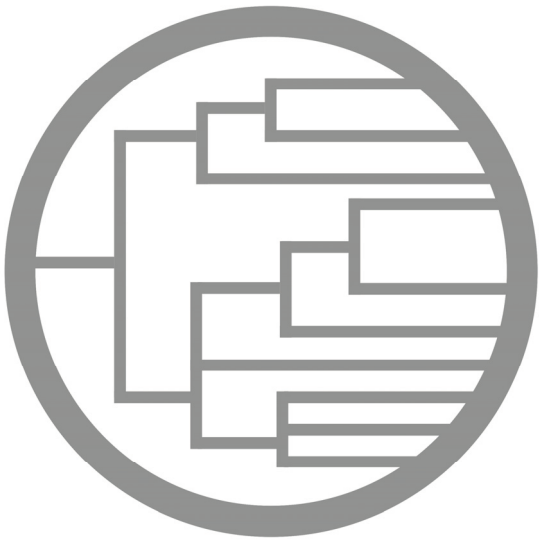
Chapter 3

Typing methods for ESBL-producing *Enterobacteriaceae*



Chapter 3.1

Evaluation of the DiversiLab typing method in a multicenter study assessing horizontal spread of highly resistant Gram-negative rods

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Abstract

The worldwide prevalence of highly resistant Gram-negative rods is increasing rapidly. Reliable typing methods are needed to detect and control outbreaks and to monitor the effectiveness of infection control programs in endemic situations. In this study, we investigated the performance of the ©DiversiLab typing method in comparison with the amplified fragment length polymorphism (AFLP) typing method.

Six hundred fifty-three highly resistant Gram-negative rods, which were obtained during a 6-month prospective survey in 18 Dutch hospitals, were typed by AFLP and DiversiLab. Subsequently, the sensitivity and specificity of DiversiLab were calculated, using AFLP as the reference method. In addition, results were compared by means of epidemiological linkage, and Cohen's kappa for agreement was calculated.

DiversiLab considered significantly more isolates (275) to belong to a cluster than AFLP (198; $P<0.001$). In direct comparison, the sensitivity was 83.8%, and the specificity was 78.6%. When epidemiological linkage was included in the analysis, DiversiLab considered eight isolates as secondary cases, which were considered unique in AFLP. Only two secondary cases, according to AFLP, were missed by DiversiLab. This results in a Kappa for agreement of 0.985.

In daily practice a typing method has to be used in combination with epidemiological information. When this was done, DiversiLab was shown to be a reliable method for the typing of highly resistant Gram-negative rods. This, in combination with the ease of use and the speed, makes DiversiLab an appropriate method for screening in routine clinical practice. When a cluster is suspected and the consequences of these findings are substantial, a confirmatory analysis should be performed.

Introduction

Worldwide, the prevalence of highly resistant Gram-negative rods is increasing. In the annual report of 2008, the European Antimicrobial Resistance Surveillance System concluded that resistance of *Escherichia coli* to four antimicrobial classes, including broad-spectrum cephalosporins, is already among the four most common resistance patterns encountered in Europe (http://www.rivm.nl/earss/result/Monitoring_reports/Annual_reports.jsp). In *Klebsiella spp.* even 14% of invasive isolates are resistant to three classes of antimicrobial drugs, including broad-spectrum cephalosporins.
This increase can be caused by patients carrying resistant pathogens on admission, by horizontal transfer between patients, by selection of resistance caused by antimicrobial use, by transfer of resistance genes between microorganisms or by combination of two or more of these mechanisms.[1-3]
In the Netherlands, national guidelines have been defined that describe control measures to avoid the spread of resistant bacteria between patients.[4] In case of a suspected outbreak, typing methods are essential to assess the presence and scale of the outbreak. The most commonly used typing methods for aerobic Gram-negative rods are amplified fragment length polymorphism (AFLP) and pulsed-field gel electrophoresis (PFGE). These methods are very reliable but also relatively expensive and time-consuming, and they require a high level of technical skill. Therefore, they cannot be used in most clinical laboratories. We tested a commercially available typing method, ©DiversiLab, on a well-defined collection of highly resistant Gram-negative rods from a recent study in Dutch hospitals.[5] The objective of this study was to determine the reliability of DiversiLab in comparison with the AFLP typing method for the typing of highly resistant Gram-negative rods in a hospital setting.

