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Summary

Background

ESBL-producing *Enterobacteriaceae* (eESBL) have a high prevalence in hospitals but real-time monitoring of nosocomial acquisition through conventional typing methods is challenging. Moreover, patient-to-patient transmission varies between the main species, namely *Escherichia coli*, and *Klebsiella pneumoniae*, then questioning the relevance of applying identical preventive measures.

Aim

To detect eESBL cross-transmission events (CTE) using combination of quantitative antibiogram with epidemiological data (combined-QA), and to rule on the effectiveness of standard or contact precautions for eESBL species.

Methods

First, a validation set was used to confirm the relevance of the combined-QA by comparison to a combination of pulsed-field gel electrophoresis and epidemiological data (combined-PFGE). Secondly, a four-year retrospective analysis was conducted to detect eESBL-CTE in hospitalized patients. Two species were screened *i.e*. ESBL-*E. coli* (ESBL-Ec), and ESBL-*K. pneumoniae* (ESBL-Kp). During the study, only standard precautions were applied to ESBL-Ec patients whereas contact precautions were retained for ESBL-Kp.

Findings

As a proof of concept, results between the two combined methods for the detection of CTE were identical for *E. coli*, and similar to at least 75% for *K. pneumoniae*. During the retrospective analysis, 722 patients with ESBL-Ec isolates and 280 with ESBL-Kp isolates were included. Nine CTE were identified for *E. coli* and 23 for *K. pneumoniae*, implying 20 (2.7%) and 36 (12.8%) patients, respectively.

Conclusion

The QA-combined method constitutes a rapid tool for epidemiological surveillance to detect CTE. In our hospital, standard precautions are sufficient to prevent acquisition of ESBL-Ec whereas contact precautions must be implemented to prevent acquisition of ESBL-Kp.

Introduction

Antimicrobial resistance (AMR) is an alarming public health threat. Recent reports are not optimistic and highlight a high level of morbidity / mortality all over the world attributable to AMR [1,2]. In 2017, the WHO published a detailed list of priority pathogens including *Enterobacteriaceae* producing extended-spectrum β-lactamase (eESBL). Since the 1980s, eESBL have emerged and spread worldwide [3]. Public health strategy to reduce impact of eESBL relies on antibiotic stewardship and infection control by hygiene measures, especially in healthcare settings. Among eESBL, ESBL-producing *Escherichia coli* (ESBL-Ec) and ESBL-producing *Klebsiella pneumoniae* (ESBL-Kp) are the two main species of eESBL phenotype [4], even though they display different epidemiological traits. ESBL-Ec are endemic in community settings but exhibit paradoxically a low risk of cross-transmission in hospitals [5]. In contrast, ESBL-Kp are almost confined to hospital settings, and have a significant epidemic capacity, as evidenced by multiple nosocomial outbreaks [6–9]. To date, the type of hygiene measures to apply for controlling eESBL, *i.e.* standard or contact precautions, is still under debate [10]. One of the main issues is whether contact precautions should be systematically applied to colonized or infected patients [11–14]. Since 2015, based on the Belgian recommendations [15], and our own expertise of epidemiological events in university hospital of Rennes, contact precautions remain applied in case of ESBL-Kp while standard precautions are applied in case of ESBL-Ec colonization or infection.

Typing methods are useful to monitor diffusion of multi-resistant bacteria and changing epidemiological trends, particularly in hospital settings. Pulsed-field gel electrophoresis (PFGE), the gold standard for decades [16] has been progressively replaced by recent molecular typing methods such as multilocus sequence typing (MLST) and whole genome sequencing (WGS), which are now becoming the reference methods. Beside these sophisticated methods, simpler approaches have been described such as the quantitative antibiogram (QA) method [17]. This technique allows to discriminate epidemiological clusters based on the determination of the Euclidian distance using inhibition zone diameters for several antibiotics. This technique was successfully used to discriminate *Staphylococcus aureus* isolates [18–20] and ESBL-producing *Proteus mirabilis* [21], emphasizing a general concordance between QA typing and ribotyping, PFGE or RAPD typing respectively. In this work, we aimed to evaluate QA typing as a rapid and economic method to assess nosocomial transmission of eESBL isolates.

