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Lack of dissemination of acquired resistance to β -lactam in small wild mammals around an isolated village in the Amazonian forest

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Summary

In this study we quantitatively evaluated the spread of resistance to β -lactams and of integrons in small rodents and marsupials living at various distances from a point of antibiotic's use. Rectal swabs from 114 animals were collected in Trois-Sauts, an isolated village in French Guiana, and along a 3 km transect heading through the nonanthropized primary forest. Prevalence of ticarcillin resistant enterobacteria was 36% (41/114). *Klebsiella* spp., naturally resistant to ticarcillin, were found in 31.1% (23/73) of animals from the village and in an equal ratio of 31.7% (13/41) of animals trapped along the transect. By contrast *E. coli* with acquired resistance to ticarcillin were found in 13.7% (10/73) of animals from the village and in only 2.4% (1/41) of those from the transect (600 m from the village). There was a huge diversity of *E. coli* and *K. pneumoniae* strains with very unique and infrequent sequence types. The overall prevalence of class 1 integrons carriage was 19.3% (22/114) homogenously distributed between animals from the village and the transect, which suggests a co-selection by a non-antibiotic environmental factor. Our results indicate that the anthropogenic acquired antibiotic resistance did not disseminate in the wild far from the point of selective pressure.

Introduction

Antibiotic resistance is a threat to public health worldwide. Its rapid growth combined with the lack of antibiotic innovation leads to increase of infections due to totally resistant bacteria (Boucher *et al.*, 2009). We know a great deal about the genetics and biochemistry of bacterial resistance to antibiotics but much less on the paths of dissemination of resistance genes in nature. Ancestral resistance genes have been present in environmental bacteria for very long period of time where their actual role is not fully understood (Bhullar *et al.*, 2012). They disseminated to human bacteria only very recently. This is believe to be because resistance confers bacteria a decisive advantage to confront the antibiotic residues that are often present in the environmental and commensal ecosystems as a consequence of the human use of antibiotic, not only in medicine but also in the food-chain industry (Marshall and Levy, 2011; Lupo *et al.*, 2012). In these ecosystems bacteria of various origins can mix and exchange resistance genes. Concerning enterobacteria which are intestinal commensal but also major potential pathogens, resistance levels are extremely alarming worldwide (Woerther *et al.*, 2013b) and the intestinal microbiota is considered to be the “epicenter” where bacteria of various origins can exchange resistance genes and disseminate (Carlet, 2012). For instance, the transfer of bacterial resistance from animals, particularly food-chain ones, to humans through the intestinal microbiota is extensively studied to assess the risk associated with animal use of antibiotics for human health (Levy *et al.*, 1976; Van den Bogaard *et al.*, 1997). The reverse transfer of resistance from humans to wild animals is considered a marker of the impact of human usage of antibiotics on the environment (Skurnik *et al.*, 2006).

Wild animals are not normally exposed to antimicrobials, but they might come in contact with resistant bacteria through direct and indirect interactions with humans and domestic animals (Marshall *et al.*, 1990). Several studies showed higher bacterial resistance rates in wild

animals living in close proximity to humans or agriculture compared to those living in more isolated areas (Rolland *et al.*, 1985; Cole *et al.*, 2005; Skurnik *et al.*, 2006; Kozak *et al.*, 2009; Allen *et al.*, 2011). The presence of integrons has also been used as a marker of the level of selection pressure (Kang *et al.*, 2005; Skurnik *et al.*, 2006; Stalder *et al.*, 2012). The level of bacterial resistance in the intestinal microbiota of wild animals seems to depend on the intensity of their direct contacts with human populations and secondary transmission seems rare (Sjolund *et al.*, 2008). However, controlled studies are lacking and it is unknown how far resistance can spread in the wild through animal transmission due to the selective pressure that follows human use of antibiotics. Here we took advantage that we were studying bacterial resistance in a population of Wayampi Amerindians living isolated in the village of Trois-Sauts in the midst of the Amazonian forest. Resulting from the care they receive in the village health post, they are exposed to well characterized quantities of antibiotics (Woerther *et al.*, 2010; Woerther *et al.*, 2013a). In order to gather data on the consecutive dissemination of resistance genes we trapped small wild rodents and marsupials at various distances from the village and analyzed their feces. We explored as a model the dissemination of β -lactam resistance because it was the most commonly used class of antibiotics in the village of Trois-Sauts (58% of all treatments) followed by metronidazole (21%) and macrolides (11%). Others antibiotics (ofloxacin, doxycycline, cotrimoxazole and nitroxoline) were anecdotal (Woerther *et al.*, 2010). β -lactam resistance was analyzed in *E. coli* on one hand and in *Klebsiella* spp. on the other, taking advantage that it is an acquired trait in *E. coli* whereas it is a natural trait in *Klebsiella* spp.

Results and discussion

The sampling was performed during three campaigns (October 2006, June 2008, and October 2010) that we carried out in the village of Trois-Sauts (in the municipality of Camopi, French Guiana: 02°15' N, 52°52' W) where we were studying bacterial resistance in the microbiota of the villagers (Skurnik *et al.*, 2008; Ruimy *et al.*, 2010; Woerther *et al.*, 2010; Catzefflis, 2012; Lebeaux *et al.*, 2012; Angebault *et al.*, 2013; Lescat *et al.*, 2013; Woerther *et al.*, 2013a). The setting is rather unique because it combines the presence of an isolated and stable human population still living in a traditional manner in a completely wild forest environment. However, this population is exposed to antibiotics due to the presence in the village of a health post where a resident paramedic treats villagers with antibiotics when necessary. In all, we collected 114 small wild mammals (rodents and marsupials) known to have a limited perimeter of life of few hectares (Catzefflis F., personal communication) by setting traps in the village (73 animals) and every 300 m from the village in a standardized manner (Mauffrey *et al.*, 2007; Catzefflis, 2012) along a 3000m transect that we draw in the forest in non anthropized zones (41 animals) (Fig. 1 and Table S1). There were 100 rodents and 14 marsupials, speciated as described (Voss *et al.*, 2001; Wilson and Reeder, 2005; Weksler *et al.*, 2006; Voss and Jansa, 2009), including 38 *Nectomys rattus*, 22 *Proechimys cuvieri*, 13 *Oecomys bicolor*, 13 *Hylaeamys megacephalus*, 4 *Oecomys rutilus*, 4 *Proechimys guyanensis*, 2 *Euryoryzomys macconnelli*, 2 *Makalata didelphoides*, 1 *Neacomys paracou*, 1 *Neusticomys oyapocki*, 8 *Marmosa murina*, 2 *Philander opossum*, 1 *Marmosops pinheiroi*, 1 *Didelphis imperfecta*, 1 *Didelphis marsupialis* and 1 *Marmosa demerarae*. A rectal swab was performed on each captured animal before it was released after being marked to avoid sampling twice the same animal.