Methods

Strain collection

The highly resistant Gram-negative rods isolates were part of a collection from a multicentre study performed in five university hospitals, eight teaching hospitals, and five general hospitals during a 6-month study period in 2007. All isolated strains, whether from clinical cultures or from screening cultures of clinical patients, were included.[5] In total 653 highly resistant Gram-negative rods were available and were included in this analysis. All isolates were sent to a central laboratory where they were stored, using Microbank vials, at -70˚C until further testing. Identification (GN) and antimicrobial susceptibility testing (AST-NO48) were performed by using the VITEK2 system (bioMérieux, Marcy l'Etiole, France). If the identification was considered 'unacceptable' (probability below 85%) or if the result differed from the identification from the participating hospital, API 20E/20NE (bioMérieux, Marcy l'Etiole, France) was

performed as a confirmation test, according to the manufacturer's recommendations. The indol spot test was used as complementary test if recommended by the Vitek2 expert system.
If more than one highly resistant Gram-negative rod was recovered from one patient, the second strain was only included if this was another species or the same species with a different susceptibility pattern. The criteria for highly resistant Gram-negative rods used are described in the Dutch national guideline for the control of highly resistant microorganisms.[4] Table 3.1.1 shows a summary of these criteria.

Table 3.1.1. Definition of highly resistant Gram-negative rods.

Species	Type of resistence to						
	ESBL	CTZ	QUI	AMG	CAR	PIP	COT
Enterobacteriaceae							
Escherichia coli	A		B	B	A		
Klebsiella spp.	A		B	B	A		
Other *Enterobacteriaceae*	A		B	B	A		B
Nonfermenters							
Acinetobacter spp.		B	B	B	A		
Stenotrophomonas Maltophilia							A
Other non-fermenters		C	C	C	C	C	C

ESBL=extended-spectrum β-lactamases, CTZ=ceftazidime, CAR=carbapenems, QUI=fluoroquinolones, AMG=aminoglycosides, PIP=piperacillin, COT=co-trimoxazole. A: resistance against an antibacterial agent of the carbapenem group, and/or the presence of ESBL-production, and/or resistance against co-trimoxazol is sufficient to define the microorganism as highly resistant, B: resistance against antibacterial agents from at least two of the indicated groups or specified antibacterial agent is required to define the microorganism as highly resistant, C: resistance against antibacterial agents from at least three of the indicated groups or specified antibacterial agents is required to define the microorganism as highly resistant.

AFLP typing method

For molecular typing, chromosomal DNA was isolated using the easyMag system (bioMérieux, Marcy l'Etoile, France). Amplified fragment length polymorphism (AFLP) was performed as described by Savelkoul et al..[6] Subsequent to restriction, ligation, and amplification, the DNA fragments were separated on an ABI Prism 3130XL genetic analyser (Applied Biosystems). Genetic relatedness was determined on basis of both visual and computerized interpretation of AFLP patterns.

DiversiLab typing method

The DiversiLab Microbial Typing System (bioMérieux, Marcy l'Etoile, France) is based on repetitive sequence-based PCR (rep-PCR), which amplifies the regions between the noncoding repetitive sequences in bacterial genomes.
The DNA was isolated by the DiversiLab Mo Bio UltraClean microbial DNA isolation kit, as recommended by the manufacturer. The DNA concentration was measured and set between 25 ng/µl and 30 ng/µl. Subsequently, the DNA was amplified using the

DiversiLab fingerprinting kit for *Escherichia spp*., *Klebsiella spp*. and other species, according to the manufacturer's instructions. PCR was performed using the following parameters: initial denaturation (94°C) for 2 minutes and then 35 cycles of 30 sec denaturation (94°C), 30 sec of annealing (50-55°C depending on the species), and 90 sec of extension (70°C), followed by 3 minutes of final extension (70°C), and ending at 4°C. The amplification products were separated with the Agilent B2100 bioanalyzer. Five microliters of DNA standard markers (used for normalization of sample runs) and 1 µl of the DNA product were used. All data were entered in the DiversiLab software system.

Definition of epidemiological linkage

Highly-resistant Gram-negative rods recovered from a specimen obtained from a patient more than 48 hours after admission were classified as hospital-associated; highly-resistant Gram-negative rods recovered less than 48 hours after admission were potential index cases. Nosocomial transmission was considered present if genotypically related strains were detected in two or more patients who had been in the same hospital ward within a maximum time window of four weeks before cultures turned positive with highly-resistant Gram-negative rods. The isolates from patients with hospital-associated highly-resistant Gram-negative rods were considered secondary cases.

