# 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

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

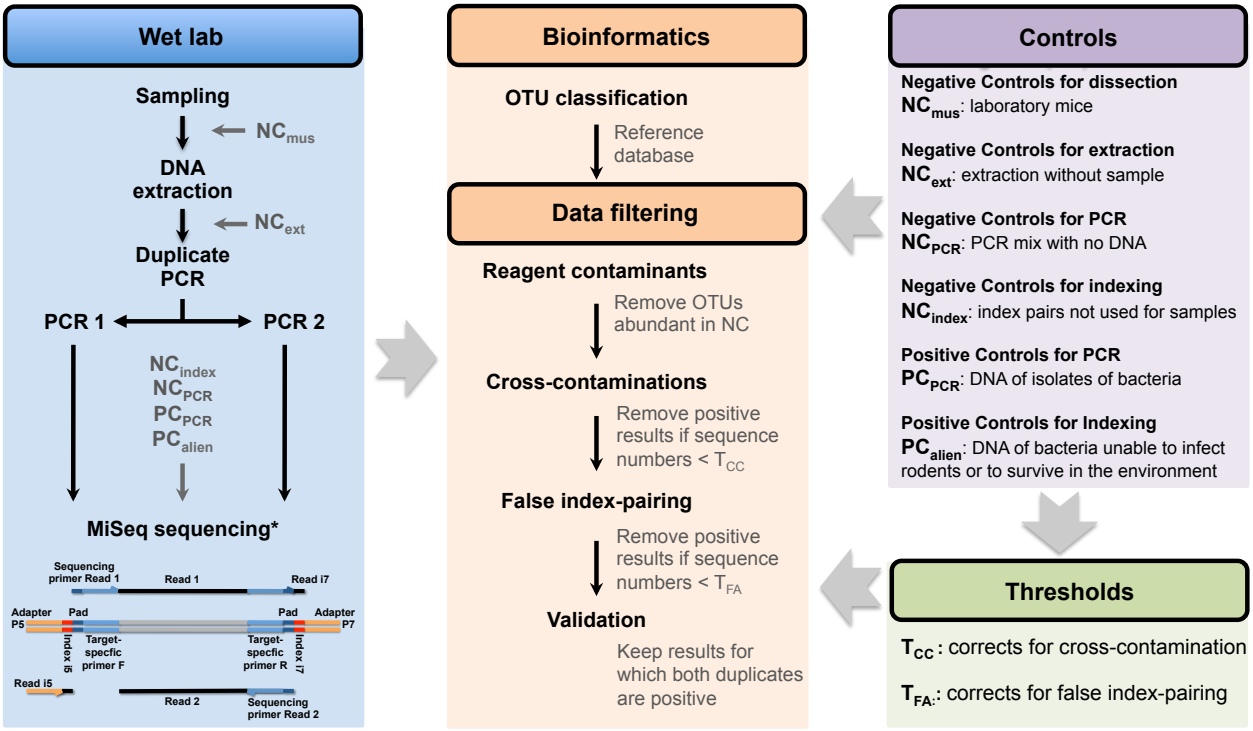



**Figure 1. Workflow of the wet laboratory, and for bioinformatics and data filtering procedures**, and a list of controls and thresholds included in the process of data filtering for the elimination of false-positive results for 16S rRNA amplicon sequencing. Reagent contaminants were detected by analyzing 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 $PC_{index}$; $T_{FA}$: 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. *see Kozich et al 2013 for details on the sequencing.

***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 experimental steps and data processing listed in Table 1 made it impossible to infer
217 prevalence and co-occurrence directly from the table of sequence presence/absence
218 in the PCR products. We suggest filtering the data with data-based estimates of the
219 different biases calculated from the multiple controls introduced during the process.
220 This strategy involves calculating sequence number thresholds from our bias
221 estimates. Two different thresholds were set for each of the 12 OTUs and two MiSeq

222 runs. We then discarded positive results associated with numbers of sequences
223 below the 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 This phenomenon is essentially due to mixed clusters during the sequencing [27].
242 We used two kinds of controls to detect incorrect assignments (Figure 1).