121 **Spatial distribution of β -lactam resistance in small wild mammal microbiota**

122 All samples analyzed contained viable enterobacteria. Ticarcillin resistant enterobacteria were
123 detected in 41/114 (36%) samples. The resistant species were *Klebsiella* spp. only in 30/114
124 (26.3%) samples, *E. coli* only in 5/114 (4.4%) samples and both in 6/114 (5.3%) samples.

125 However, there were notable differences in the spatial distribution of the two species.

126 Naturally resistant *Klebsiella* spp. strains were found in 23/73 (31.5%) samples from animals
127 trapped in the village and in a very close ratio of 13/41 (31.7%) from those from animals
128 trapped elsewhere along the transect (Tables 1, and S1). Moreover, the percentages did not
129 vary significantly with increasing distance from the village, being 2/14 (14.3%), 4/12 (33.3%)
130 and 7/15 (46.7%) on the 1st, the 2nd and the 3rd kilometers of the transect respectively (Fig. 2).

131 By contrast, *E. coli* isolates with acquired resistance to ticarcillin were found in 10/73 (13.7%)
132 samples from animals trapped in the village, but in only 1/41 (2.4%) of those from elsewhere
133 along the transect, at 600 m from the village. As a reminder, the rate of this type of resistance
134 in the dominant *E. coli* from the villagers was 20.4% (Lescat *et al.*, 2013).

135 Our current results indicate that acquired resistance to β -lactams did not spread far from the
136 point of selective pressure in the wild. This probably results from cost fitness of acquired
137 antibiotic resistance in the absence of selective pressure. Several studies have already shown
138 in a qualitative way that the level of antibiotic resistance in the microbiota of wild animals is
139 higher in those living in close proximity to human's activity than in those without contact
140 with humans (Rolland *et al.*, 1985; Cole *et al.*, 2005; Skurnik *et al.*, 2006; Kozak *et al.*, 2009;
141 Allen *et al.*, 2011). However, to our knowledge, our study is the first to assess quantitatively
142 that acquired resistance to β -lactam does not disseminate far in the wild in absence of
143 selective pressure.

144 Resistance to third generation cephalosporins was not detected in any sample.

145

146 **Characteristics of *E. coli* and *Klebsiella* spp. strains**

147 A total of 16 ticarcillin resistant *E. coli* strains were isolated from the 11 ticarcillin resistant *E.*
148 *coli* positive samples. A TEM-1 penicillinase conferring resistance to amoxicillin and
149 ticarcillin was present in all strains. Five (31.2%) were susceptible to all the others antibiotics
150 tested (Table 2) while 7 (43.7%), including the one from the 600 m point (Ec-600-1), were co-
151 resistant to trimethoprim and sulfonamides. The 4 remaining strains had each a unique pattern
152 of co-resistance, combining sulfonamides and/or trimethoprim and/or tetracycline and/or
153 kanamycin. Nine strains belonged to the two phylogroups most often shared by commensal *E.*
154 *coli*, i.e. groups A (4 strains) and B1 (5 strains) while 3 strains were from group C, 2 from
155 group D and 2 from group E (Table 2). Of note, no B2 strain was found. A high genetic
156 diversity was observed. Indeed, except for 2 sequence type (ST) 2690 strains that were
157 indistinguishable by rep-PCR (Ec-0-5 and Ec-0-12 from the village), each strain had a distinct
158 rep-pattern (Fig. 3 (a)). Strains Ec-0-1 and Ec-0-15, both isolated from animals trapped in the
159 village, were from ST155 but had different rep-patterns and antibiotic susceptibilities. The 12
160 other strains had each a unique ST and half of them had never been described. Furthermore,
161 the *gyrB* allele of Ec-0-4 had not been reported before. In all, 42.8% (6/14) of the ST found in
162 these animal strains had never been described, in accordance with a previous work in the
163 same region (Lescat *et al.*, 2013). The mean virulence score in these 16 strains was low at 1.3
164 (range 0 to 4), with mainly virulence determinants involved in iron capture. The most frequent
165 virulence genes were *fyuA* and *irp2*, which belong to the high pathogenicity island (HPI)
166 found in enterobacteria. A great diversity was also observed among plasmid carrying *bla*_{TEM-1}.
167 The most commonly occurring TEM-1 plasmid replicon was IncHI1 (5 strains), followed by
168 IncX (4 strains), IncFII (3 strains), IncFIA (1 strain) and IncB/O (1 strain). The replicons
169 could not be determined for 5/16 (31.2%) strains. All incompatibility groups detected had
170 been previously described in human and animal enterobacteria strains. However, the

171 distribution was unusual: no IncFIB and few IncFII were found in favor of IncHI1 (Johnson *et*
172 *al.*, 2007; Marcade *et al.*, 2009). The diversity of plasmid replicons could also be explained by
173 the diversity and connectivity of gamma-proteobacteria in the wild forest environment,
174 resulting in highly promiscuous exchange of mobile genetic elements. The huge genetic
175 diversity among the 16 ticarcillin resistant *E. coli* strains and their plasmids suggests that the
176 dissemination of ticarcillin resistance was more likely due to *bla*_{TEM-1}, maybe through Tn3-type
177 transposon (Marcade *et al.*, 2009), than bacterial strains or plasmids spread.