Statistical analysis

All data generated by AFLP were analysed with Pearson's correlation coefficient and clustered by unweighted-pair group matrix analyses using BioNumerics software, v5.10 (Applied-Maths, Sint-Martens-Latum, Belgium). Clusters were formed according to the biological similarity of the strains.[6]

Results of the DiversiLab typing method were analysed with the DiversiLab software (version 3.4), which uses the Pearson correlation coefficient and the unweighted-pair group method with arithmetic averages to determine distance matrices and to create dendrograms. Reports were automatically generated. Isolates with a similarity of at least 95% were considered as a cluster.

We compared the results of both typing methods in two distinctive ways. First, we compared the typing methods directly, with AFLP as the gold standard. If DiversiLab was in agreement with the gold standard and considered an isolate to be part of a cluster that was also found using AFLP, the result was scored as true positive. If DiversiLab and AFLP both considered an isolate to be unique, the result was scored as true negative. If the gold standard considered an isolate to be unique and DiversiLab considered it as part of a cluster, the result was scored as false positive. On the contrary, if the gold standard considered an isolate to be part of a cluster and DiversiLab did not or clustered it with other isolates not belonging to the AFLP-cluster,

the result was considered false negative. Sensitivity was calculated by dividing the number of true positives by the sum of true positives and false negatives. Specificity was calculated by dividing the number of true negatives by the sum of true negatives and false positives. This calculation was done for each participating hospital separately.
Secondly, we included epidemiological linkage in the analysis and compared DiversiLab and AFLP with each other. This was done by calculation of the transmission index. The transmission index was defined as the number of patients with a nosocomial transmission (secondary cases) divided by the number of patients with a highly-resistant Gram-negative rod not acquired by nosocomial transmission (potential index cases).[5] This was calculated for both AFLP and DiversiLab, and for each hospital separately. Also, a Cohen's kappa for agreement was calculated by dividing the number of strains where AFLP and DiversiLab were in agreement by the total number of strains. This was done for each hospital separately.

Results

Strain collection

Of a total of 892 highly-resistant Gram-negative rods included in the initial study,[5] 653 isolates (559 *Enterobacteriaceae* and 94 nonfermentative Gram-negative rods) were available for further typing. As shown in Table 3.1.2, *E. coli* was the most prevalent species included in this study (296 isolates; 53.0%). Of the nonfermentative Gram-negative rods, *Pseudomonas spp.* were the most prevalent (38 isolates; 40.4%).

Table 3.1.2 Distribution of species.

Species		AFLP		DiversiLab	
	Number of isolates	Number in cluster (%)	Number of secondary cases (%)	Number in cluster (%)	Number of secondary cases (%)
Enterobacteriaceae [a]					
Citrobacter spp.	28	0 (0.0)	0 (0.0)	6 (21.4)	0 (0.0)
Enterobacter spp.	68	24 (37.5)	8 (33.3)	41 (59.4)	11 (26.8)
Escherichia coli	296	119 (40.2)	11 (9.2)	158 (53.4)	13 (8.2)
Klebsiella spp.	110	34 (30.9)	13 (38.2)	40 (36.4)	13 (32.5)
Proteus spp.	37	11 (29.7)	2 (18.2)	14 (37.8)	2 (14.3)
Nonfermentative Gram-negative rods [b]					
Acinetobacter spp.	15	2 (13.3)	0 (0.0)	0 (0.0)	0 (0.0)
Chryseobacterium spp.	8	2 (25.0)	1 (50.0)	4 (50.0)	1 (25.0)
Pseudomonas spp.	38	6 (15.8)	0 (0.0)	9 (23.7)	1 (11.1)
Stenotrophomonas spp.	26	0 (0.0)	0 (0.0)	3 (11.5)	1 (33.3)

[a] Of 20 other *Enterobacteriaceae*, no clusters were found. This group consists of nine *Morganella Morganii* isolates, two *Providencia* isolates, two *Salmonella* isolates, five *Serratia* isolates, and two *Shigella* isolates. [b] Of 7 other nonfermentative Gram-negative rods, no clusters were found. This group consists of five *Achromobacter* isolates, one *Ochrobactrum anthropic* isolate, and one *Sphingomonas paucimobilis* isolate.

