Use of technical replicates to validate sample positivity Positive controls for PCR (extraction from infected tissue and/or bacterial isolates)
Library preparation	Cross-contamination between PCRs/libraries [22]	False-positive samples	Rigorous processing (use of a sterile hood, filter tips and sterile bacterial-free consumables, electophoresis and gel excision, separation of pre and post-PCR steps) Use of a protocol with an indexing step during target amplification Negative controls for indices (changing well positions between library preparation sessions)
	Chimeric recombinations by jumping PCR [27]	False-positive samples due to inter-individual recombinations	Avoiding PCR library enrichment in pooled samples. Positive controls for alien DNA, i.e. a bacterial strain that should not be identified in the sample (e.g. a host-specific bacterium unable to persist in the environment)
MiSeq sequencing (Illumina)	Sample sheet errors [21]	False-positive and negative samples	Negative controls (wells without PCR reagents for a particular index combination)
	Run-to-run carryover (Illumina Technical Support Note No. 770-2013-046)	False-positive samples	Washing of the MiSeq with dilute sodium hypochlorite solution
	Poor quality of reads due to flowcell overloading [34]	False-negative due to low quality of sequences	qPCR quantification of the library before sequencing.
	Poor quality of reads due to low-diversity libraries (Illumina Technical Support Note No. 770-2013-013)		Decreasing cluster density. Creation of artificial sequence diversity at the flowcell surface (e.g., by adding 5 to 10% PhiX DNA control library)
	Small number of reads per sample [35,36]	False-negative due to low depth of sequencing	Decreasing the level of multiplexing Discard the sample with a low number of reads
	Too sort overlapping read pairs [18]	False-negative due to low quality of sequences	Increasing paired-end sequence length or decreasing the length of the target sequence
	Mixed clusters on the flowcell [27]	False-positive due to false index-pairing	Use of a single barcode sequence for both the i5 and i7 indices for each sample (when possible, e.g. small number of samples) Positive controls for alien DNA, i.e., a bacterial strain highly unlikely to be found in the rodents studied (e.g., a host-specific bacterium unable to persist in the environment)
Bioinformatics and taxonomic classification	Poor quality of reads	False-negative samples due to poor taxonomic resolution	Removal of low-quality reads
	Errors during processing (sequence trimming, alignment) [18,37,38]	False-positive and negative samples	Use of standardized protocols and reproducible workflows
	Incomplete reference sequence databases [39]	False-negative samples	Selection of an appropriate database for the selected target region and testing of the database for bacteria of particular interest
	Error of taxonomic classification [40]	False-positive samples	Positive controls for PCRs (extraction from infected tissue and/or bacterial isolates and/or mock communities) Checking of taxonomic assignments by other methods (e.g., Blast analyses on different databases)

133

134 estimates of bacterial prevalence in wildlife populations, and could be used to
135 analyze the co-occurrence of different bacterial species within individuals.

136

137 Results & Discussion

138 ***Raw sequencing results***. The sequencing of 1569 PCR products (from rodent
139 samples and controls, see details in Table S1) in two MiSeq runs generated a total of
140 23,698,561 raw paired-end sequence reads (251-bp) of the V4 region of the
141 16SrRNA gene. Overall, 99% of wild rodent PCRs generated more than 3,000 raw
142 reads (mean: 11,908 reads; standard deviation: 6,062). The raw sequence read files
143 in FASTQ format are available for each PCR and each MiSeq run on request to the
144 corresponding author. Using mothur v1.34 [42] and the MiSeq standard operating
145 procedure (http://www.mothur.org/wiki/MiSeq_SOP), we removed 20.1% of paired-
146 end reads because they were misassembled, 1.5% of sequences because they were
147 misaligned, 2.6% because they were chimeric and 0.2% because they were non-
148 bacterial. The remaining reads were grouped into operational taxonomic units
149 (OTUs) with a divergence threshold of 3%. Bioinformatics analysis identified 13,296
150 OTUs, corresponding to a total of 7,960,533 sequences in run 1 and 6,687,060
151 sequences in run 2.

152 ***Taxonomic assignment of sequences.*** We used the Bayesian classifier
153 (bootstrap cutoff = 80%) implemented in mothur with the Silva SSU Ref database
154 v119 [43] as a reference, for the taxonomic assignment of OTUs. The 50 most
155 abundant OTUs accounted for 89% (min: 15,284 sequences; max: 2,206,731
156 sequences) of the total sequence dataset (Table S2). The accuracy of taxonomic
157 assignment (to genus level) was assessed with positive controls for PCR,
158 corresponding to DNA extracts from laboratory isolates of *Bartonella taylorii*, *Borrelia*
159 *burgdorferi* and *Mycoplasma mycoides* ($PC_{Bartonella_t}$, $PC_{Borrelia_b}$ and $PC_{Mycoplasma_m}$,
160 respectively), which were correctly assigned to a single OTU corresponding to the
161 appropriate genuine sequences (Table 2). Note that the sequences of $PC_{Mycoplasma_m}$
162 were assigned to Entomoplasmataceae rather than Mycoplasmataceae because of a
163 frequent taxonomic error reflected in most databases, including Silva [44]. This
164 problem might also affect other taxa. We therefore recommend systematically
165 carrying out a blast analysis against the sequences of taxa of interest in GenBank to

confirm the taxonomic assignment obtained with the 16S databases. Finally, we assumed that the small number of sequences per sample might limit the completeness of bacterial detection [36]. For this reason, we discarded seven rodent samples (2 *M. erythroleucus* and 5 *M. domesticus*) yielding fewer than 500 sequences for at least one of the two PCR replicates. This threshold corresponds to 99% of the distribution of the numbers of sequences between PCR products.

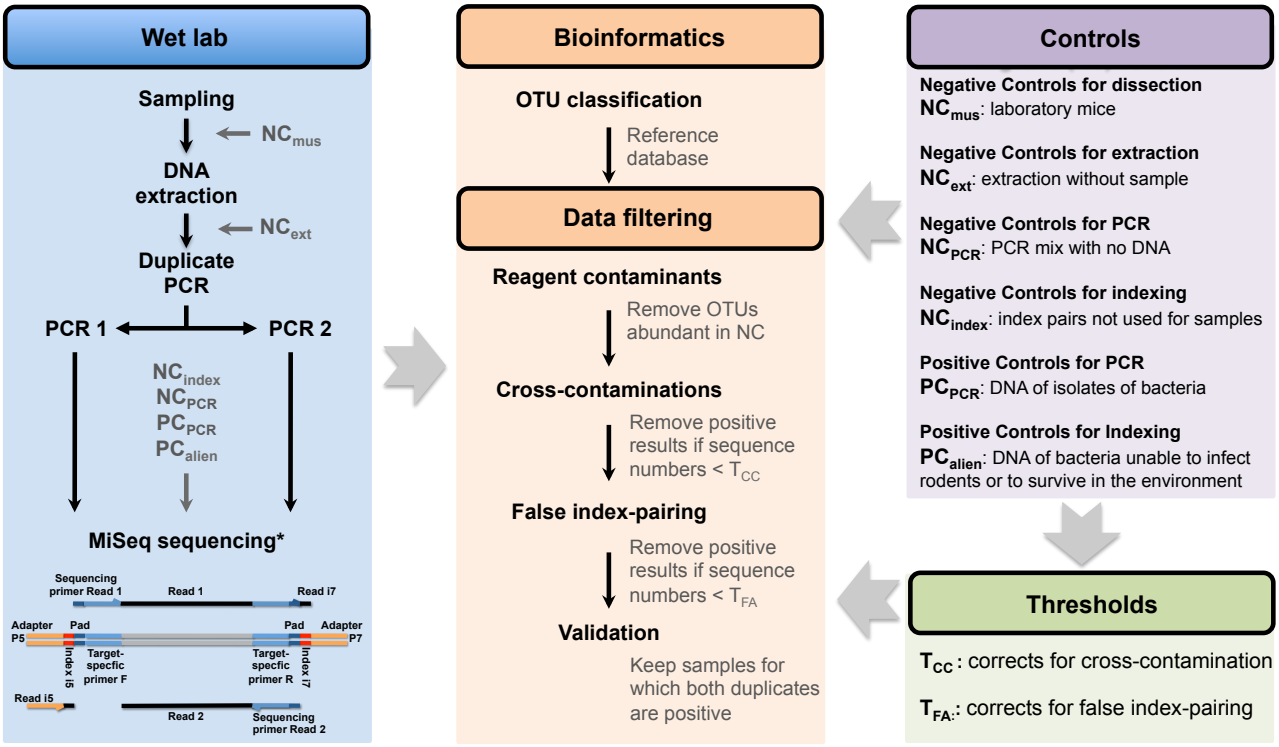


Figure 1. Workflow of the wet laboratory, and for bioinformatics and data filtering procedures, and a list of controls and thresholds included in the process of data filtering for the elimination of false-positive results for 16S rRNA amplicon sequencing. Reagent contaminants were detected by analyzing the sequences in the NC_{ext} and NC_{PCR}; T_{CC}: sequence number threshold for correcting for cross-contamination. T_{CC} values are OTU- and run-dependent and were estimated by analyzing the sequences in the controls, NC_{mus}, NC_{ext}, NC_{PCR} and PC_{PCR}; T_{FA}: sequence number threshold for correcting for false index-pairing. T_{FA} values are OTU- and run-dependent and were estimated by analyzing the sequences in the NC_{index} and PC_{alien}. A result was considered positive if the number of sequences was > T_{CC} and > T_{FA}. Samples were considered positive if a positive result was obtained for both PCR replicates.