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

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

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) | | $NC_{PCR}$ | | $NC_{ext}$ | | $NC_{mus}$ | | $PC_{Bartonnela_t}$ | | $PC_{Borrelia_b}$ | | $PC_{Mycoplasma_m}$ | | | |
| | | 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 | $T_{cc}$* | $T_{FA}$** |
| Run 1 | **Whole dataset** | 7960533 | 7149444 | 64722 | 45900 | 8002 | 39308 | 8741 | 68350 | 26211 | 137424 | 73134 | 239465 | 120552 | 280642 | 82933 | / | / |
| | ***Mycoplasma*_OTU_1** | 1410218 | 1410189 | 61807 | 2 | 1 | 3 | 2 | 9 | 5 | 3 | 3 | 8 | 6 | 4 | 3 | 6 | 282 |
| | ***Mycoplasma*_OTU_ 3** | 507376 | 507369 | 36335 | 2 | 1 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 101 |
| | ***Ehrlichia*_OTU** | 649451 | 649423 | 63137 | 4 | 2 | 3 | 2 | 7 | 4 | 1 | 1 | 1 | 1 | 12 | 6 | 6 | 130 |
| | ***Borrelia*_OTU** | 345873 | 345845 | 28528 | 4 | 4 | 7 | 4 | 9 | 4 | 1 | 1 | 0 | 0 | 7 | 3 | 4 | 69 |
| | ***Orientia*_OTU** | 279965 | 279957 | 29503 | 1 | 1 | 4 | 1 | 0 | 0 | 2 | 2 | 0 | 0 | 1 | 1 | 2 | 56 |
| | ***Bartonella*_OTU** | 202127 | 67973 | 16145 | 1 | 1 | 1 | 1 | 1 | 1 | 134124 | 71163 | 7 | 4 | 20 | 9 | 9 | 40 |
| | ***M. mycoides****** | 280151 | 338 | 28 | 0 | 0 | 0 | 0 | 2 | 2 | 34 | 20 | 24 | 18 | 279753 | 82767 | / | / |
| | ***B. burgdorferi****** | 238772 | 420 | 43 | 0 | 0 | 0 | 0 | 0 | 0 | 38 | 21 | 238238 | 119586 | 76 | 23 | / | / |
| Run 2 | **Whole dataset** | 6687060 | 6525107 | 42326 | 61231 | 9145 | 53334 | 7669 | / | / | 12142 | 7518 | 13378 | 7164 | 21868 | 6520 | / | / |
| | ***Mycoplasma*_OTU_1** | 155486 | 155486 | 7703 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 31 |
| | ***Mycoplasma*_OTU_2** | 1036084 | 1035890 | 23588 | 1 | 1 | 192 | 115 | / | / | 0 | 0 | 0 | 0 | 1 | 1 | 115 | 207 |
| | ***Mycoplasma*_OTU_ 3** | 127591 | 127590 | 5072 | 1 | 1 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 26 |
| | ***Mycoplasma* _OTU_4** | 85596 | 85583 | 20146 | 0 | 0 | 13 | 13 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 17 |
| | ***Mycoplasma*_OTU_ 5** | 56324 | 56324 | 10760 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 11 |
| | ***Mycoplasma*_OTU_ 6** | 13356 | 13356 | 1482 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| | ***Ehrlichia*_OTU** | 74017 | 74017 | 19651 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 |
| | ***Borrelia*_OTU** | 21636 | 21636 | 3085 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| | ***Orientia*_OTU** | 307 | 307 | 181 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ***Bartonella*_OTU** | 1559028 | 1547652 | 14515 | 1 | 1 | 2 | 2 | / | / | 11297 | 6714 | 2 | 2 | 74 | 59 | 59 | 312 |
| | ***Streptobacillus*_OTU** | 32399 | 32399 | 6245 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| | ***Rickettsia*_OTU** | 589 | 589 | 329 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ***M. mycoides****** | 16854 | 2 | 1 | 0 | 0 | 0 | 0 | / | / | 0 | 0 | 0 | 0 | 16852 | 5766 | / | / |
| | ***B. 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

