An outpatient clinic as a potential site of transmission for an outbreak of NDM-producing *Klebsiella pneumoniae* ST716: a study using whole-genome sequencing

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Running title: NDM-producing K. pneumoniae outbreak

40-word summary: Combining whole genome sequencing data with epidemiological data, we investigated an outbreak of NDM-producing *K. pneumoniae* across two Belgian hospitals. We identified the outpatient clinic as the likely common site of transfer between the two institutions.

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Abstract

Background.

The incidence of nosocomial infections due to carbapenem-resistant *Klebsiella pneumoniae* is increasing worldwide. Whole genome sequencing (WGS) can help elucidate the transmission route of nosocomial pathogens.

Methods.

We combined WGS and epidemiological data to analyze an outbreak of NDM-producing *K. pneumoniae* that occurred in two Belgian hospitals situated about 50 miles apart. We characterized 74 NDM-producing *K. pneumoniae* isolates [Hospital A (n=9); Hospital B (n=24) and 41 contemporary isolates from 15 other Belgian hospitals] using pulsed-field gel electrophoresis and WGS.

Results.

A *K. pneumoniae* ST716 clone was identified as being responsible for the outbreak with 9/9 strains from Hospital A and 20/24 strains from Hospital B sharing a unique pulsotype and being clustered together on WGS (compared with 1/41 isolates from other Belgian hospitals). We identified the outpatient clinic of Hospital B as the probable bridging site between the hospitals after combining epidemiological, phylogenetic and resistome data. We also identified the patient who probably caused the transmission. In fact, all but one strain from Hospital A carried a *Tn*1331-like transposon, whereas none of the Hospital B isolates did. The patient from Hospital A who did not have the *Tn*1331-like transposon was treated at the outpatient clinic of Hospital B on the same day as the first NDM-producing *K. pneumoniae* positive patient from Hospital B.

Conclusions.

The results from our WGS-guided investigation highlight the importance of implementing adequate infection control measures in outpatient settings, especially when healthcare delivery moves from acute care facilities to outpatient clinics.

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Keywords: nosocomial outbreak, carbapenemase, infection control, whole genome MLST, ambulatory care

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Introduction

Klebsiella pneumoniae is a leading nosocomial pathogen, which is well recognized as a major public health problem [1-3]. *K. pneumoniae* isolates easily acquire and transmit determinants of antimicrobial resistance, including extended-spectrum beta-lactamases and carbapenemases. In particular, the spread of carbapenemase-producing *K. pneumoniae,* largely driven by mobile genetic elements carrying carbapenemase genes, is of particular concern [4].

New-Delhi metallo-beta-lactamase (NDM) is an Ambler class B carbapenemase that efficiently hydrolyzes carbapenems and most beta-lactams, with the exception of aztreonam [5]. NDM-producing *K. pneumoniae* isolates have disseminated worldwide and are endemic in some areas of the world, including the Indian subcontinent [4]. Multiple nosocomial outbreaks of NDM-producing *K. pneumoniae* have been reported in Asia, Europe and America [4, 6-16]. NDM-producing *K. pneumoniae* isolates responsible for outbreaks may belong to various sequence types (STs). ST11, ST15, and ST17 in particular are frequently associated with multi-drug resistance [6, 7, 9, 11, 16-18].

Powerful molecular typing techniques are important to enable rapid recognition of nosocomial outbreaks caused by multi-drug resistant organisms (MDRO), and to enable strategies to be implemented to prevent the transmission of the involved pathogens [19, 20]. Whole genome sequencing (WGS) offers many advantages for investigation of such outbreaks, including the resolution power to explore the route and direction of transmission [21-23]. However, the analysis of WGS-based typing results is still challenging and requires carefully calibrated parameters for accurate interpretation.

