Characterization of Klebsiella pneumoniae isolated from patients suspected of pulmonary or bubonic plague during the Madagascar epidemic in 2017

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Research Article

Keywords: Klebsiella pneumoniae, pneumopathy, bubo, community-acquired pneumonia, virulent clone.

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Characterization of *Klebsiella pneumoniae* isolated from patients suspected of pulmonary or bubonic plague during the Madagascar epidemic in 2017

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Abstract

Klebsiella pneumoniae can lead to a wide range of diseases including pneumonia, bloodstream and urinary tract infections. During a short period of a plague epidemic in October 2017 in Madagascar, 12 *K. pneumoniae* isolates were identified in ten sputum and two buboes aspirate samples. These isolates were from 12 patients suspected of plague, without epidemiological relationships, but were negative for *Yersinia pestis* in culture. Data were collected from the plague national surveillance system. The isolates were characterized by antimicrobial susceptibility testing and whole-genome sequencing. Real-time PCR was performed to confirm the presence of *K. pneumoniae* DNA in buboes. All isolates were identified as *K. pneumoniae* sensu stricto. Five isolates were extended-spectrum ß-lactamases producers; eight different sequence types were identified. Five isolates belonged to known hypervirulent sequence types. Our results demonstrate community-acquired pneumonia caused by *K. pneumoniae* isolates in patients suspected of plague, showing that plague epidemics can hide other etiologies.

Keywords: *Klebsiella pneumoniae*, pneumopathy, bubo, community-acquired pneumonia, virulent clone.

Background

Between August 1 and November 26, 2017; a total of 2.414 clinically suspected plague cases were reported to the Central Laboratory for Plague (CLP) at the Institut Pasteur de Madagascar, including 1.878 (78%) pulmonary plague (PP), 395 (16%) bubonic plague (BP), one (<1%) septicaemia and 140 (6%) cases with unspecified clinical form[1]. This predominantly urban plague epidemic was characterised by a large volume of notifications in two major urban areas (Antananarivo and Toamasina) and by an unusually high proportion of pneumonic forms. According to the 2006 WHO standard plague case definitions and using the results of three types of diagnostic tests assessed (rapid F1-antigen diagnostic test, (RDT), molecular amplification method, and culture)[2], 386/1.878 (21%) were probable and 32/1.878 (2%) were confirmed cases among the notified PP cases. The magnitude of this pneumonic plague outbreak is likely to have been smaller that suggested by notified suspected cases[1]; and its severity indicated by the case fatality rate among confirmed plus probable (about 9%) was substantially lower than observed the last previous 18 years (25%)[3]. Over-reporting of PP cases due to limited clinical experience two most affected areas, and the difficulty to clinically diagnose PP through respiratory signs was speculated. The clinical diagnosis of pneumonic plague from polymicrobial sputum associated with other potential causes of pneumonia remains a challenge because the isolation of *Yersinia pestis* (*Y. pestis*) is more complicated compared to other bacteria.

Klebsiella pneumoniae (K. pneumoniae) is a Gram-negative bacterium naturally resistant to amoxicillin and carbenicillin. *Klebsiella pneumoniae* complex members comprise 7 phylogroups (Kp1 to Kp7) with *K. pneumoniae* sensu stricto, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *variicola*, *K. variicola* subsp. *tropica*, *K. quasivariicola*, and *K. africana*, respectively[4]. It has become an important MDR pathogen of the last decade with multiple resistance determinants, mostly for aminoglycosides, cephalosporins and carbapenems[5]. It has been

isolated from hospital-acquired infections including pneumonia, bloodstream infection, urinary tract infection, and community acquired infections such as hepatic abscess and meningitis and pneumonia. The capsule is an important virulence factor that protects *K. pneumoniae* from phagocytosis, with over 79 defined capsular serotypes. Isolates with K1 and K2 capsular serotypes are associated with virulent infections. However, not all K2 capsular isolates are virulent. In this regard, virulence was confirmed in murine models[6]. Virulence factors associated with hypervirulent *Klebsiella* infections include siderophores, virulence plasmid encoding aerobactin, salmochelin, yersiniabactin, and the hypermucoviscosity factor *rmpA/mpA2* gene [7-12].