Filtering for reagent contaminants. Metagenomics data may be affected by the contamination of reagents [23]. We therefore filtered the data, using negative controls for extraction (NC_{ext}), corresponding to extraction without the addition of a tissue sample, and negative controls for PCR (NC_{PCR}), corresponding to PCR

189 mixtures to which no DNA was added. This made it possible to identify the most
190 abundant contaminants, including *Pseudomonas*, *Acinetobacter*, *Herbaspirillum*,
191 *Streptococcus*, *Pelomonas*, *Brevibacterium*, *Brachybacterium*, *Dietzia*,
192 *Brevundimonas*, *Delftia*, *Comamonas*, *Corynebacterium*, and *Geodermatophilus,*
193 which accounted for 29% of the sequences in the dataset (Table S3). The bacterial
194 contaminants detected differed between MiSeq runs: *Pseudomonas*, *Pelomonas* and
195 Herbaspirillum predominated in run 1, whereas *Brevibacterium*, *Brachybacterium* and
196 *Dietzia* predominated in run 2. This difference probably reflects the use of two
197 different PCR kits manufactured at several months apart (Qiagen technical service,
198 pers. com.). Other taxa, such as *Streptococcus,* most originated from the DNA
199 extraction kits used, as they were detected in abundance in the negative controls for
200 extraction (NC_{ext}). These results highlight the importance of carrying out systematic
201 negative controls to filter the taxa concerned, to prevent inappropriate data
202 interpretation, particularly for the *Streptococcus* genus, which contains a number of
203 important pathogenic species. The use of DNA-free reagents would improve the
204 quality of sequencing data without affecting the depth of sequencing of the samples.

205 After filtering for the above reagent contaminants, the seven most relevant
206 pathogenic bacterial genera, *Bartonella*, *Borrelia*, *Ehrlichia*, *Mycoplasma*, *Orientia*,
207 *Rickettsia* and *Streptobacillus,* accounted for 66% of the sequences identified in wild
208 rodent samples. Six different OTUs were obtained for *Mycoplasma*
209 (*Mycoplasma*_OTU_1 to *Mycoplasma*_OTU_6), with one OTU each for the other
210 genera (Table 2). The other 34% of sequences probably corresponded to commensal
211 bacteria (Bacteroidales, Bacteroides, Enterobacteriaceae, *Helicobacter*,
212 *Lactobacillus*), undetected contaminants and rare taxa of unknown function.

213 ***Filtering for false-positive results.*** Mothur analysis produced a table of
214 abundance, giving the number of sequences for each OTU in each PCR product
215 (data available on request to the corresponding author). The multiple biases during
216 data processing listed in Table 1 made it impossible to infer prevalence and co-
217 occurrence directly from the table of sequence presence/absence in the PCR
218 products. We suggest filtering the data with data-based estimates of the different
219 biases calculated from the multiple controls introduced during the process. This
220 strategy involves calculating sequence number thresholds from our bias estimates.
221 Two different thresholds were set for each of the 12 OTUs and two MiSeq runs. We

222 then discarded positive results associated with numbers of sequences below the
223 thresholds (Figure 1).

224 ***Threshold T_{cc}: Filtering for cross-contamination.*** One source of false positives is
225 cross-contamination between samples processed in parallel (Table 1). Negative
226 controls for dissection (NC_{mus}), consisting of the spleens of healthy laboratory mice
227 manipulated during sessions of wild rodent dissection, and negative controls for
228 extraction (NC_{ext}) and PCR (NC_{PCR}) were used, together with positive controls for
229 PCR ($PC_{Bartonella_t}$, $PC_{Borrelia_b}$ and $PC_{Mycoplasma_m}$), to estimate cross-contamination.
230 For each sequencing run, we calculated the maximal number of sequences for the 12
231 pathogenic OTUs in the negative and positive controls. These numbers ranged from
232 0 to 115 sequences, depending on the OTU and the run considered (Table 2), and
233 we used them to establish OTU-specific thresholds (T_{CC}) for each run. The use of
234 these T_{CC} led to 0% to 69% of the positive results being discarded, corresponding to
235 only 0% to 0.14% of the sequences, depending to the OTU considered (Figure 2,
236 Table S4). A PCR product may be positive for several bacteria in cases of
237 coinfection. In such cases, the use of a T_{CC} makes it possible to discard the positive
238 result for one bacterium whilst retaining positive results for other bacteria.

239 ***Threshold T_{FA}: Filtering out incorrectly assigned sequences.*** Another source of
240 false positives is the incorrect assignment of sequences to a PCR product (Table 1).
241 We used two kinds of controls to detect incorrect assignments (Figure 1).

242 First, negative control index pairs (NC_{index}), corresponding to particular index pairs
243 not used to identify our samples, were included to check for cross-contamination
244 between indices or for errors during completion of the Illumina sample sheet. NC_{index}
245 returned very few read numbers (1 to 12), suggesting that there was little or no cross-
246 contamination between indices in our experiment.

247 Second, we used "alien" positive controls (PC_{alien}) in the PCR amplification step:
248 $PC_{Mycoplasma_m}$, corresponding to the DNA of *Mycoplasma mycoides,* which cannot
249 infect rodents, and $PC_{Borrelia_b}$, corresponding to the DNA of *Borrelia burgdorferi,*
250 which is not present in Africa. Neither of these bacteria can survive in abiotic
251 environments, so the presence of their sequences in African rodent PCR products
252 indicates a misassignment of sequences due to false index-pairing [27]. Using
253 $PC_{Mycoplasma_m}$, we obtained an estimate of the global false index-pairing rate of
254 0.14% (i.e. 398 of 280,151 sequences of the *Mycoplasma mycoides* OTU were

assigned to samples other than $PC_{Mycoplasma_m}$). Using $PC_{Borrelia_b}$, we obtained an estimate of 0.22% (534 of 238,772 sequences of the *Borrelia burgdorferi* OTU were assigned to samples other than $PC_{Borrelia_b}$). These values are very close to the estimate of 0.3% obtained by Kircher *et al*. [27]. Close examination of the distribution of misassigned sequences within the PCR 96-well microplates showed that all PCR products with misassigned sequences had one index in common with either $PC_{Mycoplasma_m}$ or $PC_{Borrelia_b}$ (Figure S1).

Table 2. Number of sequences for 12 pathogenic OTUs observed in wild rodents, in negative controls and in positive controls, together with T_{CC} and T_{FA} threshold values. Data are given for the two MiSeq runs separately. NC_{PCR}: negative controls for PCR; NC_{ext}: negative controls for extraction; NC_{mus}: negative controls for dissection; $PC_{Bartonella_t}$: positive controls for PCR; $PC_{Borrelia_b}$ and $PC_{Mycoplsma_m}$: positive controls for PCR and positive controls for indexing; T_{CC} and T_{FA}: thresholds for positivity for a particular bacterium according to bacterial OTU and MiSeq run (see also Figure 1).

	OTUs	Total	Wild rodents		Negative controls						Positive controls						Thresholds	
			(*n*=711)		NC_{PCR}		NC_{ext}		NC_{mus}		$PC_{Bartonnella_t}$		$PC_{Borrelia_b}$		$PC_{Mycoplasma_m}$			
		Total no. of sequences	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	Total no. of sequences	Maximum no. of sequences in one PCR	T_{cc}*	T_{FA}**
	Whole dataset	7960533	7149444	64722	45900	8002	39308	8741	68350	26211	137424	73134	239465	120552	280642	82933	/	/
Run 1	***Mycoplasma*_OTU_1**	1410218	1410189	61807	2	1	3	2	9	5	3	3	8	6	4	3	6	282
Run 1	***Mycoplasma*_OTU_ 3**	507376	507369	36335	2	1	0	0	0	0	2	2	1	1	2	2	2	101
Run 1	***Ehrlichia*_OTU**	649451	649423	63137	4	2	3	2	7	4	1	1	1	1	12	6	6	130
Run 1	***Borrelia*_OTU**	345873	345845	28528	4	4	7	4	9	4	1	1	0	0	7	3	4	69
Run 1	***Orientia*_OTU**	279965	279957	29503	1	1	4	1	0	0	2	2	0	0	1	1	2	56
Run 1	***Bartonella*_OTU**	202127	67973	16145	1	1	1	1	1	1	134124	71163	7	4	20	9	9	40
Run 1	***Mycoplasma mycoides******	280151	338	28	0	0	0	0	2	2	34	20	24	18	279753	82767	/	/
Run 1	***Borrelia burgdorferi******	238772	420	43	0	0	0	0	0	0	38	21	238238	119586	76	23	/	/
	Whole dataset	6687060	6525107	42326	61231	9145	53334	7669	/	/	12142	7518	13378	7164	21868	6520	/	/
Run 2	***Mycoplasma*_OTU_1**	155486	155486	7703	0	0	0	0	/	/	0	0	0	0	0	0	0	31
Run 2	***Mycoplasma*_OTU_2**	1036084	1035890	23588	1	1	192	115	/	/	0	0	0	0	1	1	115	207
Run 2	***Mycoplasma*_OTU_ 3**	127591	127590	5072	1	1	0	0	/	/	0	0	0	0	0	0	1	26
Run 2	***Mycoplasma* _OTU_4**	85596	85583	20146	0	0	13	13	/	/	0	0	0	0	0	0	13	17
Run 2	***Mycoplasma*_OTU_ 5**	56324	56324	10760	0	0	0	0	/	/	0	0	0	0	0	0	0	11
Run 2	***Mycoplasma*_OTU_ 6**	13356	13356	1482	0	0	0	0	/	/	0	0	0	0	0	0	0	3
Run 2	***Ehrlichia*_OTU**	74017	74017	19651	0	0	0	0	/	/	0	0	0	0	0	0	0	15
Run 2	***Borrelia*_OTU**	21636	21636	3085	0	0	0	0	/	/	0	0	0	0	0	0	0	4
Run 2	***Orientia*_OTU**	307	307	181	0	0	0	0	/	/	0	0	0	0	0	0	0	0
Run 2	***Bartonella*_OTU**	1559028	1547652	14515	1	1	2	2	/	/	11297	6714	2	2	74	59	59	312
Run 2	***Streptobacillus*_OTU**	32399	32399	6245	0	0	0	0	/	/	0	0	0	0	0	0	0	6
Run 2	***Rickettsia*_OTU**	589	589	329	0	0	0	0	/	/	0	0	0	0	0	0	0	0
Run 2	***Mycoplasma mycoides******	16854	2	1	0	0	0	0	/	/	0	0	0	0	16852	5766	/	/
Run 2	***Borrelia burgdorferi******	12197	0	0	0	0	0	0	/	/	0	0	12197	6426	0	0	/	/