Between 2012 and 2016, the Belgian National Reference Center (NRC) of antibiotic resistant gram-negative bacilli noted an increase in the number of NDM-producing *K. pneumoniae* isolates. Currently, NDM is the second most prevalent carbapenemase among isolates referred and confirmed as carbapenemase-producing Enterobacteriaceae by our NRC [24]. In particular, two Belgian hospitals had outbreaks of NDM-producing *K. pneumoniae* in 2014-2015. In this study, we analyzed

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these NDM-producing *K. pneumoniae* outbreaks using a unique combination of WGS-based approaches to identify the link and direction of transmission between the hospitals.

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Methods

Hospital settings

Hospital A is an academic tertiary care center of 864 beds including 36 intensive care beds divided into five intensive care units (ICU). In Hospital A, there are around 30,000 admissions per year of which 1,000 admissions are to the ICU. Hospital B is a general 211-bed institution (including nine ICU beds) and is part of a multi-site general healthcare institution of 838 beds. Around 6,000 patients are admitted each year to Hospital B. Hospitals A and B are located in two different Belgian regions and are situated approximately 50 miles apart.

Definitions and data collection

An outbreak case was defined as a patient colonized or infected by carbapenem-non susceptible *K. pneumoniae* between August 2014 and December 2015. *Post hoc*, we classified outbreak cases as proven (defined as a patient with a NDM producing-*K. pneumoniae* of identical Pulsed-Field Gel Electrophoresis [PFGE] profile and ST) or possible (patient with a carbapenem-non susceptible *K. pneumoniae* but without molecular NDM detection or typing analysis). A sporadic case was defined as a patient carrying a molecularly-identified NDM-producing *K. pneumoniae* strain not belonging to the same PFGE- and ST-type as the proven outbreak case strains. For each hospital, the incidence rate, expressed as the number of cases/100,000 patient-days, was compared between the outbreak period and one year earlier. In addition, we collected the history of the hospital stay, history of transfers between study hospitals, and date and type of sample regarding first evidence of carbapenem-non susceptible *K. pneumoniae*. Colonization was defined as the carriage of a carbapenem-non susceptible *K. pneumoniae* not treated with active antibiotics, whereas patients treated with active antibiotics were considered to be infected.

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Screening policy

K. pneumoniae isolates were collected from inpatients of the two study hospitals, using cultures of either clinical or rectal screening samples. At the time of the outbreak, a screening strategy for gram-negative MDRO was applied systematically upon patient admission in four high-risk departments (ICU, hematology, geriatric and gastroenterology wards) in Hospital A. Additionally, weekly surveillance was carried out during the hospital stay in the ICU and the Department of Hematology. Contact precautions were preemptively used in high-risk patients (i.e., patients with a travel history and/or previously hospitalized in another institution) until rectal swabs culture results. This screening policy was continued in Hospital A during the study period without additional measures (i.e., no cohorting with dedicated nursing staff).

In Hospital B, there was no systematic active screening surveillance for gram-negative MDRO until the detection of three patients positive for a NDM-producing *K. pneumoniae* in the Department of Internal Medicine. From that moment onwards, the infection control team started screening for MDRO among (1) contact patients (i.e., patients sharing the same room as possible outbreak cases) and (2) inpatients staying ≥15 days in Hospital B. In parallel, inpatients from all departments of Hospital B were screened during three point-prevalence surveys. Positive patients were placed on contact precautions and were cohorted in a single ward with dedicated nursing staff.

Bacterial isolates and conventional microbiology techniques

A total of 74 NDM-producing *K. pneumoniae* isolates were analyzed in this study (**Figure 1**). This included nine isolates from Hospital A and 24 isolates from Hospital B. For comparison, we also included 41 NDM-producing *K. pneumoniae* isolates collected elsewhere in Belgium and sent to the NRC between 2014 and 2015. Antimicrobial susceptibility testing (AST) was carried out using disk diffusion method (BioRad, Marnes-la-Coquette, France) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org/). The

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presence of bla_{NDM} was confirmed by polymerase chain reaction (PCR), as described elsewhere [25]. Molecular typing was performed by genomic macrorestriction (*Xba*I) followed by PFGE [26].