Here we report on the characterization of a series of 12 *K. pneumoniae* isolated from the sputum and bubo aspirates from clinically suspected plague patients during the plague outbreak in Madagascar, 2017.

Material and methods

Patients and bacterial isolates

Patients with confirmed *K. pneumoniae*, isolated during nine days of the plague epidemic (from the 6th of October 2017 till the 14th of October 2017), were included in this sub-study. Epidemiological, clinical and lab data of patients were extracted from the plague national surveillance system database of Institut Pasteur de Madagascar between August 1st and November 26th in 2017[1]. *Y. pestis* was isolated from biological samples (bubo aspirates for bubonic plague, sputum for pneumonic plague) by direct culture on *Yersinia* selective Cefsulodin-Irgasan-Novobiocin (CIN) agar medium (Oxoid Ltd., United Kingdom). All methods were carried out according to the 2006 WHO recommendations[13]. Culture incubation was done at 26–28?C for 48?h or longer as *Y. pestis* grows slower than other bacteria. Colonies obtained within 24 h on CIN medium and which did not have *Y. pestis* morphology were identified on MALDI-TOF MS (Biotyper version 3.3, Bruker Daltonics,

Champs-sur-Marne, France). Colonies identified as *K. pneumoniae* were further purified on Simmons Citrate Agar Inositol (SCAI) medium[14].

Phenotype detection

The hypermucoviscosity phenotype of the *K. pneumoniae* isolates was determined using the string test, in which a standard bacteriological loop is used to stretch a mucoviscous string from each colony cultured on SCAI medium. The formation of a viscous string > 5 mm in length was regarded as a positive test result[12].

Bacterial susceptibility testing

Antibiotic resistance profiles were determined by the standard disc diffusion method according to CASFM-EUCAST V2-0-May2017 guidelines and using breakpoints for *Enterobacteriaceae* (http://www.sfm-microbiologie.org). Isolates were tested against 17 commonly used antimicrobial agents, namely amoxicillin, amoxicillin-clavulanate, piperacillin/tazobactam, cefalotin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, ertapenem, tobramycin, gentamicin, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole and tetracycline. In addition, extended spectrum ß-lactamase (ESBL) production was tested using the standard double disc synergy test.

Genome sequencing and analysis

Genomic DNA of the *K. pneumoniae* isolates was extracted using DNeasy Blood & Tissue kit (Qiagen, Germany) and was subjected to whole genome sequencing. Genomic libraries were constructed using the Nextera XT DNA library preparation kit with dual indexing (Illumina, San Diego, USA). The libraries were sequenced on an Illumina NextSeq-500. Genome assembly was performed *de novo* using Spades Genome Assembler (Version 3.10.0). Genome analyses for core genome MLST (cgMLST) on 632 core gene were performed,

sequence types (ST), virulence genes, and capsular serotypes (K-types) were assigned using the *Klebsiella pneumoniae* database hosted through the BIGSdb web application of the Institut Pasteur in Paris (https://bigsdb.pasteur.fr/klebsiella). Antimicrobial resistance genes were identified from genome sequences using the Resfinder (version 3.2). Plasmid replicon types were determined by using the Plasmidfinder (version 2.0) tool at https://genomicepidemiology.org/. RAxML and parsnp were used to draw the tree. The Gubbins software tool was used to remove the single nucleotide polymorphisms (SNPs) on recombined regions and to create a phylogenetic tree using Maximum Likelihood. The tree was subsequently annotated with iTOL.