*: Threshold T_{cc} is based on the maximum number of sequences observed in a negative or positive control for a particular OTU in each run
**: Threshold T_{FA} is based to the false assignment rate (0.02%) weighted by the total number of sequences of each OTU in each run
***: Bacterial isolates added as positive controls for PCR and indexing (i.e., PC_{ALIEN} see Figure 1)

We then estimated the impact of false index-pairing for each PCR product, by calculating the maximal number of sequences of “alien” bacteria assigned to PCR products other than the corresponding PC. These numbers varied from 28 to 43, depending on the positive control for run 1 (Table 2) — run 2 was discarded because of the low values of the numbers of sequences, which is likely due to the fact that DNAs of PC of were hundred-fold diluted in run 2 (Table S1) —. We then estimated a false-assignment rate for each PCR product (R_{fa}), by dividing the above numbers by the total number of sequences from “alien” bacteria in the sequencing run 1. R_{fa} was estimated for $PC_{Mycoplasma_m}$ and $PC_{Borrelia_b}$ separately. R_{fa} reached 0.010% and 0.018% for $PC_{Mycoplasma_m}$ and $PC_{Borrelia_b}$, respectively. We adopted a conservative

approach, by fixing the R_{fa} value to 0.020%. This number signifies that each PCR product may receive a maximum 0.020% of sequences of an OTU present in the PCR plate due to false index-pairing. Moreover, the number of misassigned sequences for a specific OTU into a PCR product should increase with the total number of sequences of the OTU in the MiSeq run. We therefore defined the second threshold (T_{FA}) as the total number of sequences in the run for an OTU multiplied by R_{fa}. T_{FA} values varied with the abundance of each OTU in the sequencing run (Table 2). Because the abundance of each OTU varied from one sequencing run to another, T_{FA} also varied according to the sequencing run. The use of the T_{FA} led to 0% to 87% of positive results being discarded. This corresponded to 0% to 0.71% of the sequences, depending on the OTU (Figure 2, Table S4).

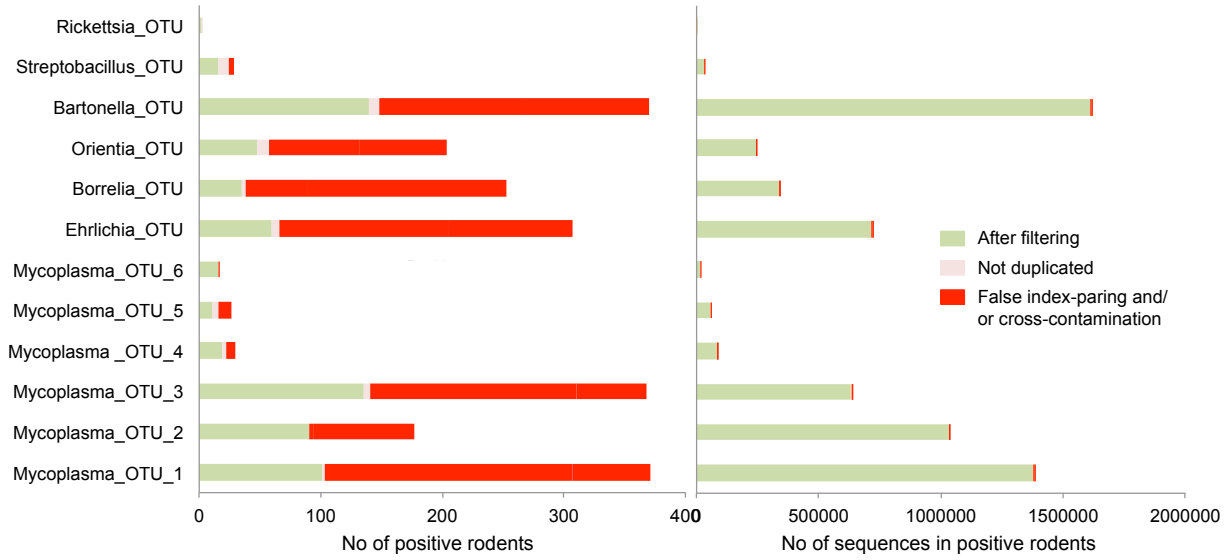


Figure 2. Numbers of positive rodents, and of sequences in positive rodents, removed for each OTU at each step in data filtering. These findings demonstrate that the positive rodents filtered out corresponded to only a very small number of sequences. (A) The histogram shows the number of positive rodents discarded because of likely cross-contamination, false index-pairing and for a negative result in a replicate PCR, and, finally the positive results retained at the end of data filtering in green. (B) The histogram shows the number of sequences corresponding to the same class of positive rodents. Note that several positive results may be recorded for the same rodent in cases of co-infection.

Validation with PCR replicates. Random contamination may occur during the preparation of PCR 96-well microplates. These contaminants may affect some of the wells, but not those for the negative controls, leading to the generation of false-positive results. We thus adopted a conservative approach, in which we considered rodents to be positive for a given OTU only if both PCR replicates were considered

positive after the filtering steps described above. The relevance of this strategy was supported by the strong correlation between the numbers of sequences for the two PCR replicates for each rodent (R^2>0.90, Figure 3 and Figure S2). At this stage, 673 positive results for 419 rodents were validated for both replicates (note that a rodent

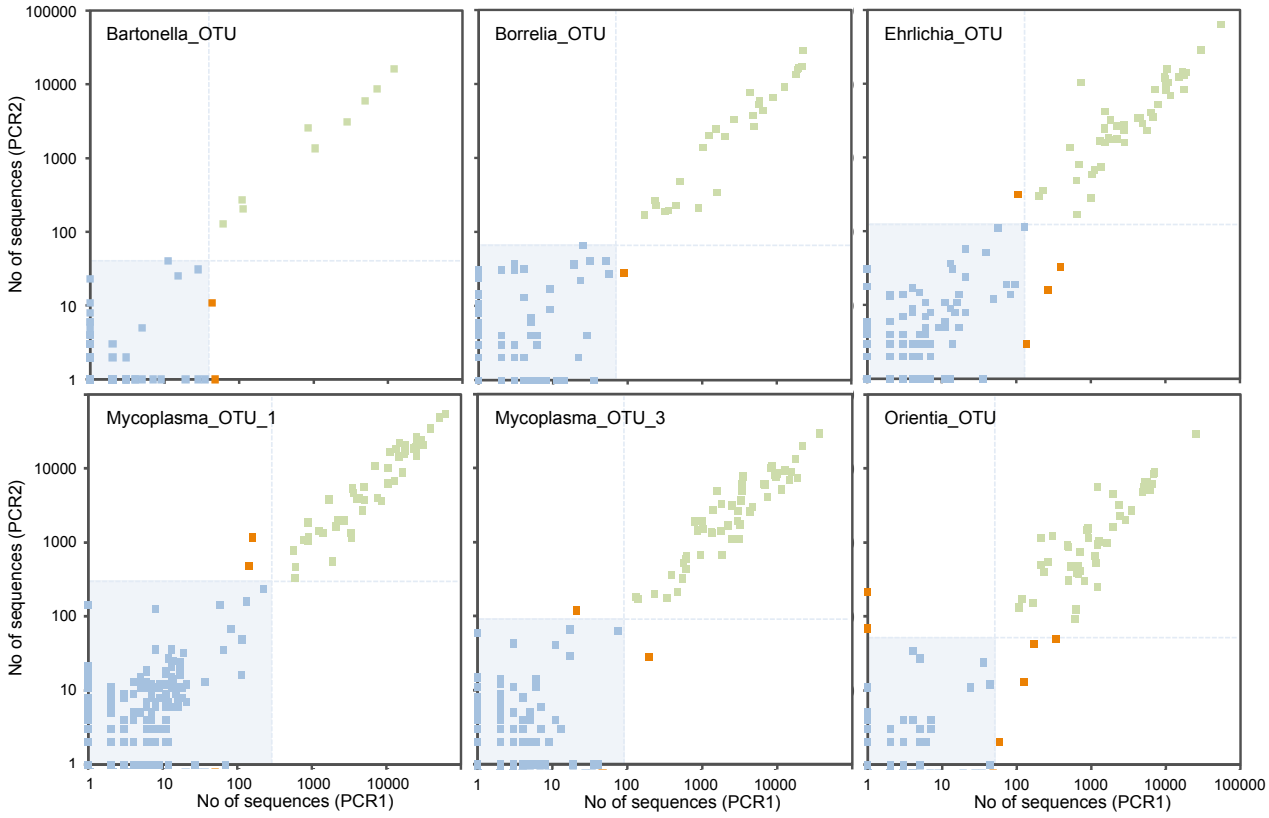


Figure 3. Plots of the number of sequences (log (x+1) scale) from bacterial OTUs in both PCR replicates (PCR1 & PCR2) of the 348 wild rodents analyzed in the first MiSeq run. Note that each rodent was tested with two replicate PCRs. Green points correspond to rodents with two positive results after filtering; red points correspond to rodents with one positive result and one negative result; and blue points correspond to rodents with two negative results. The light blue area and lines correspond to the threshold values used for the data filtering: samples below the lines are filtered out. See Figure S2 for plots corresponding to the second MiSeq run.

may be positive for several bacteria, and may thus be counted several times), whereas only 52 positive results were discarded because the result for the other replicate was negative. At this final validation step, 0% to 60% of the positive results for a given OTU were discarded, corresponding to only 0% to 7.17% of the sequences (Figure 2, Table S4 and Table S5). Note that the number of replicates may be increased, as described in the strategy of Gómez-Díaz *et al* [45].

326 ***Post-filtering results.*** Finally, the proportion of rodents positive for a given OTU
327 filtered out by the complete filtering approach varied from 6% to 86%, depending on
328 the OTU, corresponding to only 1% of the total sequences (Figure 2). Indeed, our
329 filtering strategy mostly excluded rodents with a small number of sequences for the
330 OTU concerned. These rodents were considered to be false-positive.