As a proof of concept, we first evaluated a combined-QA method as a tool for epidemiological surveillance by comparing results on a panel of ESBL-Ec and ESBL-Kp to the combined-PFGE method. Secondarily, we conducted a four-year retrospective analysis in University hospital of Rennes using the combined-QA method to detect nosocomial transmission of these two species. In this report, we provide evidence that (i) combined-QA is an easily applicable alternative method to time- and cost-consuming molecular methods for surveillance of nosocomial epidemiology of ESBL, (ii) standard precautions are sufficient to prevent cross-transmission of ESBL-Ec whereas contact precautions must be preserved and reinforced to prevent cross-transmission of ESBL-Kp.

Material and methods

1. Proof-of-concept study: comparison of the QA method to PFGE gold standard method

1.1. Context

The University Hospital of Rennes is a 1,800-bed teaching hospital with five facilities. All bacterial isolates were recovered from hospitalized patients. A total of 48 ESBL-Ec and 66 ESBL-Kp isolates from either colonized or infected patients were included to constitute two data sets. EBSL-Ec isolates were collected between 2011 and 2012 during a prospective study of inpatients from a unique intensive care unit whereas ESBL-Kp were recovered between 2012 and 2014 whether during cluster investigations, or routine testing in different wards.

1.2. Bacterial isolates

All bacterial isolates were identified by MALDI-TOF MS (Microflex; Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibility testing was performed using agar disc diffusion method and interpreted following the CA-SFM/EUCAST guidelines. For cefotaxime and/or ceftazidime resistant *E. coli* and *K. pneumoniae* isolates, we looked routinely for ESBL production by a double-disc synergy test between clavulanic acid and at least one of these two cephalosporins. When no synergic effect was evidenced for an isolate, it was further tested by the clavulanic acid-cephalosporin combined-discs method. Isolates were stored at -80°C. Molecular and phenotypic characterization PFGE was performed after DNA restriction by *Xba*I enzyme, as previously described [20,22]. DNA restriction patterns were compared by using the Dice similarity index. Isolates were considered related if the Dice index was ≥ 80%.

For QA-typing, six antibiotics were selected: cefotaxime, ceftazidime, gentamicin, nalidixic acid, ciprofloxacin and trimethoprim. For each one, inhibition zone diameters were collected and used to calculate Euclidian distance as follow [18,19]:

$E_{jk} = \sqrt{\sum_{i=1}^{n}\left(x_{ij} - x_{ik}\right)^2}$ where *E* is Euclidian distance expressed in mm, *j* and *k* represent two strains, *i* is rank of test, *x* is inhibition zone diameter, and *n* is the number of antibiotics.

The maximal uncertainty of measure was estimated at 2 mm for each diameter, so the maximal Euclidian Distance (ED) between two antibiograms performed for a same strain was theoretically equal to $\sqrt{(6 \times 2)}$ = 3.5mm, rounded up to 4mm as a cut-off. Consequently, two strains were considered as related when the ED between them was ≤ 4 mm.

1.3. Bioanalysis and statistical analysis

PFGE patterns and ED were analysed separately using BioNumerics© software (Biomérieux, Marcy-l'Etoile, France). For both methods, cluster analysis was performed using the Unweighted Pair Group Method of Analysis (UPGMA). Respective distributions of ESBL-Ec and ESBL-Kp isolates were compared using the Chi-square test in respect to the clonality. Results were considered statistically significant if $p < 0.05$.

Then, we combined QA or PFGE results with epidemiological criteria to get combined-typing data (combined-PFGE or combined-QA). Epidemiological criteria were based on spatio-temporal data that allow to estimate the probability of cross-transmission between 2 patients *i.e.* hospitalization in a same ward during a same period.

2. Four-year retrospective study

2.1. Design study

Since January 2015, infection control policy in University hospital of Rennes recommends to implement different precautions according to species of *Enterobacteriaceae*. Standard precautions are applied for patients known as colonized or infected by ESBL-Ec while contact precautions are implemented for patients carrying EBSL-Kp.