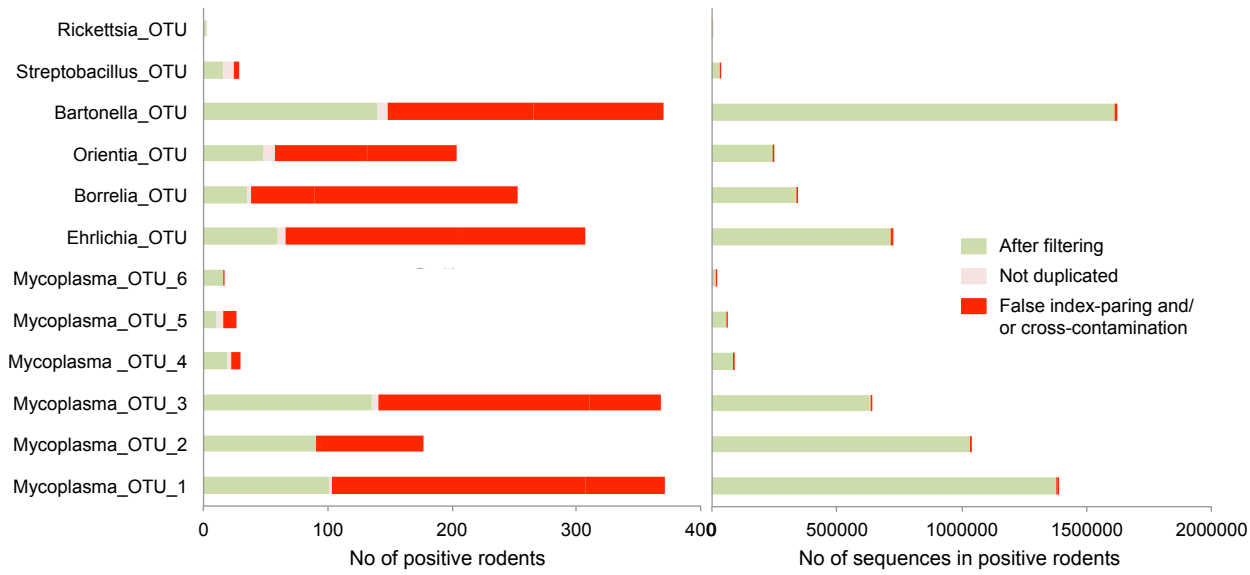
**: Threshold $T_{FA}$ is based to the false assignment rate (0.02%) weighted by the total number of sequences of each OTU in each run

***: *Mycoplasma mycoides* and *Borrelia burgdorferi* 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

279 estimated for $PC_{Mycoplasma_m}$ and $PC_{Borrelia_b}$ separately. $R_{fa}$ reached 0.010% and
280 0.018% for $PC_{Mycoplasma_m}$ and $PC_{Borrelia_b}$, respectively. We adopted a conservative
281 approach, by fixing the $R_{fa}$ value to 0.020%. This number signifies that each PCR
282 product may receive a maximum 0.020% of the total number of sequences of an
283 OTU present in a run due to false index-pairing. Moreover, the number of
284 misassigned sequences for a specific OTU into a PCR product should increase with
285 the total number of sequences of the OTU in the MiSeq run. We therefore defined the
286 second threshold ($T_{FA}$) as the total number of sequences in the run for an OTU
287 multiplied by $R_{fa}$. $T_{FA}$ values varied with the abundance of each OTU in the
288 sequencing run (Table 2). Because the abundance of each OTU varied from one
289 sequencing run to another, $T_{FA}$ also varied according to the sequencing run. The use
290 of the $T_{FA}$ led to 0% to 87% of positive results being discarded. This corresponded to
291 0% to 0.71% of the sequences, depending on the OTU (Figure 2, Table S4).