331 ***Refining bacterial taxonomic identification.*** We refined the taxonomic
332 identification of the 12 bacterial OTUs through phylogenetic and blast analyses. We
333 were able to identify the bacteria present down to genus level and, in some cases,
334 we could even identify the most likely species (Table 3 and Figure S3). For instance,
335 the sequences of the six *Mycoplasma* OTUs were consistent with three different
336 species — *M. haemomuris* for OTU_1 and 3, *M. coccoides* for OTU_4, 5 and 6, and
337 *M. species novo* [46] for OTU_2 — with high percentages of sequence identity
338 (≥93%) and strong bootstrap support (≥80%). All three of these species belong to the
339 Haemoplasma group, which is known to infect mice, rats and other mammals [47,48],
340 and is thought to cause anemia in humans [49,50]. The *Borrelia* sequences grouped
341 with three different species of the relapsing fever group (*crocidurae*, *duttonii* and
342 *recurrentis*) with a high percentage of identity (100%) and a reasonably high
343 bootstrap value (71%). In West Africa, *B. crocidurae* causes severe borreliosis, a
344 rodent-borne disease transmitted by ticks and lice [51]. The *Ehrlichia* sequences
345 were 100% identical to and clustered with the recently described Candidatus
346 *Ehrlichia khabarensis* isolated from voles and shrews in the Far East of Russia [52].
347 The *Rickettsia* sequences were 100% identical to the sequence of *R. typhi*, a species
348 of the typhus group responsible for murine typhus [53], but this clade was only
349 weakly differentiated from many other *Rickettsia* species and had only moderate
350 bootstrap support (61%). The most likely species corresponding to the sequences of
351 the *Streptobacillus* OTU was *S. moniliformis*, with a high percentage of identity
352 (100%) and a high bootstrap value (100%). This bacterium is common in rats and
353 mice and causes a form of rat-bite fever, Haverhill fever [54]. The *Orientia* sequences
354 corresponded to *O. chuto*, with a high percentage of identity (100%) and a high
355 bootstrap value (77%). This species was recently isolated from a patient infected in
356 Dubai [55]. Finally, accurate species determination was not possible for *Bartonella*,
357 as the 16S rRNA gene does not resolve the species of this genus well [56]. Indeed,

358 **Table 3. Detection of 12 bacterial OTUs in the four wild rodent species (*n*=704)**
359 **sampled in Senegal, and the biology and pathogenicity of the corresponding**
360 **bacterial genus.** *n*= number of rodents analyzed.

OTUs of interest (genus level)	Closest species* (% identity in GenBank)	Number of positive wild rodents				Biology & epidemiology
		Mastomys erytholeucus (*n*=219)	*Mastomys natalensis* (*n*=93)	*Mus musculus* (*n*=203)	*Rattus rattus* (*n*=189)	
Bartonella	undetermined	60	68	1	6	*Bartonella* spp. are intracellular fastidious hemotropic gram-negative organisms identified in a wide range of domestic and wild mammals and transmitted by arthropods. Several rodent-borne *Bartonella* species have emerged as zoonotic agents, and various clinical manifestations are reported, including fever, bacteremia and neurological symptoms [83].
Borrelia	*crocidurae* (100%) *duttonii* (100%) *recurrentis* (100%)	21	0	8	6	*Borrelia* is a genus of spiral gram-negative bacteria of the spirochete phylum. These bacteria are obligate parasites of animals and are responsible for relapsing fever borreliosis, a zoonotic disease transmitted by arthropods (tick and lice). This disease is the most frequent human bacterial disease in Africa. *B. crocidurae* is endemic to West Africa, including Senegal, and *B. duttonii* and *B. recurrentis* have been reported in Central, southern and East Africa [51].
Ehrlichia	*khabarensis* (100%)	40	0	12	8	*The genus Ehrlichia* includes five species of small gram-negative obligate intracellular bacteria. The life cycle includes the reproduction stages taking place in both ixodid ticks, acting as vectors, and vertebrates. *Ehrlichia* spp. can cause a persistent infection in the vertebrate hosts, which thus become reservoirs of infection. A number of new genetic variants of *Ehrlichia* have been recently detected in rodent species (e.g., *Candidatus Ehrlichia khabarensis* [52]).
Mycoplasma OTU_1	*haemomuris* (96%)	28	41	30	1	*Mycoplasma* is a genus including over 100 species of bacteria that lack of a cell wall around their cell membrane. *Mycoplasma coccoides* and *Mycoplasma haemomuris* are blood parasites of wild and laboratory rodents. A new closely related species was recently isolated from brown rats (AB752303 [46]). These species are commonly referred as "hemoplasmas". Hemoplasmas have been detected within the erythrocytes of cats, dogs, pigs, rodents and cattle, in which they may cause anaemia. There have been sporadic reports of similar infections in humans, but these infections have been poorly characterized [50].
Mycoplasma OTU_2	*sp. novo (100%) GenBank AB752303*	0	0	0	90	
Mycoplasma OTU_3	*haemomuris* (93%)	93	23	1	1	
Mycoplasma OTU_4	*coccoides* (96%)	0	0	0	18	
Mycoplasma OTU_5	*coccoides* (95%)	3	7	0	0	
Mycoplasma OTU_6	*coccoides* (97%)	3	14	0	0	
Orientia	*chuto* (100%) *tsutsugamushi* (98%)	0	3	46	0	*Orientia* is a genus of obligate intracellular gram-negative bacteria found in mites and rodents. *Orientia tsutsugamushi* is the agent of scrub typhus in humans. This disease, one of the most underdiagnosed and underreported febrile illnesses requiring hospitalization, has an estimated 10% fatality rate unless treated appropriately. A new species, *Orientia chuto,* was recently characterized in sick patients from the Arabian Peninsula, and new *Orientia* haplotypes have been identified in France and Senegal [9].
Rickettsia	*typhi* (100%)	1	0	0	1	*Rickettsia* is a genus of obligate intracellular gram-negative bacteria found in arthropods and vertebrates. *Rickettsia* spp. are symbiotic species transmitted vertically in invertebrates, and some are pathogenic invertebrates. *Rickettsia* species of the typhus group cause many human diseases, including murine typhus, which is caused by *Rickettsia typhi* and transmitted by fleas [53].
Streptobacillus	*moniliformis* (100%)	10	1	0	5	*Streptobacillus* is a genus of aerobic, gram-negative facultative anaerobe bacteria, which grow in culture as rods in chains. *Streptobacillus moniliformis* is common in rats and mice and is responsible of the Streptobacillosis form of rat-bite fever, the Haverhill fever. This zoonosis begins with high prostrating fevers, rigors (shivering), headache and polyarthralgia (joint pain). Untreated, rat-bite fever has a mortality rate of approximately 10% [54].

*based on phylogenetic analysis, see Figure S3
n: number of rodents screened

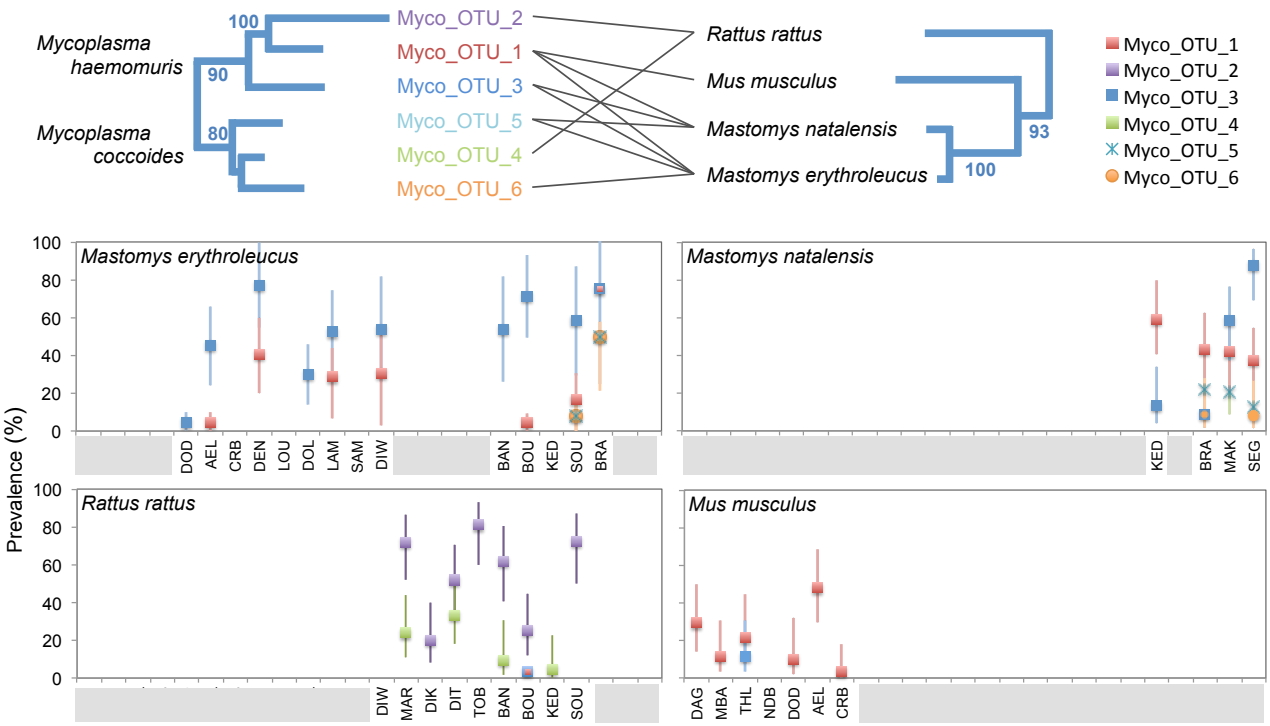
361

362 the sequences from the *Bartonella* OTU detected in our rodents corresponded to at
363 least seven different species (*elizabethae, japonica, pachyuromydis, queenslandis,*
364 *rattaustraliani, tribocorum, vinsonii*) and a putative new species recently identified in
365 Senegalese rodents [57].