Buboes *K. pneumoniae* screening by real-time PCR

We performed a real-time PCR targeting the *zur-khe* intergenic region[15] on the bubo samples in order to confirm the presence of *K. pneumoniae* DNA in the bubo and to exclude any technical contamination during culture. Bacterial DNA was extracted from the bubo samples using DNeasy Blood & Tissue kit (Qiagen, Germany). The real-time PCR assay was performed as previously described with the difference that we used 10 µl of Sso Advanced universal SYBR Green Supermix (Bio-Rad, USA)[15]. Amplifications were performed using the CFX-96 (BIO-RAD, USA) platform. The positive controls consisted of DNA from *K. pneumoniae* UAA2239 and UAA2016 which are reference strains from the National Reference Center for Antibiotics from the Institut Pasteur in Paris, the negative control was plain molecular grade water.

Ethics statement

No additional ethical clearance was requested because the analysis was carried out within the framework of the national surveillance system. No additional data was collected.

All patients provided oral consent and voluntarily agreed for sampling for diagnostic purposes.

Nucleotide sequence accession numbers

WGS data have been deposited at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA565154.

Results

Case presentation and *Klebsiella pneumoniae* antimicrobial susceptibility

K. pneumoniae was isolated from 12 clinical samples out of 496 collected between 06 and 14 October 2017 in Antananarivo (N=362) and Toamasina (N=134) and screened for *Y. pestis* presence by culture. This period was reported as the peak of the plague epidemic curve, with essentially pneumonic plague cases. Patients had early clinical signs suggesting pulmonary, secondary pulmonary or bubonic plague in an epidemic setting.

The samples containing *K. pneumoniae* were 10 sputum samples and two bubo aspirates and were negative for *Y. pestis* colonies. The description of the 12 patients is shown in (Table 1). Two-thirds of the patients (N=8) were from Antananarivo and one-third (N=4) was from Toamasina. Seven patients were men. Five patients were younger than 18 years, and six were 19 to 27 years old and one was 46 years old. Fever status was reported for 10 patients, eight of them had body temperatures >37.5°C. According to the clinical forms, nine patients were suspected of having pulmonary plague and one was defined to suffer from secondary pulmonary plague. One patient was suspected of bubonic plague and for another patient, data about the clinical form was lacking.

Four of the patients coughed for at least 5 days and complained of chest pain, although they were in an overall good state of health. Two patients had signs of hemoptysis, and one of

them was in weak health. Two patients coughed without further complaints but one was in weak health.

Four patients were under antibiotic treatment at the time of sample collection: two with trimethroprim-sulfamethoxazole; one with doxycycline and one with gentamicin. One patient who received trimethroprim-sulfamethoxazole was treated in addition with amoxicillin. There was no mortality among these patients (Table 1).

Abundant colonies with a typical *K. pneumoniae* morphology (moist, dome-shaped) were recognized after 24h culture on CIN medium. The twelve isolates were identified as *K. pneumoniae* by MALDI-TOF. The presence of *K. pneumoniae* DNA in the two bubo samples was also detected by real time-PCR. Melting curve values for the detection of *K. pneumoniae* were 79° and 80° for the positive controls and the DNAs extracted from buboes, respectively (Fig. 2). Of the 12 isolates, four had a positive string test.

Five isolates were ESBL producers. Six isolates were resistant to sulfonamides and trimethoprim. Three and two isolates were resistant to gentamycin and tobramycin, respectively. One isolate was resistant to ciprofloxacin (Table 2).

Genome analysis

Whole genome sequencing of the *K. pneumoniae* isolates allowed us to characterize cgMLST alleles, virulence genes, capsular loci and resistance genes. All *K. pneumoniae* isolates were *Klebsiella pneumoniae* sensu stricto (Kp1) (Fig 1). Among the 12 isolates eight different sequence types (STs) were identified: ST23 (N=1); ST86 (N=2); ST65 (N=1); ST280 (N=1); ST327 (N=1); ST380 (N=1); ST716 (N=1); and ST3012 (N=1), and three new STs: ST3441 (N=1), ST3442 (N=1), ST3443 (N=1) (Table 2). Comparative genomic analysis of the two Kp ST86 isolates showed that they differed from each other by 123 alleles out of 632 scgMLST gene loci.