WGS, *de novo* assembly and genome annotation

Nucleic acids were extracted using the DNA extraction ultraclean microbial DNA isolation kit (MOBIO, Carlsbad, USA). DNA libraries were prepared using Nextera XT DNA library preparation kit (Illumina, San Diego, USA), following the manufacturer's recommendations except for the normalization step that was done manually. WGS was performed using the Illumina MiSeq platform (Illumina, San Diego, USA) to generate 200bp paired-end reads. Runs were accepted according to the criteria defined by Illumina for MiSeq Reagent kit v3. *De novo* assembly was performed using the SPAdes algorithm [27]. Genomes were annotated using BioNumerics® v7.6 (Applied Maths, Sint-Martens-Latem, Belgium), and refined using the basic local alignment search tool (BLAST) when necessary (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic analysis and identification of antibiotic resistance genes

STs were assigned *in silico* by uploading assembled genomes to the multi-locus sequence typing (MLST) 1.8 tool (Center for Genomic Epidemiology (CGE), Denmark, http://www.genomicepidemiology.org/) [28]. The whole genome MLST (wgMLST) *K. pneumoniae* scheme available on BioNumerics® v7.6 was used to compare 4015 genes located on the core and accessory genome [29]. The whole genome single nucleotide polymorphism (wgSNP) analysis was done on all strains from proven outbreak cases, using Bionumerics® v7.6 [29]. For this purpose, we selected a *de novo* assembled reference genome based on sequence quality criteria for mapping, in order to determine SNP differences. Default filtering procedures were used to remove artifactual SNPs [30] and to construct minimum spanning tree (see **Supplementary data** for additional details regarding SNP analysis). Acquired antimicrobial resistant genes were identified by uploading

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assembled genomes in ResFinder 2.1 (CGE, Denmark) [31]. BLAST analysis and home-made PCRs were used to resolve discrepancies.

Ethics

Ethical approval for this study was granted by the Ethical Committee of ULB-Erasme Hospital (P2015/546).

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Results

Description of outbreaks

The timeline of proven outbreak cases from Hospitals A and B is shown in **Figures 1a** and **S1**. Metadata related to proven outbreak cases of Hospitals A and B are provided in Supplementary data (**Table S1**).

Hospital A. The incidence rate of NDM-producing *K. pneumoniae* increased from a baseline rate of 1.5 cases/100,000 patient-days (time-period August 2013 to July 2014) to 9.4 cases/100,000 patient-days during the outbreak period (August 2014 to March 2015). The first patient (Patient 1A) was detected in August 2014, one month after being admitted to the ICU. We suspected nosocomial acquisition in this first case because he had had multiple culture-negative rectal swab specimens since admission to Hospital A. However, we did not detect other concomitant NDM carrier(s) in the ICU, despite weekly surveillance screening cultures of all ICU patients. Patient 1A was then transferred to the Department of Pneumology. There, a second patient was detected with nosocomial acquisition of NDM-producing *K. pneumoniae* (Patient 2A). We later identified seven additional infected (n=4) or colonized (n=3) patients in five wards of Hospital A. All nine isolates showed the same PFGE-types and belonged to ST716 (considered as proven outbreak cases). We failed to identify a clear epidemiological link to explain the acquisition of this NDM-producing *K. pneumoniae* clone for the seven latter outbreak cases.

Hospital B. An outbreak was declared at Hospital B in January 2015 and persisted until December 2015 (end of study). The incidence rate of NDM-producing *K. pneumoniae* increased from 1 case/100,000 patient-days (2014) to 102 cases/100,000 patient-days (2015). After the identification of the first three NDM positive *K. pneumoniae* patients, an active surveillance screening program was implemented, as previously described. This demonstrated 64 additional possible outbreak cases involving the entire hospital. The most affected wards were the ICU, the Orthopedic Surgery Unit and the Departments of Geriatric and Internal Medicine. Twenty-one percent of these patients (18/67) were infected; the others were colonized. From the 67 possible outbreak cases, 24 isolates were

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available for WGS analysis, which demonstrated that 20/24 were proven outbreak cases (**Figure 2**). Of these, 15/20 patients were colonized.