178 A total of 36 *Klebsiella* spp. strains were isolated, including 34 *K. pneumoniae* and 2 *K.*
179 *oxytoca*. A high genetic diversity without clonal spread was also observed among the 34 *K.*
180 *pneumoniae* strains (Fig. 3(b) and Table 3) with only one pair sharing the same rep-patterns
181 and the same ST (Kp-2400-2 and Kp-2400-3 from 2 samples from the 2400 m point). Two
182 others pairs shared the same ST but a different rep-pattern (Kp-0-22 and Kp-600-1; Kp-0-9
183 and Kp-2400-5). Each of the 28 remaining strains had a distinct ST. Among the 31 unique
184 STs found, 24 (77.4%) were described for the first time, with new alleles for 7 of them. All
185 strains had the usual natural resistance phenotype of *Klebsiella* spp., *i.e.* resistance to
186 amoxicillin and ticarcillin, with no co-resistance. All carried the *entB* siderophore gene and
187 the *ycfM* adhesin gene, 27/34 (79.4%) the *kpn* adhesin gene, 24/34 (70.6%) the fimbriae
188 *fimHI* gene and 26/34 (76.5%) the *kfuABC* gene (Table 3). There was no phenotypically
189 hypermucoviscous strain and *magA* (specific for K1 serotype) or *rmpA* genes which are
190 associated with this phenotype were not detected in any strain. Interestingly, 3 of these
191 commensal strains from wild rodents (3 *Proechimys cuvieri*, 2 from the 2400 m point and one
192 from the village) had the *allS* gene which had been strongly associated with strains isolated
193 from liver abscess (Chou *et al.*, 2004). Furthermore, one strain had the *ybtS* gene (*Proechimys*
194 *cuvieri* from the 2100 m point), which comes from the Ybt operons of the HPI cited above
195 (Geoffroy *et al.*, 2000). Capsular serotype was determined for two strains, Kp-0-8 (K20) and

196 Kp-0-17 (K5) from two animals trapped in the village. No strain from capsular serotype K1,
197 K2, K54 or K57 was found. Altogether this showed that the *E. coli* and *K. pneumoniae* strains
198 from these wild animals were particular and unfrequently described. It also suggested that the
199 known ST-types are those prevalent in developed countries and that the number of different
200 niches allowing enterobacteria proliferation is much larger in this complex forest
201 environment, explaining enterobacteria local specialization and diversification. However, all
202 the *K. pneumoniae* strains had the *entB* siderophore gene, and frequently the *kpn* and *ycfM*
203 adhesin genes, fimbriae *fimH1* and *kfuABC* genes, just as strains of human origin.

204

205 **Detection of intestinal carriage of *bla*_{TEM}**

206 We further investigated carriage by the animals of *bla*_{TEM} gene by PCR screening of the
207 global Drigalski culture from each sample. We found only 5 positive samples, all from
208 animals trapped in the village and none from animals living in any other place on the transect
209 (data not shown). Ticarcillin resistant *E. coli* strains had indeed been isolated in these 5
210 samples. By contrast the *bla*_{TEM} PCR was negative in the 6 other samples from animals in
211 whom ticarcillin resistant *E. coli* strains had also been isolated, including the one trapped at
212 600 m from the village. This suggests that PCR on global Drigalski cultures was less sensitive
213 than further screening of these cultures on selective media and performing PCR on the
214 resulting strains for detection of *bla*_{TEM} genes.

215

216 **Prevalence of class 1, 2 and 3 integrons and gene cassettes characterization**

217 We also investigated the carriage by the animals of class 1, 2 and 3 integrons by multiplex
218 qPCR screening of the global Drigalski culture from each sample. Class 1 integrons were
219 detected in 22/114 (19.3%) of the samples, with no significant difference between those from
220 animals trapped in the village or along the transect (11/73 (15.1%) vs 11/41 (26.8%)) (Tables

221 1 and S1). There was no detectable spatial distribution along it either, the prevalence being of
222 4/14 (28.6%), 2/12 (16.7%) 5/15 (33.3%) for the 1st, 2nd and 3rd kilometers of the transect
223 respectively (Fig. 4). Class 2 integrons, which were not further studied, were detected in 3
224 samples only, coming from one animal trapped in the village, one at the 300 m point and one
225 at the 2400 m point. No class 3 integron was detected. The homogenous distribution of class 1
226 integrons between the village and the transect, independently of antibiotic selective pressure,
227 is in appearance strikingly different from the heterogeneous distribution of acquired resistance
228 described in previous studies (Cole *et al.*, 2005; Skurnik *et al.*, 2006), which found that
229 integrons were only present when antibiotic selective pressure exceeds a certain threshold.
230 The analysis of the gene cassettes in class 1 integrons showed little diversity with seven
231 different gene cassette arrays (Table 4). The gene cassettes encoded resistance to
232 trimethoprim (*dhfrA7*, *dhfrA15*, *dhfrA21*), spectinomycin and streptomycin (*aadA1*, *aadA2*,
233 *aadA6*) or chloramphenicol (*cmlA4*). *aadA6* was the most frequently found gene cassette
234 (6/22 (27.3%)). One sample (*Oecomys rutilus*) from the 2400 m trapping point had 2 different
235 integrons, one with the *dhfrA21* gene cassette and one with the *aadA1* gene cassette. However,
236 we were not able to characterize the gene cassette arrays for 8/22 (36%) class 1 integron-
237 positive samples because we failed to obtain amplification products with the endpoint PCR
238 with 5'CS and 3'CS primers. We can hypothesize that these integrons do not contain the 3'-
239 conserved segment in which the 3' CS primer hybridizes, as described especially for class 1
240 integrons detected in non-clinical strains (Gillings *et al.*, 2008). The genes cassette found in
241 these integrons were coding for resistance towards antibiotics which were not used in the
242 village. The presence of integrons carrying resistance gene cassettes in the intestinal
243 microbiota of wild animals not exposed to antibiotic selective pressure has been observed
244 before (Sunde, 2005; Goncalves *et al.*, 2013) but its significance has yet to be elucidated. All
245 genes cassette detected had been previously described in human or animal commensal strains