292




293

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

302 ***Validation with PCR replicates.*** Random contamination may occur during the
303 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 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.

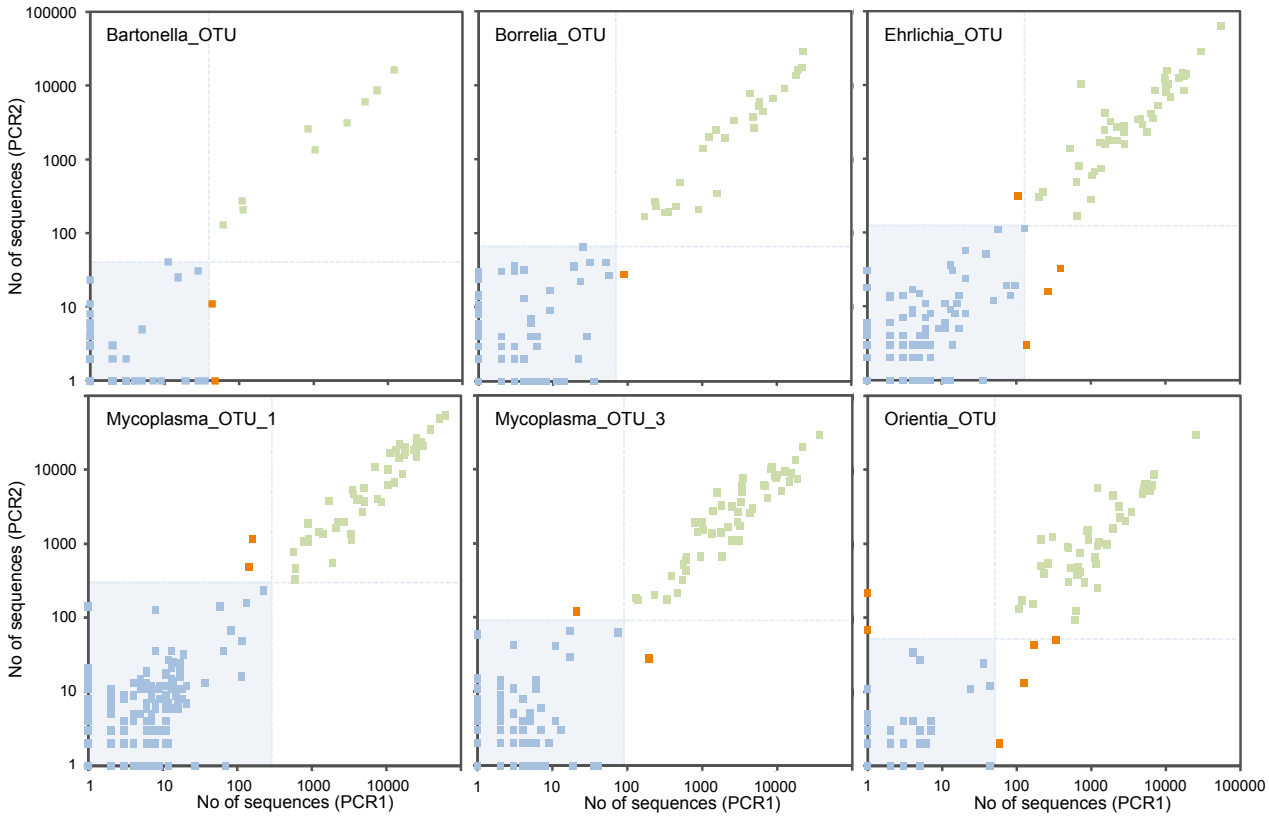



**Figure 3. Plots of the number of sequences (log (x+1) scale) from bacterial OTUs in both PCR replicates (PCR1 & PCR2) of the 348 wild rodents analyzed in the first MiSeq run.** Note that each rodent was tested with two replicate PCRs. Green points correspond to rodents with two positive results after 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.