For the study period (from January 2015 to October 2018), all ESBL-Ec and ESBL-Kp antibiogram results were collected retrospectively from the laboratory information system. Data consisted of demographic information (sex, age), ward, hospital admission day, type of sample, and inhibition zone diameters for the 6 antibiotics previously described. Hospitalized patients with one or more ESBL-Ec and/or ESBL-Kp positive samples were included. Outpatients were excluded. For patients with multiple samples positive for the same ESBL species, we considered only the first isolate for typing.

If necessary, medical records of patients were consulted to collect data on medical history.

2.2. Decision algorithm

We classified the patients according to the acquisition route of the eESBL strains following 3 definitions: an eESBL-producing isolate was considered to be (i) imported if recovered before admission or during the first 48h of hospital stay, (ii) hospital-acquired if a sample turned positive after a negative one within the first 48h, (iii) undefined if recovered after 48h of hospitalization but without sample performed during the first two days. All eESBL-producing isolates were typed according to the QA method with respect to the criteria above. Then, direct cross-transmission was assumed as probable between two patients if they were both hospitalized in the same ward, over the same time, and if at least one of them had a hospital-acquired or undefined eESBL-producing isolate. Indirect cross transmission *i.e.* environmental transmission was considered as probable if patient were hospitalized in the same ward but at a not-overlapping period (gap < 20 days).

2.3. Statistical analysis

Chi-Square and Fisher's exact tests were used to compare cross-transmission differences between ESBL-Ec and ESBL-Kp. Results were considered statistically significant if $p < 0.05$.

Results

Proof-of-concept study

With respect to the PFGE method, 6/48 ESBL-Ec isolates (12.5%) were clustered in three groups of two isolates. For ESBL-Kp isolates, a total of 37/66 (56%) were clustered into nine groups of respectively 17, 4 (2 groups), and 2 (6 groups) isolates (Figure 1A). Clonal distribution of ESBL-Kp isolates was higher than ESBL-Ec (p< 0.0001) highlighting clonal dissemination through the different wards of our hospital.

The QA method showed that 26 ESBL-Ec isolates were clustered, a higher number than with the PFGE method. Noteworthy, none of the combined methods provided evidence that ESBL-Ec clustered isolates were inter-related in space and time, thus ruling out CTE for this species.

The QA method found that 44 ESBL-Kp isolates were clustered into nine groups of respectively 19, 8, 7, 5 (2 groups), 4 and 2 (3 groups) isolates (Figure 1B). Among them, 36 isolates were also found as related by PFGE. Combined-PFGE identified 7 CTE involving 16 patients. Combined-QA enlightened 6 CTE involving 13 patients – of those 12 were also identified by combined-PFGE.

The performance of combined-QA method was compared to combined-PFGE defined as gold standard. Combined-QA typing displayed 75% sensitivity and 98% specificity. Mismatches were due to variability affecting susceptibility to fluoroquinolones (Figure 1B).

Retrospective study

A total of 1,002 patients were included over the four-year period, 722 with ESBL-Ec isolates and 280 with ESBL-Kp isolates (Figure 2). More than ninety-eight percent of eESBL isolates were identified routinely by a double-disc synergy test, and the remaining ones by the combined discs method. For both species, imported isolates were not predominant, corresponding to respectively 46% of patients for ESBL-Ec and 40% for ESBL-Kp. After applying combined-QA method, 55 ESBL-Ec- and 92 ESBL-Kp-positive patients were selected for possible implication in a CTE. Their acquisition route study highlighted higher risk of nosocomial acquisitions for ESBL-Kp-positive patients (40%) than for ESBL-Ec-positive ones (24%). Moreover, the acquisition route was significantly different by comparing these two entities (p= 0.03) (Table I). After fine-tuned analysis, 9 direct CTE were identified implying 20 ESBL-Ec-positive patients (2.7%) and 33 direct CTE involving 36 ESBL-Kp-positive patients (12.8%), showing significantly higher rates for ESBL-Kp (p<0.0001) (Table I). Average number of patients included in one CTE was similar for either ESBL-Ec or ESBL-Kp. Only one outbreak involving 6 ESBL-Kp-positive patients was identified in an intensive care unit.