246 (Kang *et al.*, 2005; Peirano *et al.*, 2006; Kadlec and Schwarz, 2008; Michael *et al.*, 2008). It is
247 of note that the method used didn't allowed to characterize the gene cassette arrays for 8/22
248 (36%) class 1 integron positive samples, thus we cannot rule out that other gene cassettes
249 could have been present there.

250

251 **Detection of *merA***

252 We hypothesized that the stable prevalence of class 1 integrons in the village and along the
253 transect could be due to a co-selection by mercury. Indeed, the *mer* operon encoding for
254 mercury resistance has often been described as associated to class 1 integron within the Tn21-
255 like transposon (Skurnik *et al.*, 2006; Skurnik *et al.*, 2010) and this region of French Guiana
256 has been heavily exposed to mercury in the past, because of gold mining prospection
257 (Laperche *et al.*, 2014).

258 This was tested by screening the global Drigalski culture from each sample and the 16
259 tetracycline resistant *E. coli* strains for *merA* gene by PCR amplification as described (Deredjian
260 *et al.*, 2011). However, we found only 1 sample positive, (from a *Makalata didelphoides*,
261 which is an arboreal echimyid Caviomorpha, trapped in the village) and none of the strains
262 were positive (data not shown). Thus, class 1 integrons might have been selected by a non-
263 antibiotic environmental factor which remains to be determined. It could also reflect other
264 opportunities in such a complex environment for spontaneous horizontal genetic transfer and
265 random drift-based enrichment of transconjugants.

266

267 Certainly our study was limited by the relatively small number of samples available from the
268 transect. This was in spite of considerable efforts of trapping (Table S2) whose yield was low.
269 The effort or trapping was indeed 7 times higher along the transect than in the village (7900 vs
270 1047 trap-nights) and provided around twice less animals. The very small density of small

271 rodents and marsupials in the forest was probably due to reduced food resources (Catzeflis F.,
272 personal communication). Another limitation of our work was that we studied the resistance
273 to only one class of antibiotic, the β -lactam. But resistance to β -lactam was chosen as a model
274 because it was by far the most frequent antibiotics used in the village. Despite these
275 limitations, our results suggest that acquired resistance genes do not diffuse when antibiotic
276 selective pressure stops which is a strong incentive to control the use of antibiotics and all
277 kind of release of these molecules in the environment.

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290

291 **Potential conflict of interest**

292 All authors: No reported conflicts.

293

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467

468

469 **Tables**

470

471

Distance from the village (m)	Number of samples	Ticarcillin resistance ^a		Integrans ^b		
		Natural n (%)	Acquired n (%)	Class 1 n (%)	Class 2 n (%)	Class 3 n (%)
0	73	23 (31.5)	10 (13.7)	11 (15.1)	1 (1.4)	0 (0)
300	4	0 (0)	0 (0)	2 (50)	1 (25)	0 (0)
600	9	2 (22.2)	1 (11.1)	2 (22.2)	0 (0)	0 (0)
900	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1200	11	3 (27.3)	0 (0)	2 (18.2)	0 (0)	0 (0)
1500	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
2100	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
2400	11	6 (54.5)	0 (0)	5 (45.5)	1 (9.1)	0 (0)
2700	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3000	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

472

473 **Table 1:** Frequencies of natural and acquired ticarcillin resistance and of class 1, 2 and 3
 474 integrans in microbiota of rodents and marsupials trapped in the village of Trois-Sauts and at
 475 specific distances from the village, i.e. the source of antibiotic selection pressure.

476 ^a Presence of ticarcillin resistant enterobacteria in the samples was screened by plating the
 477 global culture on Drigalski agar plates containing ticarcillin (32 mg/L).

478 ^b The carriage of class 1, 2, and 3 integrans by animals was detected on total DNA extracted
 479 from the global Drigalski culture by a multiplex Taqman-based qPCR with amplification of
 480 *intI1*, *intI2*, and *intI3* genes, as described (Barraud *et al.*, 2010).

481

Strain identity	Resistance Phenotype ^a	Virulence factors ^b												Virulence score	Phylogroup ^c		
		adhesins				toxins		iron capture systems			protectin						
		<i>papC</i>	<i>sfa/foc</i>	<i>ibeA</i>	<i>iha hlyC</i>	<i>kat</i>	<i>iroN ireA</i>	<i>fyuA</i>	<i>irp2</i>	<i>aer</i>	<i>neuC</i>		<i>usp</i>				
Ec-0-1	AMX, TIC, TMP, SSS	-	-	-	-	-	-	-	-	-	-	-	-	-	0	B1	
Ec-0-2	AMX, TIC, SSS, TET	-	-	-	+	-	-	-	-	+	+	+	-	-	4	A	
Ec-0-3	AMX, TIC	-	-	-	-	-	-	-	-	-	-	-	-	-	0	E	New ST (
Ec-0-4	AMX, TIC, TMP, SSS	-	-	-	-	-	-	-	-	-	-	-	-	-	0	A	New ST (6-
Ec-0-5	AMX, TIC	-	-	-	-	-	-	-	-	-	-	-	-	-	0	C	
Ec-0-6	AMX, TIC	-	-	+	+	+	+	-	-	-	-	-	-	-	3	A	
Ec-0-7	AMX, TIC, TMP, SSS	-	-	-	+	-	-	-	-	+	+	+	-	-	4	A	
Ec-0-8	AMX, TIC, TMP, SSS	-	-	-	-	-	-	-	-	-	-	-	-	+	1	E	
Ec-0-9	AMX, TIC, TMP, SSS	-	-	-	-	-	-	-	-	-	-	-	-	-	0	B1	
Ec-0-10	AMX, TIC, TMP, SSS	-	-	-	+	-	-	-	-	+	+	+	-	-	4	B1	New ST
Ec-0-11	AMX, TIC	-	-	-	-	-	-	-	-	-	-	-	-	-	0	C	New ST
Ec-0-12	AMX, TIC	-	-	-	-	-	-	-	-	-	-	-	-	-	0	C	
Ec-0-13	AMX, TIC, SSS	-	-	-	+	-	-	-	-	+	+	-	-	-	3	B1	New ST
Ec-0-14	AMX, TIC, TMP, SSS, TET	-	-	-	-	-	-	-	-	-	-	-	-	-	0	D	New ST
Ec-0-15	AMX, TIC, KAN	-	-	-	-	-	-	-	-	-	-	-	-	-	0	B1	
Ec-600-1	AMX, TIC, TMP, SSS	-	-	-	-	-	-	-	-	+	+	-	-	-	2	D	