Epidemiological link between Hospitals A and B. From the epidemiological data, Patient 2A was considered as the probable link between the hospitals. First, he was repeatedly seen at the outpatient clinic of Hospital B after discharge from Hospital A (**Figure 1b**). None of the other proven outbreak case patients from Hospital A were referred to Hospital B. Second, the first patient detected from Hospital B (Patient 1B) was treated at the outpatient clinic of Hospital B on the same day as Patient 2A (**Figure 1b**). No other relevant link could be established between patients from Hospitals A and B.

Population genomic analysis

Molecular typing using MLST, PFGE and wgMLST enabled us to separate out the 74 NDM-producing *K. pneumoniae* strains investigated in this study, as indicated in **Figure 2** (see **Supplementary data** for WGS assembly quality results). In fact, 30/74 strains (i.e., 9/9 strains from Hospital A, 20/24 strains from Hospital B and 1/41 strain from the NRC collection) were assigned to ST716, shared the same PGFE-type and clustered together on the wgMLST analysis (**Figure 2**). These 30 strains were closely related; they were separated by a mean pairwise allelic distance of only 17 alleles (out of 4015 analyzed alleles) and by no more than six SNPs (**Figure 3**). After construction of a phylogenetic tree (i.e., minimum spanning tree), the strain genome from Patient 2A was consistent with a progenitor status relative to Hospital B cases (**Figure 3**). In fact, this strain was just a single SNP away from a genome type occupying a central node position relative to all Hospital B genomes, and only three SNPs different from the Patient 1B strain genome.

Of note, the single strain from the NRC (isolated in November 2015) was detected in a hospital located close to Hospital B, and belonged to a patient who had been hospitalized in Hospital B in October 2015. In contrast, the remaining 44 strains from Hospital B (n=4/24) and the NRC (n=40/41) showed completely different pulsotypes, displayed a variety of distinct STs (ST11, ST15, ST307 and

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ST432) and a mean pairwise allelic distance of 3348 relative to the proven outbreak strains (in line with the allelic diversity previously described for *K. pneumoniae* [32]).

AST and resistome

Phenotypically, all proven outbreak strains were resistant to all tested beta-lactams, fluoroquinolones and trimethoprim-sulphamethoxazole. Most were susceptible to colistin (n=29/30), chloramphenicol (n=26/30), amikacin (n=22/30) and tigecycline (n=18/30).

Our population of proven outbreak strains had two major phenotypic and genotypic resistance patterns (**Table S3**). The first pattern (n=8/30), phenotypically characterized by amikacin-resistance and genotypically by carriage of $bla_{\text{OXA-9}}$, *aac(6´)Ib* and *aadA1* genes (by a *Tn*1331-like transposon), was found in all patients from Hospital A with the exception of Patient 2A. The second pattern (n=22/30) was phenotypically characterized by susceptibility to amikacin and genotypically by the absence of the previously listed genes due to the loss of the *Tn*1331-like element. This pattern was identified in the strain from Patient 2A (Hospital A) as well as in all 20 proven outbreak strains collected from Hospital B. We therefore concluded that Patient 2A probably introduced the ST716 NDM-producing *K. pneumoniae* into Hospital B.

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Discussion

In this study, we investigated two NDM-producing *K. pneumoniae* outbreaks occurring in two Belgian hospitals. First, we identified *K. pneumoniae* ST716, an infrequently reported clone [33], as being responsible for both outbreaks. We combined epidemiological data with WGS data –including findings from high-resolution typing methods and a resistome analysis- to identify the outpatient clinic of Hospital B as the probable transmission bridge between these outbreaks.

As a result of decreasing costs and technological improvements enabling fast and high-resolution results, WGS is increasingly being used in clinical microbiology, especially to analyze nosocomial outbreaks [23]. Unlike conventional typing methods, such as PFGE and MLST, WGS provides multiple information levels from a single technique, including data on antimicrobial resistance, genome comparison, wgMLST and SNP analysis [23].