324 At this final validation step, 0% to 60% of the positive results for a given OTU were
325 discarded, corresponding to only 0% to 7.17% of the sequences (Figure 2, Table S4
326 and Table S5). Note that the number of replicates may be increased, as described in
327 the strategy of Gómez-Díaz *et al* [45].

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

333 ***Refining bacterial taxonomic identification.*** We refined the taxonomic
334 identification of the 12 bacterial OTUs through phylogenetic and blast analyses. We
335 were able to identify the bacteria present down to genus level and, in some cases,
336 we could even identify the most likely species (Table 3 and Figure S3). For instance,
337 the sequences of the six *Mycoplasma* OTUs were consistent with three different
338 species — *M. haemomuris* for OTU_1 and 3, *M. coccoides* for OTU_4, 5 and 6, and
339 *M. species novo* [46] for OTU_2 — with high percentages of sequence identity
340 (≥93%) and strong bootstrap support (≥80%). All three of these species belong to the
341 Hemoplasma group, which is known to infect mice, rats and other mammals [47,48],
342 and is thought to cause anemia in humans [49,50]. The *Borrelia* sequences grouped
343 with three different species of the relapsing fever group (*crocidurae*, *duttonii* and
344 *recurrentis*) with a high percentage of identity (100%) and a reasonably high
345 bootstrap value (71%). In West Africa, *B. crocidurae* causes severe borreliosis, a
346 rodent-borne disease transmitted by ticks and lice [51]. The *Ehrlichia* sequences
347 were 100% identical to and clustered with the recently described Candidatus
348 *Ehrlichia khabarensis* isolated from voles and shrews in the Far East of Russia [52].
349 The *Rickettsia* sequences were 100% identical to the sequence of *R. typhi*, a species
350 of the typhus group responsible for murine typhus [53], but this clade was only
351 weakly differentiated from many other *Rickettsia* species and had only moderate
352 bootstrap support (61%). The most likely species corresponding to the sequences of
353 the *Streptobacillus* OTU was *S. moniliformis*, with a high percentage of identity
354 (100%) and a high bootstrap value (100%). This bacterium is common in rats and
355 mice and causes a form of rat-bite fever, Haverhill fever [54]. The *Orientia* sequences
356 corresponded to *O. chuto*, with a high percentage of identity (100%) and a high

357 **Table 3. Detection of 12 bacterial OTUs in the four wild rodent species (*n*=704)**
358 **sampled in Senegal, and the biology and pathogenicity of the corresponding**
359 **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%)<br>*duttonii* (100%)<br>*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%)<br>*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