193 The virulence genes identified in most isolates were colibactin locus (*clb*), siderophores
194 (*IroBCDN*), iron uptake systems and regulators (*kfu*, *kvgA*, respectively), yersiniabactin
195 (*fyuA*, *irp1/2* and *ybt*) (Supplementary data). The PP3-ST380-KL1 isolate, two ST86 isolates
196 and the PP11-ST3443-KL2 isolate were positive in string test. All the ST23, ST65, ST86 and
197 ST380 isolates had the genes *rmpA/rmpA2* associated with the hyperproduction of the
198 capsule and also carried the *iucABCD* genes coding for the synthesis of aerobactin
199 (Supplementary Data).
200 Isolates belonging to ST23, ST65, ST86 and ST380 were susceptible to all antibiotics tested,
201 with the exception of amoxicillin to which *K. pneumoniae* is intrinsically resistant.
202 A total of five isolates were ESBL producers (Table 2). A ST280 isolate and a ST3441 isolate
203 carried the $bla_{\text{CTX-M-15}}$ gene. A ST3442-KL1 isolate harboring virulence genes (*fyu*, *irp1/2*, *ybt*)
204 was ESBL producer and carried the cassette comprising *qnrB66*, *aac (3) -IIa*, $bla_{\text{SHV-27}}$ and
205 *tet* (A), as well as the IncFIB_{K} replicon marker (Table 2).
206
207 **Discussion**
208 Unexpectedly, 12 cases of *K. pneumoniae* infection were detected and identified among
209 individuals clinically suspected to have plague. No *Y. pestis* was identified from their clinical
210 samples, but three patients (PP8, PP10 and PP11) yielded a positive result on RDT.
211 However, culture is the gold standard for the identification of *Y. pestis* and RDT could provide
212 false results[16]. All patients had no epidemiological relationships and no family member or
213 contact had been recorded with plague.
214 Although the selective medium for *Yersinia* was not intended for *K. pneumoniae* isolation, *K.*
215 *pneumoniae* does grow on the CIN medium in 24h. The selectivity of this medium is reported
216 as being partial, as other Gram-negative bacilli can grow on CIN medium, including other
217 species of Enterobacterales able to ferment mannitol[17]. Therefore, full species identification
218 is recommended.

219 It is not surprising to isolate *K. pneumoniae* from pneumonia cases. Community-acquired *K.*
220 *pneumoniae* infections are common, including in Africa[18,19]. In contrast, to our best
221 knowledge, *K. pneumoniae* isolated from buboes aspirates were never reported previously.
222 Additionally to culture, the presence of *K. pneumoniae* in buboes aspirates was confirmed by
223 PCR. The advantage of melting curve analysis over Taqman based real time-PCR is its
224 lower costs without losing specificity[20]. Further studies are needed to evaluate this method,
225 which could be used in screening for *K. pneumoniae* in buboes or other suspected biological
226 samples.
227 *K. pneumoniae* has the capacity to acquire resistance genes and to become increasingly
228 more difficult to treat. One of the *K. pneumoniae* isolates detected in one of two patients who
229 was treated by combination of amoxicillin and trimethoprim-sulfamethoxazole developed
230 resistance. However, *K. pneumoniae* is known to be intrinsically resistant to ampicillin due to
231 the presence of the chromosomal ß-lactamase SHV-1 or similar[21]. At the same time, we
232 identified in this strain (ST280), the two genes *dfrA14* and *sul2*, associated to resistance to
233 trimethoprim and sulfamethoxazole, respectively. In addition, among all isolates, it was the
234 only *K. pneumoniae* isolates to be resistant to piperacillin/tazobactam (TZP), which is
235 concordant with the presence of $bla_{\text{TEM-1B}}$ gene[22].
236 Two of the five ESBL isolates harbored $bla_{\text{CTX-M15}}$. This gene was commonly found in ESBL-
237 producing *K. pneumoniae* isolated in Madagascar[19]. bla_{SHV27} and bla_{SHV101} were found in the
238 other ESBL-producer.
239 According to MLST analysis, we observed the presence of five STs known to be associated
240 with hypervirulence, including STs ST23 (N = 1), ST65 (N = 1), ST86 (N = 2) and ST380
241 (N=1). In addition, a *K. pneumoniae* isolate having the new ST3443 differs by a single locus
242 from ST86 on the *tonB* locus (allele 18 instead of 27). This strain ST3443 tested string
243 positive, as was also the case for the ST380 and two ST86 strains. Whole genome analysis
244 of *K. pneumoniae* showed that the isolates with common ST differed to each other by alleles