Using PFGE and WGS, we identified that the NDM-producing *K. pneumoniae* outbreaks observed in Hospitals A and B were due to the same NDM-producing *K. pneumoniae* clone (i.e., ST716). The first outbreak case was detected in Hospital A in August 2014. It is probable that additional colonized patients may have passed unnoticed because of the lack of a systematic active surveillance screening policy for MDRO in some units and wards of Hospital A. Regarding Hospital B, the NDM-producing *K. pneumoniae* outbreak was detected in January 2015 and involved 67 possible outbreak cases. Our findings highlight the advantages of centralizing data (e.g., at the NRC) in order to help document MDRO outbreaks and identify links between hospitals [34].

Our genomic and epidemiological data converged to identify the outpatient clinic of Hospital B as the specific site linking the two outbreaks. The second proven outbreak case (Patient 2A) was referred from Hospital A to the outpatient clinic of Hospital B, and his visit there overlapped with a visit by Patient 1B. This epidemiological suspicion was supported by two distinct lines of WGS-derived evidence. First, our SNP-based phylogenetic reconstruction identified that a single SNP separated the genome of Patient 2A's strain from a central node (Patient 9A's strain) relative to all Hospital B nodes, and the Patient 2A strain genome was only three SNPs away from the Patient 1B strain

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genome. This position is consistent with the hypothesis that Patient 2A carried a progenitor - or a microvariant of this progenitor - of the Hospital B strains. Second, resistome analysis allowed us to reinforce the hypothesis that the transmission occurred between Patients 2A and 1B. Indeed, the loss of the *Tn*1331-like transposon detected in the Patient 2A strain represents more precise evidence for the transmission from Hospital A to Hospital B via this patient. Patient 2A was the only patient from Hospital A whose strain did not carry this transposon. Conversely, this transposon was absent in all patient strains from Hospital B. The loss of this transposon served as a marker of transmission from Hospital A to Hospital B, in addition to MLST, wgMLST and SNP results. Hitherto, these different layers have rarely been used in conjunction for outbreak investigation. Our study highlights the importance of a multi-modal approach combining multiple WGS results (e.g., findings from MLST, wgMLST, SNP analysis and resistome data) with epidemiological data for outbreak investigation.

The hospitalization report for Patient 2A did not specify his NDM-*K. pneumoniae* carrier status at the time of referral to the outpatient clinic at Hospital B. Communication between healthcare facilities should be optimized by using electronic medical records and possibly by educating the patient [35]. Such measures may enable earlier appropriate infection control measures to be implemented after the interfacility transfer of patients carrying a MDRO. In this case, the fact that the source patient (Patient 2A) was seen at an outpatient clinic rather than in an acute care facility may have facilitated the dissemination of the NDM-producing *K. pneumoniae* ST716 clone. Indeed, outpatient clinics often lack appropriate resources and strategies to prevent dissemination of MDRO, as previously highlighted [36]. The implementation of adequate infection control measures in outpatient settings is crucial, because the healthcare delivery system increasingly switches between acute care facilities and outpatient clinics [35, 36].

This study has some limitations. The transmission route that we suggest (i.e., transmission from Patient 2A to Patient 1B via the outpatient clinic of Hospital B) cannot be formally proven as no screening policy was performed before the first case was detected in Hospital B. Alternate or

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additional transmission source(s) (e.g., via shared staff, or visiting relatives) cannot be excluded due to the retrospective study design. In particular, there was no systematic screening for NDM-producing *K. pneumoniae* isolates in the contact patients of outbreak cases from Hospital A. As a consequence, we may have missed identification of additional carriers of this NDM-producing ST716 *K. pneumoniae* clone who were transferred to Hospital B. However, both patient stay information and resistome analysis using WGS strongly support the role of Patient 2A in the dissemination of the NDM-producing ST716 *K. pneumoniae* clone from Hospital A to B via the outpatient clinic.