360

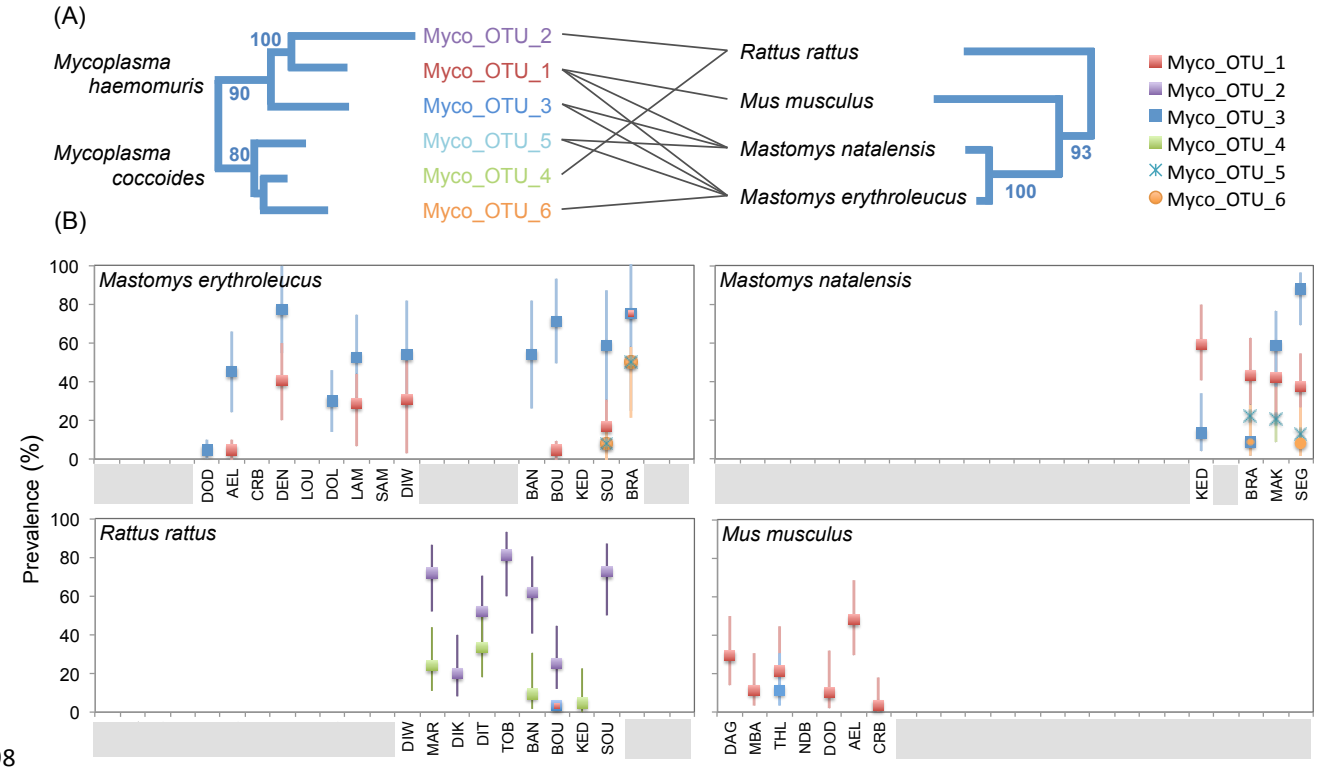
361 bootstrap value (77%). This species was recently isolated from a patient infected in
362 Dubai [55]. Finally, accurate species determination was not possible for *Bartonella*,
363 as the 16S rRNA gene does not resolve the species of this genus well [56]. Indeed,
364 the sequences from the *Bartonella* OTU detected in our rodents corresponded to at
365 least seven different species (*elizabethae, japonica, pachyuromydis, queenslandis,*
366 *rattaustraliani, tribocorum, vinsonii*) and a putative new species recently identified in
367 Senegalese rodents [57].

368 These findings demonstrate the considerable potential of 16S rRNA amplicon
369 sequencing for the rapid identification of zoonotic agents in wildlife, provided that the
370 post-sequencing data are cleaned beforehand. *Borrelia* [51] and *Bartonella* [57] were
371 the only ones of the seven pathogenic bacterial genera detected here in Senegalese
372 rodents to have been reported as present in rodents from West Africa before. The
373 other bacterial genera identified here have previously been reported to be presented
374 in rodents only in other parts of Africa or on other continents. *S. moniliformis* has
375 recently been detected in rodents from South Africa [58] and there have been a few
376 reports of human streptobacillosis in Kenya [59] and Nigeria [60]. *R. typhi* was
377 recently detected in rats from Congo, in Central Africa [61], and human seropositivity
378 for this bacterium has been reported in coastal regions of West Africa [62]. With the
379 exception of one report in Egypt some time ago [63], *Mycoplasma* has never before
380 been reported in African rodents. Several species of *Ehrlichia* (from the *E. canis*
381 group: *E. chaffeensis, E. ruminantium, E. muris, E. ewingii*) have been characterized
382 in West Africa, but only in ticks from cattle [89] together with previous reports of
383 possible cases of human ehrlichioses in this region [64]. Finally, this study reports the
384 first identification of *Orientia* in African rodents [9]. There have already been a few
385 reports of suspected human infection with this bacterium in Congo, Cameroon,
386 Kenya and Tanzania [65].