366 These findings demonstrate the considerable potential of rRNA amplicon sequencing
367 for the rapid identification of zoonotic agents in wildlife, provided that the post-
368 sequencing data are cleaned beforehand. *Borrelia* [51] and *Bartonella* [57] were the
369 only ones of the seven pathogenic bacterial genera detected here in Senegalese
370 rodents to have been reported as present in rodents from West Africa before. The
371 other bacterial genera identified here have previously been reported to be presented
372 in rodents only in other parts of Africa or on other continents. *S. moniliformis* has
373 recently been detected in rodents from South Africa [58] and there have been a few
374 reports of human streptobacillosis in Kenya [59] and Nigeria [60]. *R. typhi* was
375 recently detected in rats from Congo, in Central Africa [61], and human seropositivity
376 for this bacterium has been reported in coastal regions of West Africa [62]. With the
377 exception of one report in Egypt some time ago [63], *Mycoplasma* has never before
378 been reported in African rodents. Several species of *Ehrlichia* (from the *E. canis*
379 group: *E. chaffeensis, E. ruminantium, E. muris, E. ewingii*) have been characterized
380 in West Africa, but only in ticks from cattle [89] together with previous reports of
381 possible cases of human ehrlichioses in this region [64]. Finally, this study reports the
382 first identification of *Orientia* in African rodents [9]. There have already been a few
383 reports of suspected human infection with this bacterium in Congo, Cameroon,
384 Kenya and Tanzania [65].

385 ***Estimating prevalence and coinfection.*** After data filtering, we were able to
386 estimate the prevalence in rodent populations and to assess coinfection in individual
387 rodents, for the 12 bacterial OTUs. Bacterial prevalence varied considerably between
388 rodent species (Table 3). *Bartonella* was highly prevalent in the two multimammate
389 rats *M. natalensis* (73%) and *M. erythroleucus* (27%); *Orientia* was prevalent in the
390 house mouse *M. musculus* (22%) and *Ehrlichia* occurred frequently in only one on
391 the two multimammate rats *M. erythroleucus* (18%). By contrast, the prevalence of
392 *Streptobacillus* and *Rickettsia* was low in all rodent species (<5%). Coinfection rates
393 were high, as 184 rodents (26%) were found to be coinfected with bacteria from two
394 (19%), three (5%), four (2%) or five (0.1%) different bacterial pathogens.

395



396

397

398 **Figure 4. Prevalence of six *Mycoplasma* OTUs detected in Senegalese rodents, by**
399 **site, and phylogenetic associations between *Mycoplasma* lineages and rodent**
400 **species.** (A) Comparison of phylogenetic trees based on the 16S rRNA V4-sequences of *Mycoplasma*,
401 and on the mitochondrial cytochrome *b* gene and the two nuclear gene fragments (IRBP exon 1 and
402 GHR) for rodents (rodent tree redrawn from [91]). Lines link the *Mycoplasma* lineages detected in the
403 various rodent species (for a minimum site prevalence exceeding 10%). The numbers next to branches
404 are bootstrap values (only shown if >70%). (B) Plots of OTU prevalence with 95% confidence intervals
405 calculated by Sterne's exact method [92] by rodent species and site (see [67] for more information about
406 site codes and their geographic locations). The gray bars in the X-legend indicate sites from which the
407 rodent species concerned is absent.

408

409 Interestingly, several *Mycoplasma* OTUs appeared to be specific to a rodent genus
410 or species (Table 3, Figure 4). OTU_2, putatively identified as a recently described
411 lineage isolated from brown rat, *Rattus norvegicus* [46], was specifically associated
412 with *R. rattus* in this study. Of the OTUs related to *M. coccoides*, OTU_4 was found
413 exclusively in *R. rattus,* whereas OTUs_5 and 6 seemed to be specific to the two
414 multimammate rats (*M. erytholeucus and M. natalensis*). Comparative phylogenies of
415 *Mycoplasma* OTUs and rodents showed that *R. rattus*, which is phylogenetically
416 more distantly related to the other three rodents, contained a *Mycoplasma*
417 community different from that in the *Mus-Mastomys* rodent clade (Figure 4).
418 Pathogen prevalence also varied considerably between sites, as shown for the six
419 *Mycoplasma* OTUs (Figure 4). This suggests that the infection risks for animals and

420 humans vary greatly according to environmental characteristics and/or biotic features
421 potentially related to recent changes in the distribution of rodent species in Senegal
422 [66,67]

423

424 Perspectives

425 ***Improving HTS for epidemiological surveillance.*** The screening strategy
426 described here has the considerable advantage of being non-specific, making it
427 possible to detect unanticipated or novel bacteria. Razzauti *et al*. [8] recently showed
428 that the sensitivity of 16S rRNA amplicon sequencing on the MiSeq platform was
429 equivalent to that of whole RNA sequencing (RNAseq) on the HiSeq platform for
430 detecting bacteria in rodent samples. However, little is known about the comparative
431 sensitivity of HTS approaches relative to qPCR with specific primers, the current gold
432 standard for bacterial detection within biological samples. Additional studies are
433 required to address this question. Moreover, as 16S rRNA amplicon sequencing is
434 based on a short sequence, it does not yield a high enough resolution to distinguish
435 between species in some bacterial genera, such as *Bartonella*. Whole-genome
436 shotgun or RNAseq techniques provide longer sequences, through the production of
437 longer reads or the assembly of contigs, and they might therefore increase the
438 accuracy of species detection [68]. However, these techniques would be harder to
439 adapt for the extensive multiplexing of samples [8]. Other methods could be used to
440 assign sequences to bacterial species for individuals found positive for a bacterial
441 genera following the 16S rRNA screening. For example, positive PCR assays could
442 be carried out with bacterial genus-specific primers, followed by amplicon
443 sequencing, as commonly used in MLSA (multilocus sequence analysis) strategies
444 [69] or high-throughput microfluidic qPCR assays based on bacterial species-specific
445 primers could be used [70]. High-throughput amplicon sequencing approaches could
446 be fine-tuned to amplify several genes for species-level assignment, such as the *gltA*
447 gene used by Gutierrez *et al*. [71] for the *Bartonella* genus, in parallel with the 16S
448 rRNA-V4 region.

449 This strategy could also easily be adapted for other microbes, such as protists, fungi
450 and even viruses, provided that universal primers are available for their detection
451 (see [72,73] for protists and fungi, and [74] for degenerate virus family-level primers

452 for viruses). Finally, our filtering method could also be translated to any other post-
453 sequencing dataset of indexed or tagged amplicons in the framework of
454 environmental studies (e.g. metabarcoding for diet analysis and biodiversity
455 monitoring [75], the detection of rare somatic mutations [76] or the genotyping of
456 highly polymorphic genes (e.g. MHC or HLA typing, [77,78]).

457 ***Monitoring the risk of zoonotic diseases.*** Highly successful synanthropic
458 wildlife species, such as the rodents studied here, will probably play an increasingly
459 important role in the transmission of zoonotic diseases [79]. Many rodent-borne
460 pathogens cause only mild or undifferentiated disease in healthy people, and these
461 illnesses are often misdiagnosed and underreported [54,80-83]. The information
462 about pathogen circulation and transmission risks in West Africa provided by this
463 study is important in terms of human health policy. We show that rodents carry seven
464 major pathogenic bacterial genera: *Borrelia*, *Bartonella*, *Mycoplasma, Ehrlichia*,
465 *Rickettsia*, *Streptobacillus* and *Orientia.* The last five of these genera have never
466 before been reported in West African rodents. The data generated with our HTS
467 approach could also be used to assess zoonotic risks and to formulate appropriate
468 public health strategies involving the focusing of continued pathogen surveillance and
469 disease monitoring programs on specific geographic areas or rodent species likely to
470 be involved in zoonotic pathogen circulation, for example.

471

472 Materials & Methods

473 ***Ethics statement.*** Animals were treated in accordance with European Union
474 guidelines and legislation (Directive 86/609/EEC). The CBGP laboratory received
475 approval (no. B 34-169-003) from the Departmental Direction of Population
476 Protection (DDPP, Hérault, France), for the sampling of rodents and the storage and
477 use of their tissues. None of the rodent species investigated in this study has
478 protected status (see UICN and CITES lists).

479 ***Sample collection.*** Rodents were killed by cervical dislocation, as recommended
480 by Mills *et al.* [84] and dissected as described in Herbreteau *et al.* [85]. Rodent
481 species were identified by morphological and/or molecular techniques [67]. Cross-
482 contamination during dissection was prevented by washing the tools used

483 successively in bleach, water and alcohol between rodents. We used the spleen for
484 bacterial detection, because this organ is a crucial site of early exposure to bacteria
485 [86]. Spleens were placed in RNAlater (Sigma) and stored at 4°C for 24 hours and
486 then at -20°C until their use for genetic analyses.

487 ***Target DNA region and primer design.*** We used primers with sequences
488 slightly modified from those of the universal primers of Kozich *et al*. [18] to amplify a
489 251-bp portion of the V4 region of the 16S rRNA gene (16S-V4F:
490 GTGCCAGCMGCCGCGGTAA; 16S-V4R: GGACTACHVGGGTWTCTAATCC). The
491 ability of our primers to hybridize to the DNA of bacterial zoonotic pathogens was
492 assessed by checking that there were low numbers of mismatched bases over an
493 alignment of 41,113 sequences from 79 zoonotic genera inventoried by Taylor et al
494 [1], extracted from the Silva SSU database v119 [43] (Table S6). The FASTA file is
495 available on request to the corresponding author. We used a slightly modified version
496 of the dual-index method of Kozich *et al*. [18] to multiplex our samples. The V4
497 primers included different 8-bp indices (i5 in the forward and i7 in the reverse
498 position) and Illumina adapters (i.e. P5 in the forward and P7 in the reverse position)
499 in the 5' position. The combinations of 24 i5-indexed primers and 36 i7-indexed
500 primers made it possible to identify 864 different PCR products loaded onto the same
501 MiSeq flowcell. Each index sequence differed from the others by at least two
502 nucleotides, and each nucleotide position in the sets of indices contained
503 approximately 25% of each base, to prevent problems due to poor index design
504 (Table 1).