In conclusion, a multimodal analysis (i.e., combination of multiple results from WGS with epidemiological data) enabled us to conclude that a multi-hospital outbreak of NDM-producing *K. pneumoniae* was most likely due to the same NDM-producing *K. pneumoniae* ST716 clone. More specifically, we identified the outpatient clinic as the probable transmission site bridging the two outbreaks. Our findings highlight the importance of implementing adequate infection control measures in outpatient settings, especially as healthcare delivery moves from acute care facilities to outpatient clinics. Further studies are required to determine the importance of outpatient facilities in the dissemination of MDRO, and possibly to identify measures to prevent the spread of these organisms in such settings.

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Conflict of interest

None declared. P.S. reports consultancy fees from Genoscreen.

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Figures

Figure 1. (a) Timeline of detection of NDM-producing *K. pneumoniae* in proven outbreak cases of Hospitals A and B. Patients are ordered according to the date of detection of NDM-producing *K. pneumoniae* isolate (indicated as a black line). For each patient, the time from hospital admission to discharge is represented as a grey box. **(b) Timeline regarding the probable transmission from Patient 2A to Patient 1B via the outpatient clinic of Hospital B.** For each patient, the hospital stay is represented as a box, with colours and sizes depending on exact location (Hospital A vs. Hospital B vs. outpatient clinic of Hospital B) and length of stay, respectively.

Figure 2. Flowchart of included cases and analysis performed.

[a]Proven outbreak case: defined as a patient colonized or infected with a NDM-producing *K. pneumoniae* of identical PFGE- and ST-type. [b]Possible outbreak case: defined as a patient colonized or infected with a *K. pneumoniae* resistant to carbapenems but no molecular analysis of the isolate. [c]Sporadic case: defined as a patient carrying a molecularly-identified NDM-producing *K. pneumoniae* strain not belonging to the same PFGE- and ST-type as the proven outbreak case strains. MLST, multi locus sequence type; NDM, New-Delhi metallo-beta-lactamase; SNP, single nucleotide polymorphism; wgMLST, whole genome multi locus sequence type; WGS, whole genome sequencing.

Figure 3. Minimum spanning tree based on whole genome single nucleotide polymorphism (wgSNP) analysis of the NDM-producing *K. pneumoniae* isolates of the proven outbreak cases of Hospital A (n=9), Hospital B (n=20), and the National Reference Center collection (n=1). Each circle represents an isolate belonging to a proven outbreak case. The codes in the circles refer to case numbers and are ordered chronologically. The numbers on the branches indicate the number of single nucleotide polymorphisms. [a]Proven outbreak case: defined as a patient colonized or infected with a NDM-producing *K. pneumoniae* of identical PFGE- and ST-type. NRC, Belgian National Reference Center of antibiotic resistant gram-negative bacilli.