483

484 **Table 2:** Resistance phenotype, virulence factors and score, phylogroup and sequence type (ST) of *E. coli* strains485 ^a The susceptibility of the following antibiotics were tested: amoxicillin (AMX), ticarcilline (TIC), amoxicillin

486 ceftazidime, cefepime, ceftazidime, cefepime, ceftazidime, cefepime, ceftazidime, cefepime, ceftazidime, cefepime, ceftazidime, cefepime, ceftazidime, cefepime,

487 sulfonamide (SSS) and tetracycline (TET)

488 ^b *E. coli* virulence factors were detected by PCR as described elsewhere (Johnson *et al.*, 2006) and virulence factors

489 (2011).

490 ^c Phylogroups of *E. coli* strains were determined by quadruplex PCR as described elsewhere (Clermont *et al.*,491 ^d Multilocus sequence typing (MLST) was performed using one of the MLST schemes developed for *E. coli*492 (<http://www.mlst.ucc.ie/mlst/dbs/Ecoli>).

493 ^e T, transfer by electroporation; C, transfer by mating. The transferability of TEM-1 genes was assessed by ma
494 (Bakour *et al.*, 1983). When mating failed, transformation into *E. coli* DH5 α (Invitrogen) was attempted by el
495 DNA.

496 ^f Resistance genes *bla*_{TEM} were amplified with specific primers, as described elsewhere (Saladin *et al.*, 2002),

497 ^g NT, Not Typed. Plasmid replicons from transconjugants and transformants were typed by PCR, as described

498

499

Strain identity	Resistance phenotype ^a	Virulence factors ^b										ST ^c
		Adhesins					Iron capture systems					
		<i>rmpA</i>	<i>allS</i>	<i>ycfM</i>	<i>mrKD</i>	<i>kpn</i>	<i>fimH1</i>	<i>entB</i>	<i>iroN</i>	<i>ybtS</i>	<i>kfuABC</i>	
Kp-0-1	AMX, TIC	-	-	+	+	+	+	+	-	-	-	New ST (2-3-1-1-7-1-1)
Kp-0-2	AMX, TIC	-	-	+	-	-	-	+	-	-	+	New ST (16-24-36-27-47-17-67)
Kp-0-3	AMX, TIC	-	-	+	-	-	+	+	-	-	+	New ST (18-23-25-96-79-20-51)
Kp-0-4	AMX, TIC	-	-	+	-	-	+	+	-	-	+	New ST (18-23-56-New allele-11-43-New allele)
Kp-0-5	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (16-24-21-27-54-22-105)
Kp-0-6	AMX, TIC	-	-	+	-	+	-	+	-	-	+	New ST (28-24-21-53-137-22-67)
Kp-0-7	AMX, TIC	-	-	+	-	+	-	+	-	-	-	682
Kp-0-8	AMX, TIC	-	-	+	-	+	-	+	-	-	-	1294
Kp-0-9	AMX, TIC	-	-	+	+	+	-	+	-	-	+	New ST (2-1-2-1-27-1-39)
Kp-0-10	AMX, TIC	-	-	+	-	+	-	+	-	-	+	New ST (16-24-43-27-47-17-New allele)
Kp-0-11	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (16-24-21-53-47-17-215)
Kp-0-12	AMX, TIC	-	-	+	-	-	-	+	-	-	+	New ST (16-24-21-106-68-59-188)
Kp-0-13	AMX, TIC	-	-	+	-	-	+	+	-	-	+	New ST (16-24-21-33-104-New allele-New allele)
Kp-0-14	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (18-22-18-22-New allele-13-50)
Kp-0-15	AMX, TIC	-	-	+	-	+	-	+	-	-	-	999
Kp-0-16	AMX, TIC	-	-	+	+	+	+	+	-	-	-	New ST (4-4-1-1-6-2-12)
Kp-0-17	AMX, TIC	-	-	+	+	+	-	+	-	-	+	New ST (2-1-5-3-4-4-8)
Kp-0-18	AMX, TIC	-	+	+	-	+	+	+	-	-	+	New ST (16-24-New allele-33-76-33-67)
Kp-0-19	AMX, TIC	-	-	+	-	+	+	+	-	-	+	1208
Kp-0-20	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (28-24-21-33-68-New allele-225)
Kp-0-21	AMX, TIC	-	-	+	+	+	+	+	-	-	-	New ST (7-1-1-1-12-1-123)
Kp-0-22	AMX, TIC	-	-	+	-	+	-	+	-	-	-	442
Kp-600-1	AMX, TIC	-	-	+	-	+	-	+	-	-	-	442
Kp-600-2	AMX, TIC	-	-	+	-	+	-	+	-	-	-	197
Kp-1200-1	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (16-18-43-27-47-93-67)
Kp-1200-2	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (16-18-21-33-50-22-67)
Kp-1200-3	AMX, TIC	-	-	+	-	+	+	+	-	-	+	New ST (18-22-18-16-25-13-165)
Kp-2100-1	AMX, TIC	-	-	+	-	+	+	+	-	+	+	New ST (16-24-36-27-47-22-67)
Kp-2400-1	AMX, TIC	-	-	+	-	+	-	+	-	-	-	347
Kp-2400-2	AMX, TIC	-	+	+	-	-	+	+	-	-	+	New ST (18-22-26-63-115-13-New allele)
Kp-2400-3	AMX, TIC	-	+	+	-	+	+	+	-	-	+	New ST (18-22-26-63-115-13-New allele)
Kp-2400-4	AMX, TIC	-	-	+	-	+	-	+	-	-	+	New ST (16-18-43-27-47-93-75)
Kp-2400-5	AMX, TIC	-	-	+	+	+	+	+	-	-	+	New ST (2-1-2-1-27-1-39)
Kp-2400-6	AMX, TIC	-	-	+	+	+	-	+	-	-	-	New ST (2-20-1-1-4-4-4)