Direct comparison of AFLP and DiversiLab

Using AFLP, 188 *Enterobacteriaceae* and 10 nonfermentative Gram-negative rods were considered to belong to a cluster. In total 71 clusters were found, with a median cluster size of 2 isolates. The largest cluster contained 11 isolates. Using DiversiLab, 259 *Enterobacteriaceae* and 16 nonfermentative Gram-negative rods belonged to a cluster. These isolates are distributed over 91 clusters. The median cluster size was 2, and the largest cluster contained 13 isolates. The number of isolates belonging to a cluster was significantly higher for *Enterobacteriaceae* in DiversiLab compared with AFLP (P<0.001). Using AFLP, the isolates of 5 hospitals were all unique. The other hospitals revealed 1 to 11 clusters. Using DiversiLab, the same 5 hospitals revealed no clusters. The other hospitals revealed between one and 16 clusters. In Table 3.1.3, the number of clusters and the number of isolates in the clusters of each hospital are shown. Also, the sensitivity and specificity, calculated by direct comparison of AFLP and DiversiLab, are calculated. The overall sensitivity was 83.8% and ranged from 57.1% to 100% in the various hospitals. The overall specificity was 78.6% with a range from 66.7% to 100%.

Table 3.1.3 Direct comparison of AFLP and DiversiLab.

Hospital	AFLP		DiversiLab		Sensitivity	Specificity
	Number of clusters	Number of isolates in clusters	Number of clusters	Number of isolates in clusters		
	N	N (% of total)	N	N (% of total)	%	%
1	8	27 (31.0)	13	44 (50.6)	100.0	71.7
2	2	6 (16.2)	4	11 (29.7)	100.0	86.7
3	7	19 (47.5)	8	21 (52.5)	94.7	85.7
4	0	0 (0.0)	0	0 (0.0)	not applicable	100.0
5	6	17 (39.5)	7	26 (60.5)	94.7	66.7
6	2	4 (33.3)	2	4 (33.3)	100.0	100.0
7	8	20 (31.3)	8	25 (39.1)	75.0	79.6
8	0	0 (0.0)	0	0 (0.0)	not applicable	100.0
9	0	0 (0.0)	0	0 (0.0)	not applicable	100.0
10	0	0 (0.0)	0	0 (0.0)	not applicable	100.0
11	0	0 (0.0)	0	0 (0.0)	not applicable	100.0
12	7	18 (43.9)	7	20 (48.8)	83.0	82.6
13	2	7 (23.3)	2	7 (23.3)	57.1	87.0
14	1	2 (66.7)	1	2 (66.7)	100.0	100.0
15	2	4 (30.8)	2	4 (30.8)	100.0	100.0
16	6	12 (23.5)	10	21 (41.2)	91.7	76.9
17	11	38 (31.4)	16	57 (48.3)	76.9	69.6
18	9	26 (28.6)	11	33 (36.3)	61.5	76.9
Overall	71	201 (30.7)	91	276 (42.1)	83.8	78.6

Comparison of transmission indices

In Table 3.1.4 the results with the addition of epidemiological linkage are shown. The number of primary and secondary cases per hospital are given, as well as the transmission index and the kappa value within each hospital.
The transmission index in 14 out of 18 hospitals was identical for AFLP and DiversiLab. AFLP and DiversiLab were not in agreement for the epidemiological linkage of 10 isolates coming from 4 hospitals; 8 isolates were considered primary cases by AFLP and secondary cases by DiversiLab, and 2 isolates were considered primary cases in DiversiLab and secondary cases by AFLP. This results in a kappa of 0.985, (range 0.884-1.000).
Organisms within a cluster did not always exhibit the same resistance phenotype, even if transmission was suspected. However, for all but 1 of the 10 isolates where AFLP and DiversiLab were not in agreement for the epidemiological linkage, the resistance pattern of the primary and secondary case were identical.

Table 3.1.4 Comparison of the number of primary and secondary cases and transmission index values.