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Figure 1a

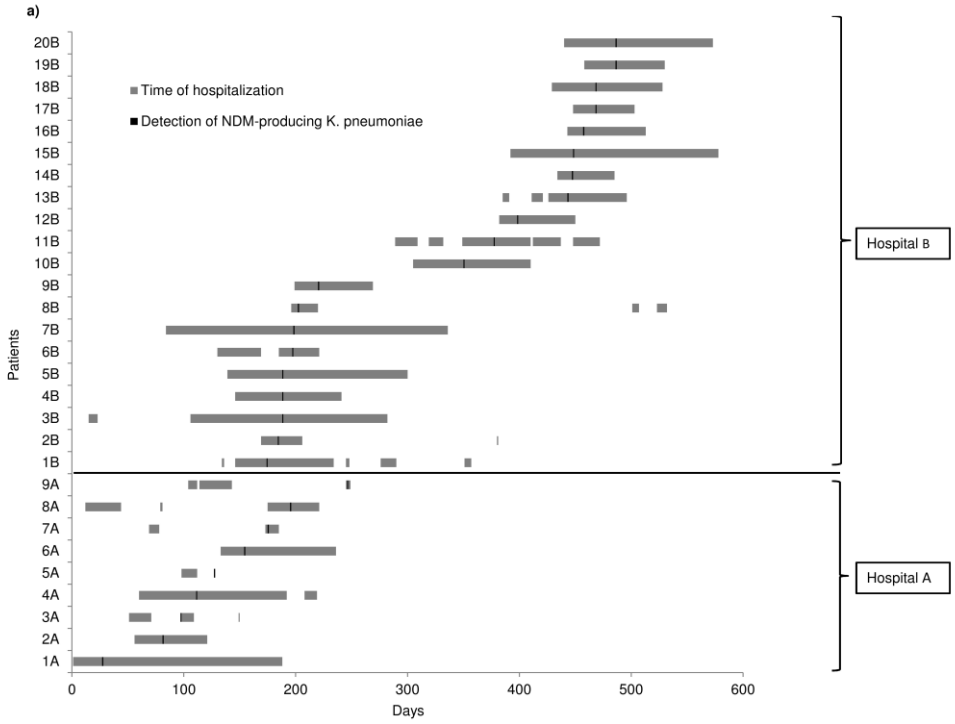

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Figure 1b

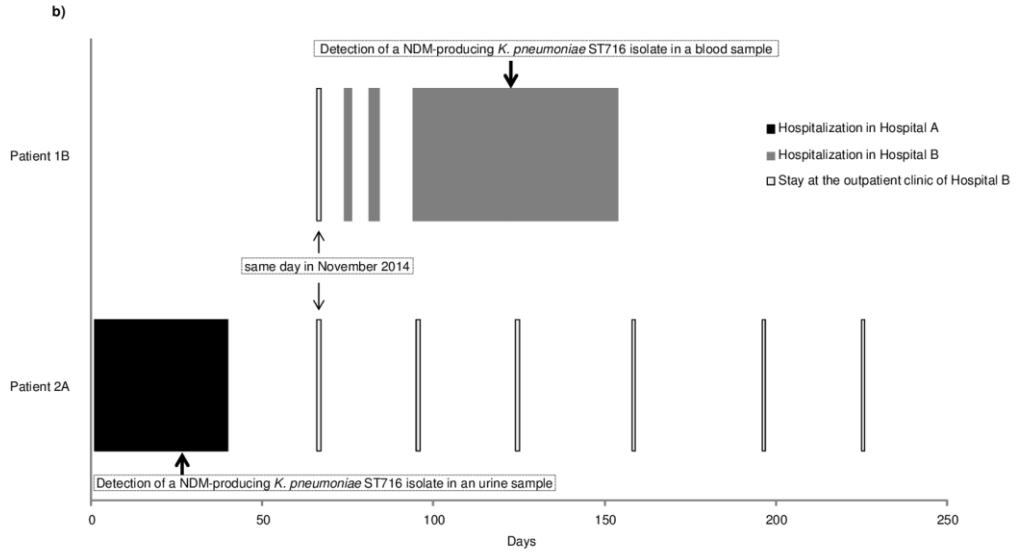