245 occurring outside the 7 household genes. The presence of common virulence genes in the
246 ST23 isolate which were ICEKp10 encoding *clb 2* sequence variants, *ybt 1* and *rmpA/rmpA2*
247 suggests its belonging to CG23 sublineage I (CG23-I)[23].
248 MLST typing of *K. pneumoniae* isolated in different countries revealed that ST23, ST65,
249 ST86 and ST380 were responsible for hepatic abscess cases and other invasive CA
250 infections[24].These isolates have been reported particularly in Asia, but their diffusion outside
251 Asia has been described[24]. Among virulence factors, *mpA* and aerobactin are the most
252 important one[25]. The presence of genes responsible for the hypermucoviscosity phenotype,
253 *rmpA/rmpA2*, plays an important role in the virulence of *K. pneumoniae* isolates. These
254 genes are often associated with serotype K1 and K2. Expression of these genes allows the
255 bacteria to escape the host's defense system and colonize the mucous membranes.
256 Epidemiological studies have shown that the majority of ST23 are related to K1 capsular
257 serotypes and liver abscesses[23,26], while K2 is the second capsular serotype resulting in
258 community-acquired pneumonia[25]. Yersiniabactin, a virulence gene (*Ybt*), detected in the
259 three *K. pneumoniae* isolates serotyped K1 has been reported as the iron absorption system
260 in highly virulent *Y. pestis*[27], but was shown to have evolved ancestrally within the *Klebsiella*
261 genus[23]. Several studies have shown isolates belonging to these STs (23, 65, 86 and 380),
262 with the same combination of virulence factors, to be virulent in mouse models[12,23].
263 During an epidemic, knowledge of the etiology is essential in order to provide the most
264 adapted treatment to patients. Microbiological diagnosis can improve the effectiveness of
265 treatments, avoid long-term complications for the infected patient, and in addition avoid
266 widespread overuse and misuse of antibiotics. Early diagnosis can help to prevent or stop an
267 outbreak too. One of the reasons for a possible treatment failure could arise during
268 inaccurate diagnoses and inappropriate treatments. Similar symptoms can lead to routine
269 treatments based on syndromic approaches which are often applied in developing countries.
270 As the physician is rarely able to make an etiological diagnosis on clinical grounds alone,

treatment should ideally be based on the result of bacteriological examination. In this case, bacteriological diagnosis could be complicated by the fact that the respiratory tract could be infected by *K. pneumoniae*[28]. Although the population we included in our study is young, the clinical signs of a few patients warned us of possible serious infections due to *K. pneumoniae* such as bloody sputum and a chest pain which were among typical signs of pestis pneumonia, however, *Y. pestis* was not found in culture. Typically, the plague is better known by its three clinical forms: bubonic, septicemic and pulmonary plague while hypervirulent *K. pneumoniae* strains are known to cause pneumonia, sepsis, liver abscesses and meningitis[29-31].

We acknowledge the following limitations of our study. First, we studied a limited number of samples in a short duration of the epidemic, which is far from being representative of all the negative samples for *Y. pestis*. Second, detailed data about clinical characteristics and outcomes were lacking due to the outbreak emergency context. Finally, we did not confirm the virulence of the *K. pneumoniae* strains using mouse models.

Conclusions

Although few samples were studied, within a short duration of inclusion (9 days from 06/10/17 to 14/10/17), our results show that the plague-suspected patients in fact acquired a pneumonia caused by *K. pneumoniae*. Bacterial identification proved useful for determining the etiology. WGS and AST results showed that among the 12 *K. pneumoniae* isolates, there were ESBL producers and virulent strains. This study shows that during epidemics of plague, clinicians and microbiologists should also consider other pathogens, especially *K. pneumoniae* given the fact that the clinical symptoms of respiratory plague can be confused with other pathologies.