505 ***DNA extraction and PCRs.*** All laboratory manipulations were conducted with
506 filter tips, under a sterile hood, in a DNA-free room. DNA was extracted with the
507 DNeasy 96 Tissue Kit (Qiagen) with final elution in 200 µl of elution buffer. One
508 extraction blank (NC_{ext}), corresponding to an extraction without sample tissue, was
509 systematically added to each of the eight DNA extraction microplates. DNA was
510 quantified with a NanoDrop 8000 spectrophotometer (Thermo Scientific), to confirm
511 the presence of a minimum of 10 ng/µl of DNA in each sample. DNA amplification
512 was performed in 5 µL of Multiplex PCR Kit (Qiagen) Master Mix, with 4 µL of
513 combined i5 and i7 primers (3.5µM) and 2 µL of genomic DNA. PCR began with an
514 initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at

515 95°C for 20 s, annealing at 55°C for 15 s and extension at 72°C for 5 minutes,
516 followed by a final extension step at 72°C for 10 minutes. PCR products (3 µL) were
517 verified by electrophoresis in a 1.5% agarose gel. One PCR blank (NC_{PCR}),
518 corresponding to the PCR mix with no DNA, was systematically added to each of the
519 18 PCR microplates. DNA was amplified in replicate for all wild rodent samples
520 (*n*=711) (Table S1).

521 ***Library preparation and MiSeq sequencing.*** Two MiSeq (Illumina) runs
522 were conducted, including PCR products from wild rodents and the positive and
523 negative controls detailed in Figure 1 and Table S1. The MiSeq platform was chosen
524 because it generates lower error rates than other HTP platforms [87]. The number of
525 PCR products multiplexed was 823 for the first MiSeq run and 746 for the second
526 MiSeq run (Table S1). Additional PCR products from other projects were added to
527 give a total of 864 PCR products per run. PCR products were pooled by volume for
528 each 96-well PCR microplate: 4 µL for rodents and controls, and 1.5 µL for bacterial
529 isolates. Mixes were checked by electrophoresis in 1.5% agarose gels before their
530 use to generate a ‘‘super-pool’’ of 864 PCR products for each MiSeq run. We
531 subjected about 100 µL of each ‘‘super-pool’’ to size selection for the full-length
532 amplicon (expected size: 375 bp including primers, indexes and adaptors), by
533 excision in a low-melting agarose gel (1.25%) to discard non-specific amplicons and
534 primer dimers. The PCR Clean-up Gel Extraction kit (Macherey-Nagel) was used to
535 purify the excised bands. DNA was quantified by using the KAPA library
536 quantification kit (KAPA Biosystems) on the final library before loading on a MiSeq
537 (Illumina) flow cell (expected cluster density: 700-800 K/mm^2) with a 500-cycle
538 Reagent Kit v2 (Illumina). We performed runs of 2 x 251 bp paired-end sequencing,
539 which yielded high-quality sequencing through the reading of each nucleotide of the
540 V4 fragments twice after the assembly of reads 1 and reads 2. The raw sequence
541 reads (.fastq format) are available on request to the corresponding author.

542 ***Bioinformatic and taxonomic classification.*** MiSeq datasets were
543 processed with mothur v1.34 [42] and with the MiSeq standard operating procedure
544 (SOP) [18]. We used the Silva SSU Reference database v119 [43] and the Silva
545 taxonomy file for taxonomic assignment. The abundance table generated by mothur
546 for each PCR product and each OTU was filtered as described in the Results section.
547 The most abundant sequence for each OTU in each sample was extracted from the

548 sequence dataset with a custom-written Perl script. The FASTA files are available
549 from GenBank (Accession Number KU697337 to KU697350). The sequences were
550 aligned with reference sequences from bacteria of the same genus available from the
551 SILVA SSU Ref NR database v119, using SeaView v4 [88]. Phylogenetic trees were
552 generated from the K2P distance with SeaView and species were identified on the
553 basis of the "closest phylogenetic species". We also used our sequences for blast
554 analyses of GenBank, to identify the reference sequences to which they displayed
555 the highest percentage identity.
556

557 ## Acknowledgments

558 This study was funded by the French National Institute for Agricultural Research
559 (INRA) Meta-omics and microbial ecosystems metaprogram (Patho-ID project:
560 Rodent and tick pathobiomes) and the ANR ENEMI (ANR-11-JSV7-0006). We would
561 like to thank Virginie Dupuy for extracting DNA from bacterial cultures and Julie
562 Sappa from Alex Edelman & Associates for improving the English writing. Analyses
563 were performed on the CBGP HPC computational platform. The funders had no role
564 in study design, data collection and analysis, the decision to publish, or preparation
565 of the manuscript.
566

567 ## Authors' contributions

568 The study was conceived and designed by MG and JFC. MG, AL, CT, LT, HV and
569 MR carried out the molecular biology procedures and validated the MiSeq data. MG,
570 EB, MB and ADG contributed to the development of bioinformatics methods and
571 validated taxonomic assignments. JFC and MTV coordinated the Patho-ID project
572 and CB and NC coordinated the ENEMI project. MG, JFC, LT, CB and NC analyzed
573 the data. MG and JFC wrote the manuscript. CB, NC, MR and MVT helped to draft
574 and to improve the manuscript. All the authors have read and approved the final
575 manuscript.
576

577 ## Supplementary materials

578 **Table S1. Numbers of samples and numbers of PCRs for wild rodents and**
579 **controls.** Negative Controls for dissection, NC_{mus} ; Negative Controls for extraction, NC_{ext} ; Negative
580 Controls for PCR, NC_{PCR} ; Negative Controls for indexing, NC_{index} ; Positive Controls for PCR, PC_{PCR} ;
581 Positive Controls for Indexing, PC_{alien}. See also Figure 1 for more details concerning negative controls
582 (NC) and positive controls (PC).

583 **Table S2. The 50 most abundant OTUs in wild rodents and controls.**

584 **Table S3. Bacterial contaminants observed in negative and positive controls.**
585 They were identified as contaminants on the basis of negative controls for extraction and PCR. Taxa
586 in bold correspond to the sequences of DNA extracted from laboratory isolates.

587 **Table S4. Proportion of sequences and proportion of positive results removed**
588 **at each step in data filtering.** Note that several positive results may be recorded for the same
589 rodent in cases of co-infection.

590 **Table S5. Proportion of positive results for both PCR products at each step in**
591 **data filtering.** Note that several positive results may be recorded for the same rodent in cases of
592 co-infection.

593 **Table S6. Number of mismatches between PCR forward and reverse primers**
594 **and 41,113 bacterial 16S rRNA V4 sequences of 79 zoonotic genera.** Data [1] was
595 extracted from the Silva SSU database v119. Numbers of mismatches > 3 correspond to
596 sequences of bad quality from different taxon. The number of mismatches in the 3' side of primers was
597 always <2.

598 **Figure S1. Numbers of sequences of the positive controls for indexing**
599 **$PC_{Borrelia_b}$ (in blue) and $PC_{Mycoplasma_m}$ (in red) in the various PCR products, with**
600 **a dual-indexing design, for MiSeq runs 1 (a) and 2 (b).** The two PCRs for $PC_{Borrelia_b}$
601 were performed with plate 9, positions A1 and E1 for run 1 and B1 and F1 for run 2, and the four
602 PCRs for $PC_{Mycoplasma_m}$ were performed with plate 9, positions C1, D1, G1 and H1 for the two runs.
603 The numbers of sequences for the other wells correspond to indexing mistakes due to false index-
604 pairing (see Table 1).

605 **Figure S2. Plots of the number of sequences (log (x+1) scale) from bacterial**
606 **OTUs in both PCR replicates (PCR1 & PCR2) for the 356 wild rodents analyzed**
607 **in the second MiSeq run.** Note that each rodent was tested with two duplicate PCRs. Green
608 points correspond to rodents with two positive results after the filtering process; orange points
609 correspond to rodents with one positive result and one negative result; and blue points correspond to
610 rodents with two negative results. The light blue area and lines correspond to the threshold values
611 used for the data filtering: samples below the lines are filtered out. See Figure S2 for plots
612 corresponding to the second MiSeq run. See Figure 3 for plots corresponding to the first MiSeq run.

613 **Figure S3. Phylogenetic trees of the sequences of the V4 hypervariable region**
614 **of the 16S rRNA gene for 12 pathogenic bacterial OTUs detected in wild**
615 **rodents from Senegal.** Sequences boxed with an orange line were retrieved from African
616 rodents and/or corresponds to positive controls (PC) for *Borellia burgdorferi*, *Mycoplasma mycoides*
617 and *Bartonella taylorii*. The other sequences were extracted from the SILVA database and GenBank.
618 Trees include all lineages collected for *Rickettsia*, *Bartonella*, *Ehrlichia* and *Orientia*, but only lineages
619 of the Spotted Fever Group for *Borrelia*, and lineages of the pneumonia group for *Mycoplasma*. The
620 numbers indicated are the bootstrap values >55%. The Fasta files used are available on request to
621 the corresponding author.
622

623 # References

624 1. **Taylor LH, Latham SM, Woolhouse ME.** 2001. Risk factors for human disease
625 emergence. Philos Trans R Soc Lond B Biol Sci 356: 983-989.

626 2. **King DA, Peckham C, Waage JK, Brownlie J, Woolhouse ME.** 2006.
627 Epidemiology. Infectious diseases: preparing for the future. Science 313:
628 1392-1393.

629 3. **Grogan LF, Berger L, Rose K, Grillo V, Cashins SD, et al.** 2014. Surveillance
630 for emerging biodiversity diseases of wildlife. PLoS Pathog 10: e1004015.

631 4. **Petrosino JF, Highlander S, Luna RA, Gibbs RA, Versalovic J. 2009.**
632 Metagenomic pyrosequencing and microbial identification. Clin Chem 55: 856-
633 866.

634 5. **Hugon P, Dufour JC, Colson P, Fournier PE, Sallah K, et al.** 2015. A
635 comprehensive repertoire of prokaryotic species identified in human beings.
636 Lancet Infect Dis 15: 1211-1219.

637 6. **Rynkiewicz EC, Hemmerich C, Rusch DB, Fuqua C, Clay K. 2015.**
638 Concordance of bacterial communities of two tick species and blood of their
639 shared rodent host. Mol Ecol 24: 2566-2579.