387 ***Estimating prevalence and coinfection.*** After data filtering, we were able to
388 estimate the prevalence in rodent populations and to assess coinfection in individual
389 rodents, for the 12 bacterial OTUs. Bacterial prevalence varied considerably between
390 rodent species (Table 3). *Bartonella* was highly prevalent in the two multimammate
391 rats *M. natalensis* (73%) and *M. erythroleucus* (27%); *Orientia* was prevalent in the
392 house mouse *M. musculus* (22%) and *Ehrlichia* occurred frequently in only one on
393 the two multimammate rats *M. erythroleucus* (18%). By contrast, the prevalence of

394 *Streptobacillus* and *Rickettsia* was low in all rodent species (<5%). Coinfection was
395 common, as 184 rodents (26%) were found to be coinfected with bacteria from two
396 (19%), three (5%), four (2%) or five (0.1%) different bacterial pathogens.

397




398

399

400 **Figure 4. Prevalence of *Mycoplasma* lineages in Senegalese rodents, by site, and**
401 **phylogenetic associations between *Mycoplasma* lineages and rodent species.** (A)
402 Comparison of phylogenetic trees based on the 16S rRNA V4-sequences of *Mycoplasma*, and on the
403 mitochondrial cytochrome *b* gene and the two nuclear gene fragments (IRBP exon 1 and GHR) for
404 rodents (rodent tree redrawn from [91]). Lines link the *Mycoplasma* lineages detected in the various
405 rodent species (for a minimum site prevalence exceeding 10%). The numbers next to branches are
406 bootstrap values (only shown if >70%). (B) Plots of OTU prevalence with 95% confidence intervals
407 calculated by Sterne's exact method [92] by rodent species and site (see [67] for more information about
408 site codes and their geographic locations). The gray bars in the X-legend indicate sites from which the
409 rodent species concerned is absent.
410

411 Interestingly, several *Mycoplasma* OTUs appeared to be specific to a rodent genus
412 or species (Table 3, Figure 4). OTU_2, putatively identified as a recently described
413 lineage isolated from brown rat, *Rattus norvegicus* [46], was specifically associated
414 with *R. rattus* in this study. Of the OTUs related to *M. coccoides*, OTU_4 was found
415 exclusively in *R. rattus,* whereas OTUs_5 and 6 seemed to be specific to the two
416 multimammate rats (*M. erytholeucus and M. natalensis*). Comparative phylogenies of
417 *Mycoplasma* OTUs and rodents showed that *R. rattus*, which is phylogenetically

418 more distantly related to the other three rodents, contained a *Mycoplasma*
419 community different from that in the *Mus-Mastomys* rodent clade (Figure 4).
420 Pathogen prevalence also varied considerably between sites, as shown for the six
421 *Mycoplasma* OTUs (Figure 4). This suggests that the infection risks for animals and
422 humans vary greatly according to environmental characteristics and/or biotic features
423 potentially related to recent changes in the distribution of rodent species in Senegal
424 [66,67]

425

## 426 Perspectives

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

449 gene used by Gutierrez *et al*. [71] for the *Bartonella* genus, in parallel with the 16S
450 rRNA-V4 region.

451 This strategy could also easily be adapted for other microbes, such as protists, fungi
452 and even viruses, provided that universal primers are available for their detection
453 (see [72,73] for protists and fungi, and [74] for degenerate virus family-level primers
454 for viruses). Finally, our filtering method could also be translated to any other post-
455 sequencing dataset of indexed or tagged amplicons in the framework of
456 environmental studies (e.g. metabarcoding for diet analysis and biodiversity
457 monitoring [75], the detection of rare somatic mutations [76] or the genotyping of
458 highly polymorphic genes (e.g. MHC or HLA typing, [77,78]).