500

501 **Table 3:** Resistance phenotype, virulence factors and sequence type (ST) of *K. pneumoniae*
502 strains.

503 ^a The susceptibility of the following antibiotics were tested: amoxicillin (AMX), ticarcilline
504 (TIC), amoxicillin + clavulanate, cefotaxime, ceftazidime, cefepime, ceftoxitin, ertapenem,
505 gentamicin, amikacin, kanamycin, nalidixic acid, ofloxacin, trimethoprim, sulfonamide and
506 tetracycline

507 ^b *K. pneumoniae* capsular serotypes K1 (*magA*), K2, K5, K20, K54 and K57 were determined
508 using multiplex PCR, as described (Turton *et al.*, 2008). Virulence genes were searched by
509 PCR. Primers used are reported in Table S3. Two reference strains of capsular serotypes K1
510 (NTUH K2044) and K2 (CG43) were used as controls (Chen *et al.*, 2004; Fang *et al.*, 2004).

511 ^c Multilocus sequence typing (MLST) was performed using the international MLST scheme
512 of the Institut Pasteur, Paris, France (<http://www.pasteur.fr/mlst>)

513

514
515

Cassette array	Number of samples					Total
	0 (Village)	300 m	600 m	1200 m	2400 m	
<i>dfrA15-cmlA4-aadA2</i>	2 - - - -					2
<i>dfrA21-aadA2</i>	1 - - - -					1
<i>aadA1</i>	- - - -	1				1
<i>aadA2</i>	2 - - - -					2
<i>aadA6</i>	1 -	1	1	3	6	6
<i>dfrA7</i>	1 - - - -					1
<i>dfrA21</i>	1 - - -		1	2		2
ND	3	2	1	1	1	8

516

517 Table 4: Gene cassettes arrays of class 1 integrons in microbiota of rodents and marsupials
518 trapped in Trois-Sauts and at specific distances from the village.

519 ND: Not Determined

520 For the construction of gene cassette array libraries, primers 5'CS and 3'CS at 0.5 μ M
521 (Levesque *et al.*, 1995) were used to amplify and sequence the variable GC-containing region
522 of class 1 integrons directly from the extracted DNA (5 μ L) as described (Stalder *et al.*,
523 2013). At least 20 clones were used for sequencing.

524

525

526

527 **Figure legends**

528

529

530 Figure 1: Study site: Trois-Sauts (in the municipality of Camopi, French Guiana: 02°15' N,
531 52°52' W).

532 Red numbers represent the number of samples per trapping point (in the village and at specific
533 distances from the village).

534 Rectal swabs of trapped animal were inoculated extemporaneously onto Drigalski agar slants
535 in screw-cup tubes and sent to France at room temperature. There, the global Drigalski culture
536 from each tube was suspended in 1.5 mL of brain-heart infusion (BHI) broth with 10%
537 glycerol and stored at -80°C.

538

539

540 Figure 2: Percentage of samples bearing natural (*Klebsiella* spp.) or acquired (*E. coli*)
541 ticarcillin resistance according to the distance from the village, i.e. the source of antibiotic
542 selection pressure.

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545 Figure 3: Dendrogram and rep-PCR fingerprints of *E. coli* (a) and *K. pneumoniae* (b) strains.
546 Strains were named by their initials (Ec for *E. coli* and Kp for *K. pneumoniae*), followed by
547 the distance from the village of the isolation site, followed by the order number. The genetic
548 relatedness was analyzed by rep-PCR DNA fingerprinting with the DiversiLab system
549 (bioMérieux, Marcy l'Etoile, France) as in Woerther *et al.*, 2013a.

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552 Figure 4: Percentage of samples bearing class 1 or class 2 integrons according to the distance
553 from the village, i.e. the source of antibiotic selection pressure.

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557 **Supporting Information**