Hospital	AFLP			DiversiLab			Cohen's kappa for agreement
	Number of primary cases N	Number of secondary cases N	Transmission Index value	Number of primary cases N	Number of secondary cases N	Transmission Index value	
1	84	3	0.036	84	3	0.036	1.000
2	36	1	0.028	36	1	0.028	1.000
3	35	5	0.143	35	5	0.143	1.000
4	4	0	0.000	4	0	0.000	1.000
5	38	5	0.132	33	10	0.303	0.884
6	12	0	0.000	12	0	0.000	1.000
7	62	2	0.032	61	3	0.049	0.984
8	5	0	0.000	5	0	0.000	1.000
9	4	0	0.000	4	0	0.000	1.000
10	7	0	0.000	7	0	0.000	1.000
11	3	0	0.000	3	0	0.000	1.000
12	38	3	0.079	38	3	0.079	1.000
13	27	3	0.111	28	2	0.071	0.900
14	3	0	0.000	3	0	0.000	1.000
15	13	0	0.000	13	0	0.000	1.000
16	45	6	0.133	45	6	0.133	1.000
17	111	7	0.063	111	7	0.063	1.000
18	90	1	0.011	89	2	0.022	0.989
Overall	617	36	0.058	611	42	0.069	0.985

Discussion

In this study, we evaluated the performance of the DiversiLab typing method. This was done by using a well-described collection of highly-resistant Gram-negative rods. Our evaluation showed that DiversiLab considered significantly more isolates to be part of a cluster compared to AFLP (P<0.001). In a direct comparison, the sensitivity was 83.8% and the specificity was 78.6%. However, when epidemiological linkage was included in the analysis, the performance of DiversiLab was comparable to AFLP. In other words, the interrelationships between the isolates in the clusters found with DiversiLab, were comparable to the interrelationships between the isolates in the clusters found with AFLP. In 14 hospitals the results were in full agreement. As a result, the overall kappa was very high (0.985), which indicated a good concordance between the two tests.

A number of other studies evaluating DiversiLab, have been published.[7-9] Most evaluate DiversiLab for only one species, and a number of reference methods have been used. Fluit et al. evaluated DiversiLab with a number of well typed species and showed that DiversiLab was a useful tool to help identify hospital outbreaks of *Acinetobacter spp*., *Stenotrophhomonas maltophila*, *Enterobacter cloacae*, *Klebsiella spp*. and *E. coli*, but was considered inadequate for *Pseudomonas aeruginosa.*[8] Our results are in line with these findings, except for the conclusions regarding *P. aeruginosa*. However, another recent study showed a good performance of DiversiLab with *P. aeruginosa* in comparison with PFGE.[7] Our results were also in line with a study by Grisold and colleagues,[9] who showed concordant results for identification of outbreak and non-outbreak-related *Acinetobacter baumannii* and extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains for PFGE and DiversiLab.

Our study is, according to us, unique because we included all resistant Gram-negative species found in several hospitals in a specific period. In addition, our analysis includes epidemiological linkage, which is mandatory to evaluate the effect of using the typing method in daily practice. The main limitation of our study was that we only included highly resistant Gram-negative rods, whereas in daily practice the method will also be used for outbreaks of highly resistant Gram-positive bacteria.

In conclusion, our study shows that the sensitivity and specificity of DiversiLab is lower than those of AFLP. However, when epidemiological linkage is included in the analysis, the performance is very good (kappa 0.985), leading to similar conclusions about horizontal spread of higly-resistant Gram-negative rods. The good performance, in combination with the ease of use and the speed, makes DiversiLab an appropriate screening method in routine clinical practice. However, when the screening indicates a severe problem that would justify extensive control measures, a more discriminatory method (e.g. AFLP) should be used to determine the true extend of the outbreak.

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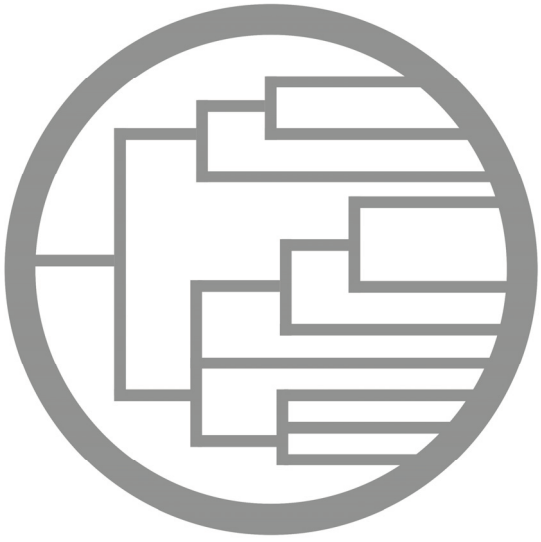
Chapter 3.2

Comparison of SpectraCell RA typing and multilocus sequence typing for extended-spectrum β-lactamase-producing *Escherichia coli*



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Abstract

Multilocus sequence typing (MLST) is one of the most reliable methods for typing of *Escherichia coli,* including extended-spectrum β-lactamase (ESBL)-producing *E. coli*. We investigated the performance of a new typing method, ©SpectraCell RA (River diagnostics, Madison, Wisconsin, USA), in comparison with MLST on a well-defined collection of ESBL-producing *E. coli* obtained from chicken meat and humans.