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299

300 **Competing interests**

301 None.

302

303 **Ethical approval**

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305

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309

310 **Author contributions**

311 A.R., T.C., V.J., S.B., M.R. and J.C. wrote the manuscript. A.R., L.M.A., S.A.R., S.H.R., V.A.,

312 S.R. performed lab experiments. S.A.R, V.A., S.R., M.R completed data collection. A.R.,

313 S.B., V.J., M.R. and J.C. conceived the study. A.R., T.C., S.B., M.R. and J.C. analysed and

314 interpreted data. All authors read and approved the final manuscript.

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Figures

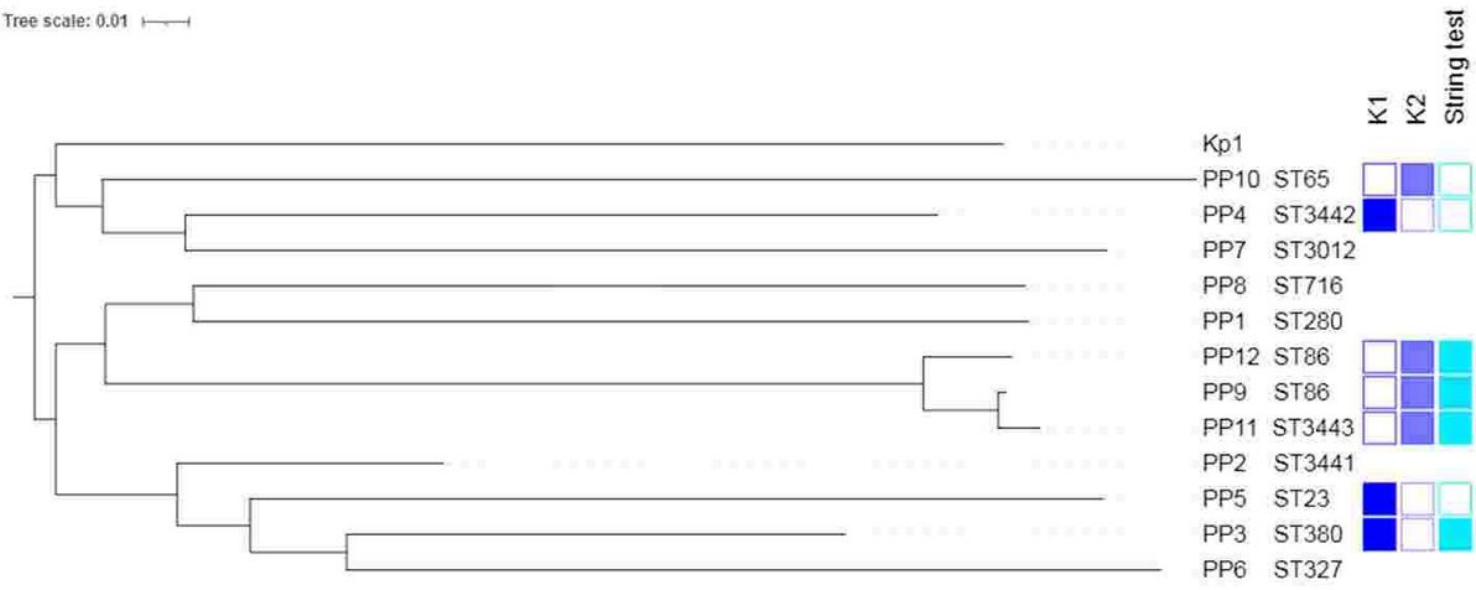

Figure 1

Phylogenetic tree of 12 K. pneumoniae isolates

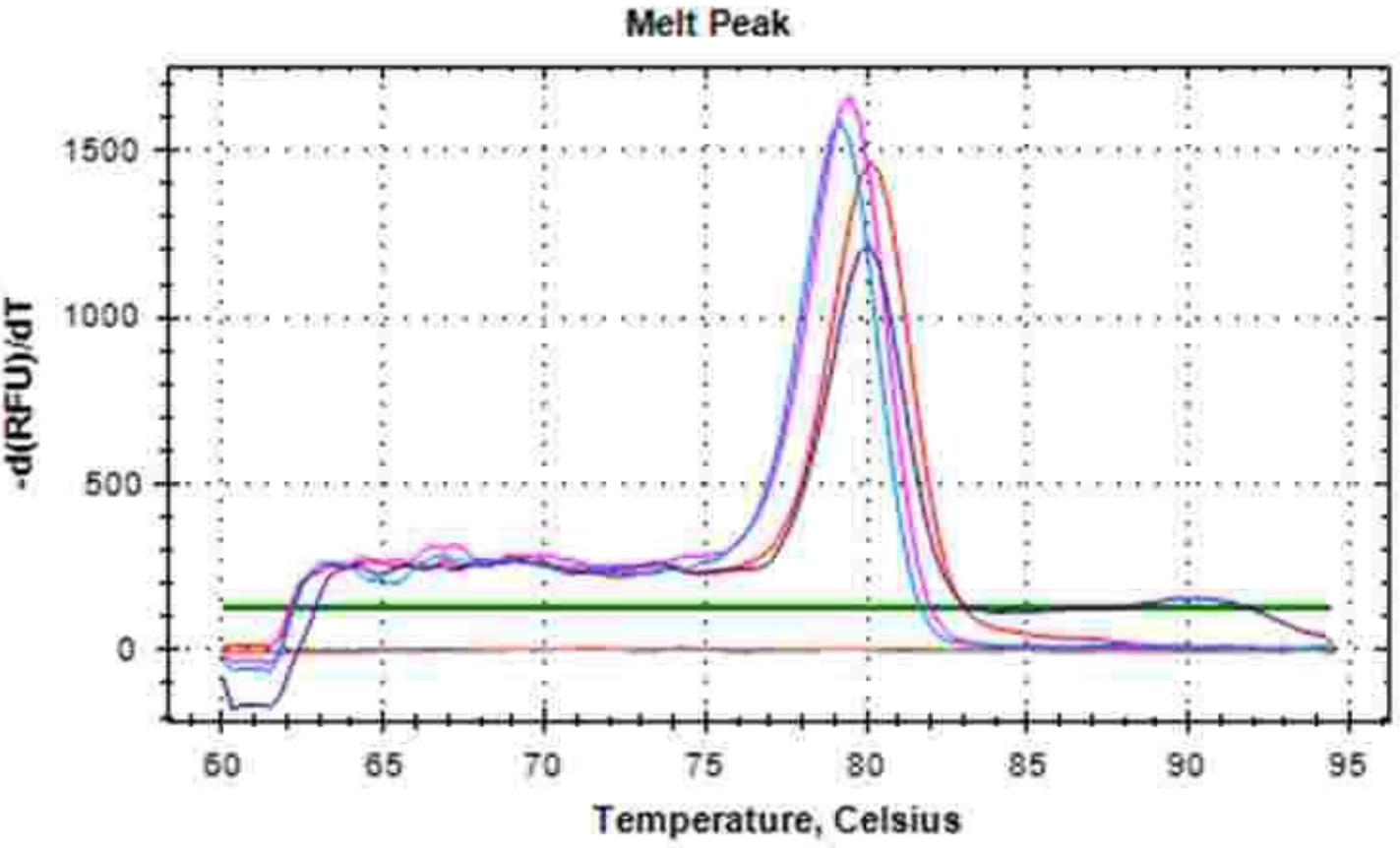

Figure 2

Melting curve results from ZKIR region detection obtained after 40 cycles on the ZKIR quantitative PCR system of Kp. DNA from two positive controls from strains blue to UUA 2239, violet to UUA 2016. DNA extracted from patients' buboes in pink to PP2 and red color to PP8. Orange color corresponds to negative control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.pdf
- TableS1.csv