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Sample	Distance from the village (m)	Animal	Rodent / Marsupial	Ticarcillin resistant <i>E. coli</i>	<i>Klebsiella</i> spp.	Class 1 integron	Class 2 integron
1	0	<i>Nectomys rattus</i>	Rodent	Ec-0-1 No		--	
2	0	<i>Nectomys rattus</i>	Rodent	No Kp-0-1		--	
3	0	<i>Nectomys rattus</i>	Rodent	No Kp-0-2		--	
4	0	<i>Nectomys rattus</i>	Rodent	No No		--	
5	0	<i>Philander opossum</i>	Marsupial	No No		--	
6	0	<i>Makalata didelphoides</i>	Rodent	No Kp-0-3		--	
7	0	<i>Nectomys rattus</i>	Rodent	No No		--	
8	0	<i>Makalata didelphoides</i>	Rodent	No Kp-0-4		--	
9	0	<i>Marmosa murina</i>	Marsupial	No Kp-0-5		--	
10	0	<i>Nectomys rattus</i>	Rodent	No No		--	
11	0	<i>Nectomys rattus</i>	Rodent	No No		--	
12	0	<i>Nectomys rattus</i>	Rodent	No No		--	
13	0	<i>Marmosa murina</i>	Marsupial	No No		--	
14	0	<i>Oecomys bicolor</i>	Rodent	No Kp-0-6		+-	
15	0	<i>Hylaeamys megacephalus</i>	Rodent	No No		--	
16	0	<i>Nectomys rattus</i>	Rodent	Ec-0-2 Kp-0-7		--	
17	0	<i>Marmosa murina</i>	Marsupial	No No		+-	
18	0	<i>Nectomys rattus</i>	Rodent No No			--	
19	0	<i>Proechimys cuvieri</i>	Rodent No No			--	
20	0	<i>Oecomys bicolor</i>	Rodent No No			--	
21	0	<i>Nectomys rattus</i>	Rodent No No			--	
22	0	<i>Nectomys rattus</i>	Rodent No No			--	
23	0	<i>Nectomys rattus</i>	Rodent Ec-0-3 No			--	
24	0	<i>Nectomys rattus</i>	Rodent No No			--	
25	0	<i>Oecomys bicolor</i>	Rodent No No			--	
26	0	<i>Oecomys bicolor</i>	Rodent No No			--	
27	0	<i>Oecomys bicolor</i>	Rodent No No			--	
28	0	<i>Hylaeamys megacephalus</i>	Rodent No No			-+	
29	0	<i>Nectomys rattus</i>	Rodent No No			--	
30	0	<i>Nectomys rattus</i>	Rodent Ec-0-4, Ec-0-5 Kp-0-8			+-	
31	0	<i>Oecomys bicolor</i>	Rodent No <i>K. oxytoca</i>			+-	
32	0	<i>Oecomys bicolor</i>	Rodent Ec-0-6, Ec-0-7 Kp-0-9			+-	
33	0	<i>Oecomys bicolor</i>	Rodent No No			--	
34	0	<i>Nectomys rattus</i>	Rodent No No			--	
35	0	<i>Neusticomys oyapocki</i>	Rodent No Kp-0-10			--	
36	0	<i>Nectomys rattus</i>	Rodent No No			--	
37	0	<i>Nectomys rattus</i>	Rodent No Kp-0-11			--	
38	0	<i>Proechimys cuvieri</i>	Rodent No No			--	
39	0	<i>Nectomys rattus</i>	Rodent Ec-0-8 No			--	
40	0	<i>Nectomys rattus</i>	Rodent No No			+-	
41	0	<i>Nectomys rattus</i>	Rodent No No			--	
42	0	<i>Nectomys rattus</i>	Rodent No Kp-0-12			--	
43	0	<i>Nectomys rattus</i>	Rodent No Kp-0-13			--	
44	0	<i>Nectomys rattus</i>	Rodent No Kp-0-14			--	
45	0	<i>Nectomys rattus</i>	Rodent No No			--	
46	0	<i>Nectomys rattus</i>	Rodent No No			--	
47	0	<i>Nectomys rattus</i>	Rodent No No			--	
48	0	<i>Marmosa murina</i>	Marsupial No Kp-0-15			+-	

49 0		<i>Nectomys rattus</i>	Rodent	No	No	--
50 0		<i>Marmosa murina</i>	Marsupial	No	Kp-0-16	+-
51 0		<i>Hylaeamys megacephalus</i>	Rodent	No	Kp-0-17	--
52 0		<i>Nectomys rattus</i>	Rodent	Ec-0-9	No	--
53 0		<i>Hylaeamys megacephalus</i>	Rodent	No	No	--
54 0		<i>Nectomys rattus</i>	Rodent	No	No	--
55 0		<i>Proechimys cuvieri</i>	Rodent	No	Kp-0-18	--
56 0		<i>Nectomys rattus</i>	Rodent	No	No	--
57 0		<i>Oecomys bicolor</i>	Rodent	No	No	--
58 0		<i>Philander opossum</i>	Marsupial	Ec-0-10	Kp-0-19	--
59 0		<i>Hylaeamys megacephalus</i>	Rodent	No	No	--
60 0		<i>Nectomys rattus</i>	Rodent	No	No	--
61 0		<i>Marmosa murina</i>	Marsupial	No	No	--
62 0		<i>Oecomys bicolor</i>	Rodent	No	No	+-
63 0		<i>Oecomys bicolor</i>	Rodent	No	No	--
64 0		<i>Oecomys bicolor</i>	Rodent	No	No	--
65 0		<i>Nectomys rattus</i>	Rodent	No	No	--
66 0		<i>Hylaeamys megacephalus</i>	Rodent	No	No	--
67 0		<i>Nectomys rattus</i>	Rodent	No	No	--
68 0		<i>Oecomys bicolor</i>	Rodent	No	No	--
69 0		<i>Oecomys bicolor</i>	Rodent	No	No	--
70 0		<i>Oecomys bicolor</i>	Rodent	No	Kp-0-20	--
71 0		<i>Nectomys rattus</i>	Rodent	No	No	--
72 0		<i>Nectomys rattus</i>	Rodent	Ec-0-11, Ec-0-12	Kp-0-21	+-
73 0		<i>Marmosa murina</i>	Marsupial	Ec-0-13, Ec-0-14, Ec-0-15	Kp-0-22	+-
74	300	<i>Marmosa murina</i>	Marsupial	No	No	--
75	300	<i>Nectomys rattus</i>	Rodent	No	No	--
76	300	<i>Proechimys cuvieri</i>	Rodent	No	No	+-
77	300	<i>Oecomys rutilus</i>	Rodent	No	No	++
78	600	<i>Didelphis marsupialis</i>	Marsupial	Ec-600-1	No	+-
79	600	<i>Hylaeamys megacephalus</i>	Rodent	No	Kp-600-1	--
80	600	<i>Neacomys paracou</i>	Rodent	No	No	--
81	600	<i>Hylaeamys megacephalus</i>	Rodent	No	No	+-
82	600	<i>Proechimys cuvieri</i>	Rodent	No	No	--
83	600	<i>Proechimys cuvieri</i>	Rodent	No	No	--
84	600	<i>Hylaeamys megacephalus</i>	Rodent	No	No	--
85	600	<i>Proechimys cuvieri</i>	Rodent	No	Kp-600-2	--
86	600	<i>Hylaeamys megacephalus</i>	Rodent	No	No	--
87	900	<i>Proechimys cuvieri</i>	Rodent	No	No	--
88	1200	<i>Proechimys cuvieri</i>	Rodent	No	Kp-1200-1	--
89	1200	<i>Proechimys cuvieri</i>	Rodent	No	No	--
90	1200	<i>Hylaeamys megacephalus</i>	Rodent	No	No	+-
91	1200	<i>Proechimys cuvieri</i>	Rodent	No	No	--
92	1200	<i>Proechimys cuvieri</i>	Rodent	No	No	--
93	1200	<i>Proechimys guyannensis</i>	Rodent	No	Kp-1200-2	--
94	1200	<i>Proechimys cuvieri</i>	Rodent	No	No	--
95	1200	<i>Euryoryzomys macconnelli</i>	Rodent	No	No	--
96	1200	<i>Hylaeamys megacephalus</i>	Rodent	No	Kp-1200-3	+-
97	1200	<i>Didelphis imperfecta</i>	Marsupial	No	No	--
98	1200	<i>Proechimys cuvieri</i>	Rodent	No	No	--
99	1500	<i>Proechimys cuvieri</i>	Rodent	No	<i>K. oxytoca</i>	--
100	2100	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2100-1	--