Ninety-two ESBL-producing *E. coli* isolates obtained from meat and 59 ESBL-producing *E. coli* isolates obtained from human rectal swabs and clinical blood cultures were typed using MLST and SpectraCell RA. The sensitivity and specificity of SpectraCell RA was calculated, using MLST as reference method. Subsequently, the results of SpectraCell RA were used to determine the relatedness of ESBL-producing *E. coli* isolates from chicken and humans.

Using MLST as the gold standard, the performance of SpectraCell RA was evaluated for 3 different cutoff values; 0.99975, 0.99955, and 0.99935. Depending on the cutoff value, the sensitivity was mediocre to unacceptably low, with values of 9.4%, 43.9% and 66.7% respectively. When sensitivity increased, the specificity decreased rapidly, from 95.6% to 69.8% and 34.4%, respectively. Also, the number of clusters containing both human and meat samples varied from 0 (0.0%) to 14 (38.9%).

Our study shows that SpectraCell RA is not a suitable typing method for ESBL-producing *E. coli* when evaluating relationships at the population level.

Introduction

The prevalence of multi-drug resistant microorganisms[1,2] is increasing. Reliable typing methods are needed to discover the sources and transmission routes for emerging pathogens, especially for tracing transmission in the hospital setting. Many typing methods are currently available. They are based on different principles, with the main division being between phenotypic and genotypic testing. It is possible for there to be little correlation between the results of genotypic and phenotypic typing or between the results of different phenotypic typing methods themselves.[3] Furthermore, the resolution is an important differentiating factor between the typing methods. The choice for a specific typing method depends on the research question that has to be answered. For example, when a possible outbreak in the hospital is investigated, a fast typing method with a high resolution is needed. On the other hand, when the question regards the linkage of strains in the population, a typing method with a lower resolution based on more conservative targets is more appropriate.

Multilocus sequence typing (MLST) is a well-known typing method for *Escherichia coli*. It is a sequence-based typing system, that targets housekeeping genes and is suitable for typing with a perspective on population epidemiology.[4] In a recent study, MLST was used to type extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolated from chicken meat and humans.[5] This study identified chicken meat as a plausible source of ESBL-producing *E. coli* in humans.

Although MLST is a very reliable method, it is also relatively expensive, time-consuming and requires a high level of technical skill. A new phenotypic typing method based on Raman spectroscopy, the ©SpectraCell RA (River diagnostics, Madison, Wisconsin USA), is now commercially available. This method is easy to use and delivers relatively fast results.

The objective of this study was to evaluate the performance of SpectraCell RA in comparison with MLST on a well defined collection of ESBL-producing *E. coli* isolates obtained from chicken meat and humans.[5]

Methods

Strain collection

The ESBL-producing *E. coli* isolates were part of a collection of ESBL-producing *Enterobacteriaceae* isolated from both meat and humans. The methods for detection and confirmation of the presence of ESBL-producing *E. coli* in the samples and typing of the samples were previously described.[5] Briefly, all samples were collected between January 2008 and December 2009 and were cultured using selective medium. Presence

of ESBL-producing *E. coli* was both phenotypical and genotypical confirmed and all ESBL-producing *E. coli* isolates were typed using MLST as described by Wirth et al..[4]