ESBL distribution was significatively different between wards, as evidenced by a higher proportion of ESBL-Kp CTE in ICUs than ESBL-Ec, and conversely more frequent ESBL-Ec CTE in medicine or surgery wards than in the ICUs (p<0.0001) (Table I). We also estimated indirect CTE assuming that for two patients hospitalized in the same ward over distinct but close periods, cross-transmission could be linked to an environmental pool, for example sink drain, as previously reported by others [23,24]. In that way, we found 6 ESBL-Ec-positive patients (0.8%) and 25 ESBL-Kp-positive patients (8.9%) possibly concerned by indirect CTE. There again, ESBL-Kp isolates displayed significant higher CTE rates (p< 0.0001) (Table I).

Table I: Significant differences between and ESBL- Ec and Kp-positive groups of patients.

	ESBL-Ec	ESBL-Kp	*p* value
Acquisition route in patients with possible CTE*			
Imported	20/55 (36%)	35/92 (38%)	*p*= 0.03
Hospital-acquired	13/55 (24%)	37/92 (40%)	
Undefined	22/55 (40%)	20/92 (22%)	
Patients involved in CTE			
Direct cross-transmission	20 / 722 (2.7%)	36 / 280 (12.8%)	*p*< 0.0001
Indirect cross-transmission	6 / 722 (0.8%)	25 / 280 (8.9%)	*p*< 0.0001
Type of units			
Intensive care units	4	28	*p*< 0.0001
Medicine and surgery units**	16	8	

* Acquisition route from suspected patients in CTE *i.e.* 55 ESBL-Ec and 92 ESBL-Kp. Possible CTE was estimated when clustering by the QA method was identical and patients were hospitalized in the same ward within a maximum of 20 days.

** Including after-care rehabilitation and long-term care units

Discussion

ESBL-Ec strains are endemic in community settings but cross-transmission in hospitals are scarce [5]. Therefore, based on previous studies, we assumed that standard precautions were sufficient to avoid CTE, and consequently modified the infection control policy in University hospital of Rennes since January 2015. From this point, only standard precautions have been applied to ESBL-Ec patients while contact precautions were maintained for all other eESBL isolates, mainly *E. cloacae* and *K. pneumoniae*, which are considered to be highly epidemic in hospitals [1]. To assess the efficacy of this infection control strategy, we aimed to develop a rapid method to detect CTE of eESBL isolates. We hypothesized that QA, a method described thirty-five years ago [17], could be useful as it would meet the convenience criteria outlined for a typing method [25]. In a preliminary study, we showed that CTE implicating ESBL-Ec-positive patients were limited by using the QA method combined with epidemiological criteria. Nevertheles, a strong limitation of this paper was the lack of comparison to a reference typing-method like PFGE [26]. In the present work, we upgraded robustness by comparing these two typing methods on two sets of ESBL-Ec and ESBL-Kp- producing isolates. In a second time, we retrospectively tested it to assess the rate of nosocomial acquisition of such strains in hospitalized patients for a 4-year period.

The QA-typing method tends to overestimate isolates clustering compared to the PFGE, which means that the estimation of the population diversity based on phenotypic characterization with six antibiotics is less important than the genotypic diversity appreciated by restriction and electrophoresis of bacterial DNAs. Thus, the QA method alone does not appear to be suitable to assess reliable discrimination in ESBL-producing isolates and detect CTE. This point has already been reported previously for other methods like high-throughput MLST or Raman spectroscopy [27]. To improve methodology, we decided to combine both methods with epidemiological criteria. In that way, results were reliable as no ESBL-Ec CTE were detected by both methods, and at least 75% of ESBL-Kp CTE identified by combined-PFGE were also retrieved by the combined-QA method. Discrepancies resulted from acquisition of resistance to fluoroquinolones, likely due to mutations on the *gyrA* gene, which strongly modified the ED between two related isolates. Interestingly, when we took into account the susceptibility to fluoroquinolones of the two isolates involved in CTE#7 detected by combined-PFGE, this event would not exist since the first one was resistant and the second one susceptible. If that was true, there would be 6, not 7, CTE among our panel, and combined-QA allowed us to identify 12 of 14 isolates (86%) detected by combined-PFGE (Figure 1B). Our validation panels allowed to confirm the usefulness of the combined-QA method to monitor intra-hospital eESBL isolates diffusion. Moreover, generating ED from antibiogram data stored in microbiology lab information system is simple, rapid and inexpensive compared to more fastidious techniques like PFGE or Raman spectroscopy. In addition, QA-typing could be applied to automated methods determining MIC values instead of inhibition zone diameters (personal communication).