101	2400	<i>Marmosa demerarae</i>	Marsupial	No	No	+ -
102	2400	<i>Oecomys rutilus</i>	Rodent	No	No	+ +
103	2400	<i>Proechimys cuvieri</i>	Rodent	No	No	- -
104	2400	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2400-1	- -
105	2400	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2400-2	+ -
106	2400	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2400-3	+ -
107	2400	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2400-4	+ -
108	2400	<i>Proechimys guyannensis</i>	Rodent	No	No	- -
109	2400	<i>Proechimys cuvieri</i>	Rodent	No	Kp-2400-5	- -
110	2400	<i>Marmosops pinheiroi</i>	Marsupial	No	Kp-2400-6	- -
111	2400	<i>Hylaeamys megacephalus</i>	Rodent	No	No	- -
112	2700	<i>Proechimys guyannensis</i>	Rodent		No No	- -
113	3000	<i>Proechimys cuvieri</i>	Rodent	No	No	- -
114	3000	<i>Euryoryzomys macconnelli</i>	Rodent	No	No	- -

560

561 Table S1: Animals trapped in the village and along the transect, with the distance of the trap
562 from the village and the carriage of tetracycline resistant *E. coli* or *Klebsiella* spp. strains and
563 class 1 and 2 integrons in the rectal swab samples.

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Distance from the village (m)	Trap-nights (n)
0	1047
300	786
600	1341
900	241
1200	1341
1500	241
1800	786
2100	241
2400	1341
2700	241
3000	1341

566 Table S2: Sampling effort (trap-nights) over the three campaigns (October 2006, June 2008,
567 and October 2010) according to the distance from the village.

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569

Name DNA sequence	Target site	Amplicon size (pb)	Reference
ycfM-F 5'-CGATTGAGCATCAGGATCAG-3' <i>ycfM</i> ycfM-R 5'-GTTGGTGCGGTTGTTAC-3'		161	This study
mrkD-1 5'-TAT(T/C)G(G/T)CTTAATGGCGCTGG-3' <i>mrkD</i> mrkD-2 5'-TAATCGTACGTCAGGTAAAGA(C/T)C-3'		920	Brisse <i>et al.</i> (Brisse <i>et al.</i> , 2009)
entB-F 5'-CGCCCAGCCGAAAGAGCAGA-3' entB-R 5'-CATCGGCACCGAATCCAGAC-3'	<i>entB</i>	508	This study
ybtS-F 5'-CAAAAATGGGCGGTGGATTC-3' <i>ybtS</i> ybtS-R 5'-CCTGACGGAACATAAACGAGCG-3'			Bachman <i>et al.</i> (Bachman <i>et al.</i> , 2011)
kfu-F 5'-ATAGTAGGCGAGCACCGAGA-3' <i>kfu</i> kfu-R 5'-AGAACCTTCCTCGCTGAACA-3'		520	Yu <i>et al.</i> (Yu <i>et al.</i> , 2008)
iroN-F 5'-GCATAGGCGGATACGAACAT-3' <i>iroN</i> iroN-R 5'-CACAGGGCAATTGCTTACCT-3'		556	Yu <i>et al.</i> (Yu <i>et al.</i> , 2008)
magA-F 5'-GGTGCTCTTTACATCATTGC-3' <i>magA</i> magA-R 5'-GCAATGGCCATTTGCGTTAG-3'		1280	Fang <i>et al.</i> (Fang <i>et al.</i> , 2004)
1416R 5'-CCGTTAGGCAATCCAGAC-3' <i>allS</i> 336F2 5'TCTGATTTA(A/T)CCCACATT-3'		1090	Chou <i>et al.</i> (Chou <i>et al.</i> , 2004)
rmpA-F 5'-ACTGGGCTACCTCTGCTTCA-3' <i>rmpA</i> rmpA-R 5'-CTTGCATGAGCCATCTTTCA-3'		535	Brisse <i>et al.</i> (Brisse <i>et al.</i> , 2009)

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Table S3: Primers used for characterization of *K. pneumoniae* virulence factors