640 7. **Gofton AW, Doggett S, Ratchford A, Oskam CL, Paparini A, et al. 2015.**
641 Bacterial Profiling Reveals Novel "Ca. Neoehrlichia", Ehrlichia, and
642 Anaplasma Species in Australian Human-Biting Ticks. PLoS One 10:
643 e0145449.

644 8. **Razzauti M, Galan M, Bernard M, Maman S, Klopp C, et al. 2015.** A
645 Comparison between Transcriptome Sequencing and 16S Metagenomics for
646 Detection of Bacterial Pathogens in Wildlife. PLoS Negl Trop Dis 9: e0003929.

647 9. **Cosson JF, Galan M, Bard E, Razzauti M, Bernard M, et al.** 2015. Detection of
648 Orientia sp. DNA in rodents from Asia, West Africa and Europe. Parasit
649 Vectors 8: 172.

650 10. **Vayssier-Taussat M, Moutailler S, Michelet L, Devillers E, Bonnet S, et al.**
651 2013. Next generation sequencing uncovers unexpected bacterial pathogens
652 in ticks in western Europe. PLoS One 8: e81439.

653 11. **Williams-Newkirk AJ, Rowe LA, Mixson-Hayden TR, Dasch GA.** 2014.
654 Characterization of the bacterial communities of life stages of free living lone
655 star ticks. Amblyomma americanum). PLoS One 9: e102130.

656 12. **Williams-Newkirk AJ, Rowe LA, Mixson-Hayden TR, Dasch GA.** 2012.
657 Presence, genetic variability, and potential significance of "Candidatus
658 Midichloria mitochondrii" in the lone star tick Amblyomma americanum. Exp
659 Appl Acarol 58: 291-300.

660 13. **Carpi G, Cagnacci F, Wittekindt NE, Zhao F, Qi J, et al.** 2011. Metagenomic
661 profile of the bacterial communities associated with Ixodes ricinus ticks. PLoS
662 One 6: e25604.

663 14. **Vaumourin E, Vourc'h G, Gasqui P, Vayssier-Taussat M.** 2015. The
664 importance of multiparasitism: examining the consequences of co-infections
665 for human and animal health. Parasit Vectors 8: 545.

666 15. **Tollenaere C, Susi H, Laine AL.** 2016. Evolutionary and Epidemiological
667 Implications of Multiple Infection in Plants. Trends Plant Sci 21: 80-90.

668 16. **Vayssier-Taussat M, Albina E, Citti C, Cosson JF, Jacques MA, et al.** 2014.
669 Shifting the paradigm from pathogens to pathobiome: new concepts in the
670 light of meta-omics. Front Cell Infect Microbiol 4: 29.

671 17. **Vayssier-Taussat M, Kazimirova M, Hubalek Z, Hornok S, Farkas R, et al.**
672 2015. Emerging horizons for tick-borne pathogens: from the 'one pathogen-
673 one disease' vision to the pathobiome paradigm. Future Microbiol 10: 2033-
674 2043.

675 18. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013.
676 Development of a dual-index sequencing strategy and curation pipeline for
677 analyzing amplicon sequence data on the MiSeq Illumina sequencing
678 platform. Appl Environ Microbiol 79: 5112-5120.

679 19. **Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, et al.** 2010.
680 Comparison of two next-generation sequencing technologies for resolving
681 highly complex microbiota composition using tandem variable 16S rRNA gene
682 regions. Nucleic Acids Res 38: e200.

683 20. **Kuczynski J, Lauber CL, Walters WA, Parfrey LW, Clemente JC, et al.** 2012.
684 Experimental and analytical tools for studying the human microbiome. Nat Rev
685 Genet 13: 47-58.

686 21. **Sinha R, Chen J, Amir A, Vogtmann E, Shi J, et al.** 2015. Collecting Fecal
687 Samples for Microbiome Analyses in Epidemiology Studies. Cancer Epidemiol
688 Biomarkers Prev.

689 22. **Kircher M, Heyn P, Kelso J. 2011.** Addressing challenges in the production and
690 analysis of illumina sequencing data. BMC Genomics 12: 382.

691 23. **Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, et al.** 2014. Reagent
692 and laboratory contamination can critically impact sequence-based
693 microbiome analyses. BMC Biol 12: 87.

694 24. **Horvath A, Peto Z, Urban E, Vagvolgyi C, Somogyvari F.** 2013. A novel,
695 multiplex, real-time PCR-based approach for the detection of the commonly
696 occurring pathogenic fungi and bacteria. BMC Microbiol 13: 300.

697 25. **Caro-Quintero A, Ochman H. 2015.** Assessing the Unseen Bacterial Diversity in
698 Microbial Communities. Genome Biol Evol 7: 3416-3425.

699 26. **Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A,**
700 **Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, Apprill A, Knight B.**
701 2015. Improved bacterial 16S rRNA gene. V4 and V4-5. and fungal internal
702 transcribed spacer marker gene primers for microbial community surveys.
703 mSystems 1:e00009-15

704 27. **Kircher M, Sawyer S, Meyer M**. 2012. Double indexing overcomes inaccuracies
705 in multiplex sequencing on the Illumina platform. Nucleic Acids Res 40: e3.

706 28. **Esling P, Lejzerowicz F, Pawlowski J.** 2015. Accurate multiplexing and filtering
707 for high-throughput amplicon-sequencing. Nucleic Acids Res 43: 2513-2524.

708 29. **Bystrykh LV. 2012.** Generalized DNA barcode design based on Hamming
709 codes. PLoS One 7: e36852.

710 30. **Meyerhans A, Vartanian JP, Wain-Hobson S.** 1990. DNA recombination during
711 PCR. Nucleic Acids Res 18: 1687-1691.

712 31. **Paabo S, Irwin DM, Wilson AC.** 1990. DNA damage promotes jumping between
713 templates during enzymatic amplification. J Biol Chem 265: 4718-4721.

714 32. **Odelberg SJ, Weiss RB, Hata A, White R.** 1995. Template-switching during
715 DNA synthesis by Thermus aquaticus DNA polymerase I. Nucleic Acids Res
716 23: 2049-2057.

717 33. **Lahr DJ, Katz LA**. 2009. Reducing the impact of PCR-mediated recombination in
718 molecular evolution and environmental studies using a new-generation high-
719 fidelity DNA polymerase. Biotechniques 47: 857-866.

720 34. **Whiteford N, Skelly T, Curtis C, Ritchie ME, Lohr A, et al.** 2009. Swift: primary
721 data analysis for the Illumina Solexa sequencing platform. Bioinformatics 25:
722 2194-2199.

723 35. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, et al.** 2012.
724 Ultra-high-throughput microbial community analysis on the Illumina HiSeq and
725 MiSeq platforms. ISME J 6: 1621-1624.

726 36. **Smith DP, Peay KG.** 2014. Sequence depth, not PCR replication, improves
727 ecological inference from next generation DNA sequencing. PLoS One 9:
728 e90234.

729 37. **Kilianski A, Haas JL, Corriveau EJ, Liem AT, Willis KL, et al.** 2015. Bacterial
730 and viral identification and differentiation by amplicon sequencing on the
731 MinION nanopore sequencer. Gigascience 4: 12.

732 38. **Callahan B, Proctor D, Relman D, Fukuyama J, Holmes S.** 2016.
733 Reproducible Research Workflow in R for the Analysis of Personalized Human
734 Microbiome Data. Pac Symp Biocomput 21: 183-194.

735 39. **Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, et al.** 2014. The SILVA
736 and "All-species Living Tree Project. LTP)" taxonomic frameworks. Nucleic
737 Acids Res 42: D643-648.

738 40. **Westcott SL, Schloss PD.** 2015. De novo clustering methods outperform
739 reference-based methods for assigning 16S rRNA gene sequences to
740 operational taxonomic units. PeerJ 3: e1487.

741 41. **Sinha R, Abnet CC, White O, Knight R, Huttenhower C.** 2015. The microbiome
742 quality control project: baseline study design and future directions. Genome
743 Biol 16: 276.

744 42. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al.** 2009.
745 Introducing mothur: open-source, platform-independent, community-supported
746 software for describing and comparing microbial communities. Appl Environ
747 Microbiol 75: 7537-7541.

748 43. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, et al.** 2013. The SILVA
749 ribosomal RNA gene database project: improved data processing and web-
750 based tools. Nucleic Acids Res 41: D590-596.

751 44. **Gasparich GE, Whitcomb RF, Dodge D, French FE, Glass J, et al.** 2004. The
752 genus Spiroplasma and its non-helical descendants: phylogenetic

753 classification, correlation with phenotype and roots of the Mycoplasma
754 mycoides clade. Int J Syst Evol Microbiol 54: 893-918.

755 45. **Gomez-Diaz E, Doherty PF, Jr., Duneau D, McCoy KD.** 2010. Cryptic vector
756 divergence masks vector-specific patterns of infection: an example from the
757 marine cycle of Lyme borreliosis. Evol Appl 3: 391-401.

758 46. **Sashida H, Sasaoka F, Suzuki J, Fujihara M, Nagai K, et** al. 2013. Two
759 clusters among Mycoplasma haemomuris strains, defined by the 16S-23S
760 rRNA intergenic transcribed spacer sequences. J Vet Med Sci 75: 643-648.

761 47. **Neimark H, Johansson KE, Rikihisa Y, Tully JG.** 2001. Proposal to transfer
762 some members of the genera Haemobartonella and Eperythrozoon to the
763 genus Mycoplasma with descriptions of 'Candidatus Mycoplasma haemofelis',
764 'Candidatus Mycoplasma haemomuris', 'Candidatus Mycoplasma haemosuis'
765 and 'Candidatus Mycoplasma wenyonii'. Int J Syst Evol Microbiol 51: 891-899.

766 48. **Neimark H, Peters W, Robinson BL, Stewart LB.** 2005. Phylogenetic analysis
767 and description of Eperythrozoon coccoides, proposal to transfer to the genus
768 Mycoplasma as Mycoplasma coccoides comb. nov. and Request for an
769 Opinion. Int J Syst Evol Microbiol 55: 1385-1391.

770 49. **Steer JA, Tasker S, Barker EN, Jensen J, Mitchell J, et al.** 2011. A novel
771 hemotropic Mycoplasma. hemoplasma. in a patient with hemolytic anemia and
772 pyrexia. Clin Infect Dis 53: e147-151.