Over the four-year period, ESBL-Ec CTE involved 2.7% of ESBL-Ec-positive patients whereas the rate was 12.6% for ESBL-Kp-positive patients. These results are of interest since contact precautions were implemented for ESBL-Kp but not for ESBL-Ec. A low CTE rate with ESBL-Ec-positive patients was previously reported [11,13], and our results confirm the adequacy of standard precautions to manage ESBL-Ec cross-transmission. In contrast, the high capacity of ESBL-Kp to disseminate justifies reinforced precautions. Our results are in concordance with those from a multicentre European study that estimated a transmission capacity of 3.7 times higher for non-ESBL-Ec (including ESBL-Kp) than ESBL-Ec [28]. Our study disclosed that ESBL-Kp CTE occur mainly in ICUs, highlighting an increased risk for ESBL-Kp nosocomial transmission as demonstrated through the MOSAR-ICU study [29]. In our hospital, patients in ICUs are hospitalized in single rooms that constitute one of the prevention measures of the

contact precaution bundle, even though it was reported in a large study that there was no difference in ESBL-CTE between contact precautions in single or multiple beds [30]. Other factors probably affect the risk of CTE such as a longer stay length in ICU, antibiotic selective pressure, and high care load [31]. Another possibility is that *K. pneumoniae* survive longer than *E. coli* in the hospital, thus emphasizing the value of environmental cleaning [32,33]. This could explain the higher indirect CTE rates for ESBL-Kp than ESBL-Ec that we observed. A French national survey in ICUs assessed the colonization rate of sink drains showing that 37% were contamined by eESBL of which 24% were ESBL-Kp and 3% of ESBL-Ec [34]. Altogether, this information demonstrates the importance of environmental hygiene through periodic decontamination of water points and the disposal of contaminated liquids.

To sum up, our results confirm that standard precautions are sufficient to prevent ESBL-Ec CTE. As proposed by the Belgian Infection Control Society and the European Society of Clinical Microbiology and Infectious Diseases [15,35], European recommendations for management of EBSL-Ec-positive patients without contact precautions could be harmonized. By contrast, precautions must be reinforced to prevent ESBL-Kp CTE by advising appropriate hygiene bundles considering the characteristics of *K. pneumoniae*.

In our hands, the combined-QA method showed similar results for detection of eESBL-CTE than other epidemiological tools such as genotypic and mathematical model analysis [6,11,13,28,29,36]. Indeed, discriminatory power and genetic relationship inference are not essential to epidemiologic linkage [37], supporting the usefulness of the combined-QA method in this context. This typing method, although less sophisticated than PFGE or WGS, can be prospectively applied in all routine microbiology laboratories and/or infection control teams. Recently, new typing methods with fast output have emerged, such as the Fourier-transform infrared (FTIR) spectroscopy, allowing to correctly and quickly cluster *K. pneumoniae* isolates [38,39]. It is obvious that the nosocomial dissemination of ESBL-Kp is not yet controlled despite reinforcement of hygiene measures in hospitals. A prospective and daily follow-up including rapid typing methods (combined-QA and FTIR) would probably manage more efficiently ESBL-Kp hospital diffusion.

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Figure captions

Figure 1. **A**: Dendrogram after calculation of Dice index and clustering by UPGMA method applied to results of PFGE analysis of *K. pneumoniae* DNAs after restriction by *Xba*I enzyme. A 80% similarity cut-off is retained to define clusters, and each one is designated by a letter. Cross-transmission events (CTE) are numbered from 1 to 7. **B**: Dendrogram after calculation of Euclidian Distances between *K. pneumoniae* isolates with a 4 mm cut-off. Letters designating isolates correspond to clusters from PFGE dendrogram. CTEs are numbered from 1 to 6. Discordant isolates are identified by ◄— or ●-● and numbers between brackets corresponding to CTE #1 and #7 from PFGE dendrogram. Range of diameter values around ciprofloxacin discs are indicated for the three clusters containing cluster "A" isolates.

Figure 2. Workflow used to estimate direct and indirect cross-transmission events. Exclusion criteria were detailed in the materials and methods section.