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

473

474 ## Materials & Methods

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

481 ***Sample collection.*** Rodents were killed by cervical dislocation, as recommended
482 by Mills *et al.* [84] and dissected as described in Herbreteau *et al.* [85]. Rodent
483 species were identified by morphological and/or molecular techniques [67]. Cross-
484 contamination during dissection was prevented by washing the tools used
485 successively in bleach, water and alcohol between rodents. We used the spleen for
486 bacterial detection, because this organ is a crucial site of early exposure to bacteria
487 [86]. Spleens were placed in RNAlater (Sigma) and stored at 4°C for 24 hours and
488 then at -20°C until their use for genetic analyses.

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

507 ***DNA extraction and PCRs.*** All laboratory manipulations were conducted with
508 filter tips, under a sterile hood, in a DNA-free room. DNA was extracted with the
509 DNeasy 96 Tissue Kit (Qiagen) with final elution in 200 µl of elution buffer. One
510 extraction blank ($NC_{ext}$), corresponding to an extraction without sample tissue, was
511 systematically added to each of the eight DNA extraction microplates. DNA was
512 quantified with a NanoDrop 8000 spectrophotometer (Thermo Scientific), to confirm

513 the presence of a minimum of 10 ng/µl of DNA in each sample. DNA amplification
514 was performed in 5 µL of Multiplex PCR Kit (Qiagen) Master Mix, with 4 µL of
515 combined i5 and i7 primers (3.5µM) and 2 µL of genomic DNA. PCR began with an
516 initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at
517 95°C for 20 s, annealing at 55°C for 15 s and extension at 72°C for 5 minutes,
518 followed by a final extension step at 72°C for 10 minutes. PCR products (3 µL) were
519 verified by electrophoresis in a 1.5% agarose gel. One PCR blank ($NC_{PCR}$),
520 corresponding to the PCR mix with no DNA, was systematically added to each of the
521 18 PCR microplates. DNA was amplified in replicate for all wild rodent samples
522 (*n*=711) (Table S1).

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

544 ***Bioinformatic and taxonomic classification.*** MiSeq datasets were
545 processed with mothur v1.34 [42] and with the MiSeq standard operating procedure

546 (SOP) [18]. We used the Silva SSU Reference database v119 [43] and the Silva
547 taxonomy file for taxonomic assignment. The abundance table generated by mothur
548 for each PCR product and each OTU was filtered as described in the Results section.
549 The most abundant sequence for each OTU in each sample was extracted from the
550 sequence dataset with a custom-written Perl script. The most abundant sequences
551 for the 12 OTUs are available from GenBank (Accession Number KU697337 to
552 KU697350). The sequences were aligned with reference sequences from bacteria of
553 the same genus available from the SILVA SSU Ref NR database v119, using
554 SeaView v4 [88]. The FASTA files used are available on request to the
555 corresponding author. Phylogenetic trees were generated from the K2P distance with
556 SeaView and species were identified on the basis of the "closest phylogenetic
557 species". We also used our sequences for blast analyses of GenBank, to identify the
558 reference sequences to which they displayed the highest percentage identity.
559

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566 were performed on the CBGP HPC computational platform. The funders had no role
567 in study design, data collection and analysis, the decision to publish, or preparation
568 of the manuscript.
569

## 570 Authors' contributions

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

577 and to improve the manuscript. All the authors have read and approved the final
578 manuscript.

579

## 580 Supplementary materials

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

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

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

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

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

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

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

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

615 **Figure S3. Phylogenetic trees of the 16S rRNA V4 sequences for 12 pathogenic**
616 **bacterial OTUs detected in wild rodents from Senegal.** Sequences boxed with an
617 orange line were retrieved from African rodents and/or corresponds to positive controls (PC) for

618 *Borellia burgdorferi*, *Mycoplasma mycoides* and *Bartonella taylorii*. The other sequences were
619 extracted from the SILVA database and GenBank. Trees include all lineages collected for *Rickettsia*,
620 *Bartonella*, *Ehrlichia* and *Orientia*, but only lineages of the Spotted Fever Group for *Borrelia*, and
621 lineages of the pneumonia group for *Mycoplasma*. The numbers indicated are the bootstrap values
622 >55%. The Fasta files used are available on request to the corresponding author.
623

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