773 50. **Pitcher DG, Nicholas RA.** 2005. Mycoplasma host specificity: fact or fiction? Vet
774 J 170: 300-306.

775 51. **Trape JF, Diatta G, Arnathau C, Bitam I, Sarih M, et al.** 2013. The
776 epidemiology and geographic distribution of relapsing fever borreliosis in West
777 and North Africa, with a review of the Ornithodoros erraticus complex. Acari:
778 Ixodida). PLoS One 8: e78473.

779 52. **Rar VA, Pukhovskaya NM, Ryabchikova EI, Vysochina NP, Bakhmetyeva**
780 **SV, et al.** 2015. Molecular-genetic and ultrastructural characteristics of
781 'Candidatus Ehrlichia khabarensis', a new member of the Ehrlichia genus.
782 Ticks Tick Borne Dis 6: 658-667.

783 53. **Perlman SJ, Hunter MS, Zchori-Fein E.** 2006. The emerging diversity of
784 Rickettsia. Proc Biol Sci 273: 2097-2106.

785 54. **Elliott SP. 2007.** Rat bite fever and Streptobacillus moniliformis. Clin Microbiol
786 Rev 20: 13-22.

787 55. **Izzard L, Fuller A, Blacksell SD, Paris DH, Richards AL, et al.** 2010. Isolation
788 of a novel Orientia species. O. chuto sp. nov.. from a patient infected in Dubai.
789 J Clin Microbiol 48: 4404-4409.

790 56. **Buffet JP, Kosoy M, Vayssier-Taussat M**. 2013. Natural history of Bartonella-
791 infecting rodents in light of new knowledge on genomics, diversity and
792 evolution. Future Microbiol 8: 1117-1128.

793 57. **Mediannikov O, Aubadie M, Bassene H, Diatta G, Granjon L, Fenollar F**. 2014.
794 Three new *Bartonella* species from rodents in Senegal. Int J Infec Dis
795 21S:335.

796 58. **Julius R, Bastos A, Brettschneider H, Chimimba C.** 2012. Dynamics of
797 Rodent-borne zoonotic diseases and their reservoir hosts: invasive *Rattus* in
798 South Africa. Proc 25th Vertebrate Pest Conference, Monterey, California,
799 USA.

800 59. **Bhatt KM, Mirza NB. 1992**. Rat bite fever: a case report of a Kenyan. East Afr
801 Med J 69: 542-543.

802 60. **Gray HH. 1967.** Squirrel bite fever. Trans R Soc Trop Med Hyg 61:857.

803 61. **Laudisoit A, Falay D, Amundala N, Akaibe D, de Bellocq JG, et al. 2014**. High
804 prevalence of Rickettsia typhi and Bartonella species in rats and fleas,
805 Kisangani, Democratic Republic of the Congo. Am J Trop Med Hyg 90: 463-
806 468.

807 62. **Dupont HT, Brouqui P, Faugere B, Raoult D.** 1995. Prevalence of antibodies to
808 Coxiella burnetti, Rickettsia conorii, and Rickettsia typhi in seven African
809 countries. Clin Infect Dis 21: 1126-1133.

810 63. **Ammar AM, Sabry MZ, Kirchhoff H.** 1980. Distribution of mycoplasmas in field
811 and laboratory rodents in Egypt. Z Versuchstierkd 22: 216-223.

812 64. **Ndip LM, Labruna M, Ndip RN, Walker DH, McBride JW.** 2009. Molecular and
813 clinical evidence of Ehrlichia chaffeensis infection in Cameroonian patients
814 with undifferentiated febrile illness. Ann Trop Med Parasitol 103: 719-725.

815 65. **Kelly DJ, Foley DH, Richards AL.** 2015. A Spatiotemporal Database to Track
816 Human Scrub Typhus Using the VectorMap Application. PLoS Negl Trop Dis
817 9: e0004161.

818 66. **Konecny A, Estoup A, Duplantier JM, Bryja J, Ba K, et al.** 2013. Invasion
819 genetics of the introduced black rat. Rattus rattus. in Senegal, West Africa.
820 Mol Ecol 22: 286-300.

821 67. **Dalecky A, Ba K, Piry S, Lippens C, Diagne CA, Kane M, Sow A, Diallo M,**
822 **Niang Y, Konecny A, Sarr N, Artige E, Charbonnel N, Granjon L,**
823 **Duplantier JM, Brouat C.** 2015. Range expansion of the invasive house
824 mouse *Mus musculus domesticus* in Senegal, West Africa: a synthesis of
825 trapping data over three decades, 1983–2014. *Mammal Review* 45: 176-190

826 68. **Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL.** 2016. Analysis of the
827 microbiome: Advantages of whole genome shotgun versus 16S amplicon
828 sequencing. Biochem Biophys Res Commun 469: 967-977.

829 69. **Glaeser SP, Kampfer P.** 2015. Multilocus sequence analysis. MLSA. in
830 prokaryotic taxonomy. Syst Appl Microbiol 38: 237-245.

831 70. **Michelet L, Delannoy S, Devillers E, Umhang G, Aspan A, et al.** 2014. High-
832 throughput screening of tick-borne pathogens in Europe. Front Cell Infect
833 Microbiol 4: 103.

834 71. **Gutierrez R, Morick D, Cohen C, Hawlena H, Harrus S.** 2014. The effect of
835 ecological and temporal factors on the composition of Bartonella infection in
836 rodents and their fleas. ISME J 8: 1598-1608.

837 72. **Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM.** 2009. A method
838 for studying protistan diversity using massively parallel sequencing of V9

839 hypervariable regions of small-subunit ribosomal RNA genes. PLoS One 4:
840 e6372.

841 73. **Mueller RC, Gallegos-Graves LV, Kuske CR.** 2016. A new fungal large subunit
842 ribosomal RNA primer for high-throughput sequencing surveys. FEMS
843 Microbiol Ecol 92.

844 74. **Anthony SJ, Epstein JH, Murray KA, Navarrete-Macias I, Zambrana-Torrelio**
845 **CM, et al.** 2013. A strategy to estimate unknown viral diversity in mammals.
846 MBio 4: e00598-00513.

847 75. **Galan M, Pages M, Cosson JF.** 2012. Next-generation sequencing for rodent
848 barcoding: species identification from fresh, degraded and environmental
849 samples. PLoS One 7: e48374.

850 76. **Robasky K, Lewis NE, Church GM.** 2014. The role of replicates for error
851 mitigation in next-generation sequencing. Nat Rev Genet 15: 56-62.

852 77. **Galan M, Guivier E, Caraux G, Charbonnel N, Cosson JF**. 2010. A 454
853 multiplex sequencing method for rapid and reliable genotyping of highly
854 polymorphic genes in large-scale studies. BMC Genomics 11: 296.

855 78. **Lange V, Bohme I, Hofmann J, Lang K, Sauter J, et al.** 2014. Cost-efficient
856 high-throughput HLA typing by MiSeq amplicon sequencing. BMC Genomics
857 15: 63.

858 79. **McFarlane R, Sleigh A, McMichael T.** 2012. Synanthropy of wild mammals as a
859 determinant of emerging infectious diseases in the Asian-Australasian region.
860 Ecohealth 9: 24-35.

861 80. **Meerburg BG, Singleton GR, Kijlstra A.** 2009. Rodent-borne diseases and their
862 risks for public health. Crit Rev Microbiol 35: 221-270.

863 81. **Civen R, Ngo V.** 2008. Murine typhus: an unrecognized suburban vectorborne
864 disease. Clin Infect Dis 46: 913-918.

865 82. **Watt G, Parola P.** 2003. Scrub typhus and tropical rickettsioses. Curr Opin Infect
866 Dis 16: 429-436.

867 83. **Vayssier-Taussat M, Moutailler S, Féménia F, Raymond P, Croce O, La**
868 **Scola B, Fournier PE, Raoult D.** 2016. Identification of new zoonotic
869 *Bartonella* species responsible for bacteremia in humans bitten by ticks.
870 Emerg Inf Dis 22:

871 84. **Mills JN, Childs J, Ksiazek TG, Peters CJ, Velleca WM.** 1995. Methods for
872 trapping and sampling small mammals for virologic testing. CDC, Atlanta.

873 85. **Herbreteau V, Rerkamnuaychoke W, Jittapalapong S, Chaval Y, Cosson JF,**
874 **Morand S.** 2011. Field and laboratory protocols for rodent studies. Kasetsart
875 University Press 46 p. http://www.ceropath.org/research/protocols

876 86. **Mebius RE, Kraal G.** 2005. Structure and function of the spleen. Nat Rev
877 Immunol 5: 606-616.

878 87. **D'Amore R, Ijaz UZ, Schirmer M, Kenny JG, Gregory R, et al.** 2016. A
879 comprehensive benchmarking study of protocols and sequencing platforms for
880 16S rRNA community profiling. BMC Genomics 17: 55.

881 88. **Gouy M, Guindon S, Gascuel O.** 2010. SeaView version 4: A multiplatform
882 graphical user interface for sequence alignment and phylogenetic tree
883 building. Mol Biol Evol 27: 221-224.

884 89. **Parola P, Inokuma H, Camicas JL, Brouqui P, Raoult D.** 2001. Detection and
885 identification of spotted fever group Rickettsiae and Ehrlichiae in African ticks.
886 Emerg Infect Dis 7: 1014-1017.

887 90. **Qiu Y, Nakao R, Ohnuma A, Kawamori F, Sugimoto C.** 2014. Microbial
888 population analysis of the salivary glands of ticks; a possible strategy for the
889 surveillance of bacterial pathogens. PLoS One 9: e103961.

890 91. **Lecompte E, Aplin K, Denys C, Catzeflis F, Chades M, Chevret P.** 2008.
891 Phylogeny and biogeography of African Murinae based on mitochondrial and
892 nuclear gene sequences, with a new tribal classification of the subfamily. BMC
893 Evol Biol 8:199

894 92. **Reiczigel J. 2003**. Confidence intervals for the binomial parameter: some new
895 considerations. Statistics in Medicine 22:611-621

896