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Figure 2

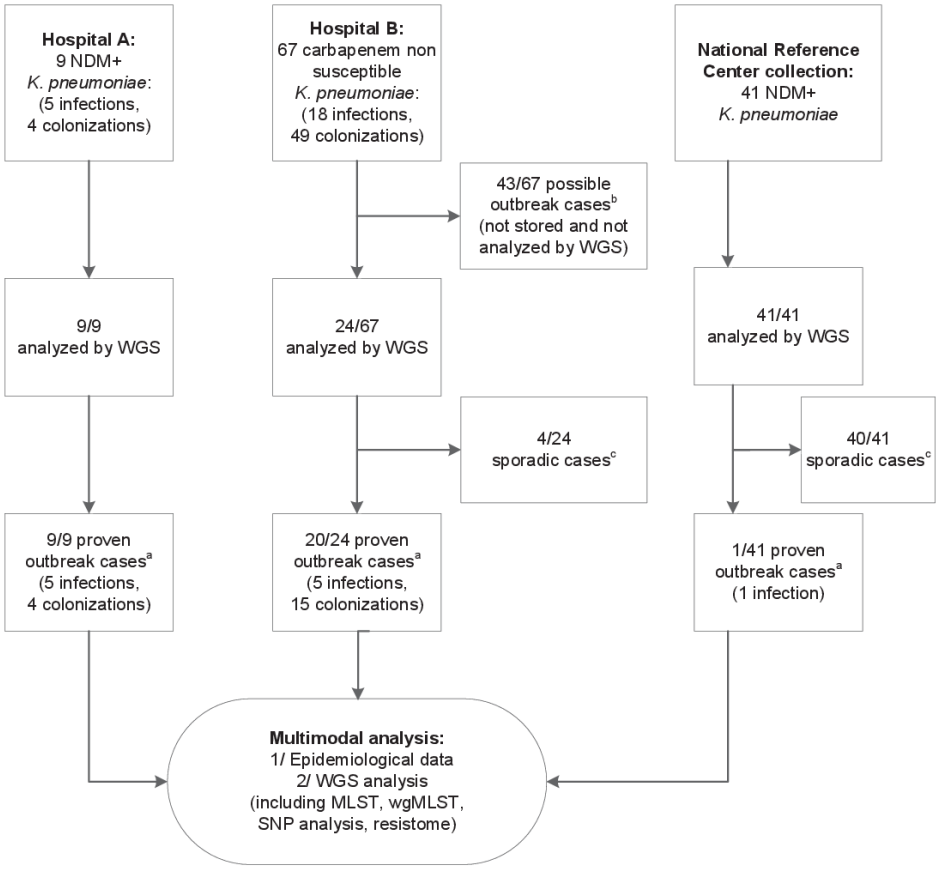

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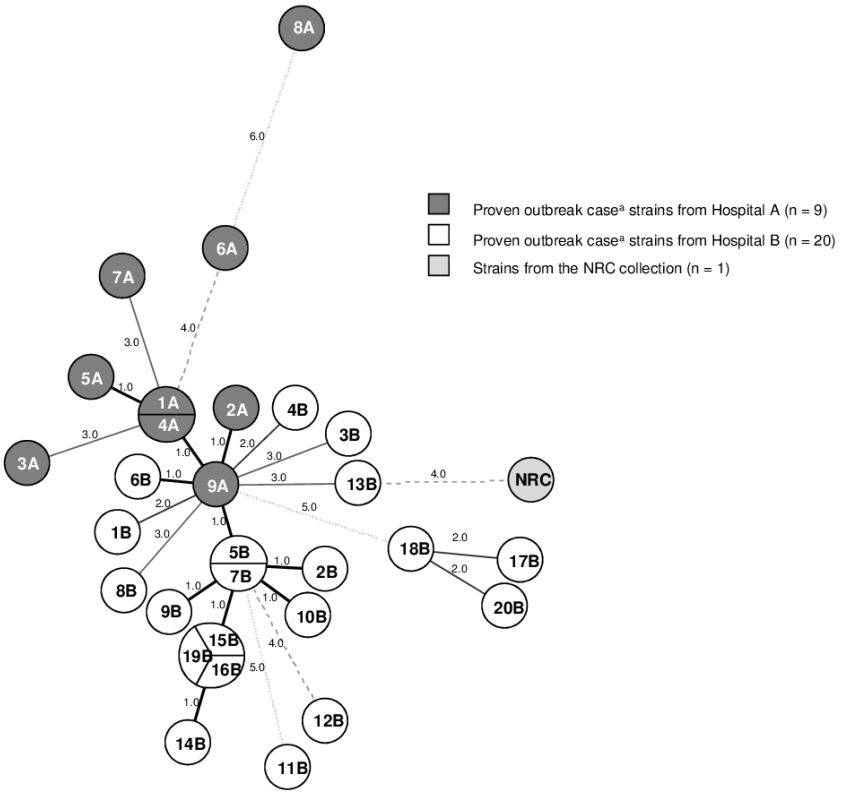
Figure 3



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