SpectraCell RA typing

All ESBL-producing *E. coli* isolates from meat, rectal swabs, and blood cultures were typed using SpectraCell RA. This typing method uses Raman spectroscopy to detect and type bacteria. The spectrometer uses a source of light with a known frequency. When the light falls through bacteria, the various proteins in the bacteria alter the frequency. Also, the amount of every protein alters the frequency in a different way. All alterations are detected and translated in a pattern. The resemblance of these patterns indicates the relatedness of two distinctive bacteria.
The procedure was done according to the manufacturer's instructions and took 3 days. On day one, a trypic soy agar (TSA) was inoculated and incubated for 18 to 24 h at 35°C. On day two, the grown isolates were suspended in 20 µl of sterilised water. Subsequently, 20 µl of the suspension was inoculated on a new TSA agar and spread over an area of 2 by 4 cm. After a 10-minute drying period, the agar was incubated during 20 h by 35°C. On the last day, a 1 µl sterile loop filled with colonies was suspended in 10 µl of demineralised water. After 3 minutes of centrifuging at high speed, 3 µl of supernatant was removed and the solution was resuspended. Then 3 µl of the suspension was inoculated on one well of the SpectraCell RA cartridge. After a drying period of 15 to 30 minutes, the cartridge was entered in the SpectraCell RA machine, and the software produced a spanning tree of the different isolates.
Thirteen randomly chosen isolates were tested twice to assess the reproducibility. Retrospectively, these isolates were unique regarding the SpectraCell RA typing results, and using MLST there were 2 clusters of 2 isolates. The information obtained from testing the isolates twice, was used by the software to generate a cutoff value (Figure 3.2.1). The software generates an optimum range for the cutoff value. In this range, the chances of indicating two unique isolates to be identical are minimal, as well as the chances of indicating two identical isolates to be unique. For our collection, the software calculated an optimum cutoff range of 0.99955 up to 0.99975.

Statistical analysis

We compared the results of MLST and SpectraCell RA, with MLST as the gold standard. If SpectraCell RA was in agreement with the gold standard and considered an isolate to be part of a cluster that was also found using MLST, or MLST found the isolates closely related, the result was scored as true positive. If SpectraCell RA and MLST both considered an isolate to be unique, the result was scored as true negative. If the gold standard considered an isolate to be part of a cluster and SpectraCell RA did not, the result was considered false negative. On the contrary, if SpectraCell RA considered an isolate to be part of a cluster and MLST did not, or SpectraCell RA clustered isolates of

different, not related, MLST clusters together, the result was considered false positive. Sensitivity was calculated by dividing the number of true positives by the sum of true positives and false negatives. Specificity was calculated by dividing the number of true negatives by the sum of true negatives and false positives. This was done for several cutoff values.
Second, we used SpectraCell RA to evaluate the relatedness between ESBL-producing *E. coli* in humans and in meat. This was done for several cutoff values.

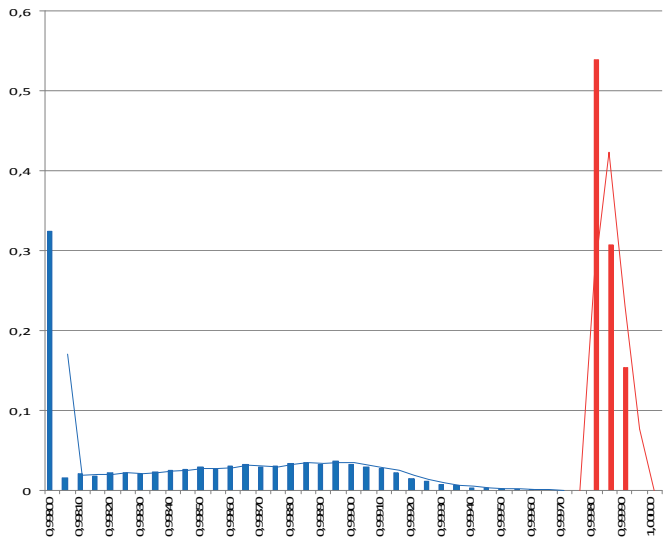

Figure 3.2.1 Graphic of performance at different cutoff values, generated by SpectraCell RA software. The red graphic shows the chance that, at a specific similarity rate, duplicate test results of one isolate are indicated as not identical. The blue graphic shows the chance that at a specific similarity rate, two unique isolates are indicated to be identical.

Results

In total, 151 ESBL-producing *E. coli* were available for testing, 92 originated from meat samples, and 59 were human. Of the meat samples, the majority, 84 isolates, originated with chicken meat; 8 isolates originated with beef, pork, or other meat types. Of the human samples, 43 came from rectal swabs and 16 came from blood cultures.
Using MLST, 74 sequence types were found, of which 28 contained more than one isolate. Using SpectraCell RA, the number of clusters differed depending on the cutoff value. We chose 3 different cutoff values. The highest cutoff value, 0.99975, was the

maximum cutoff value for this collection, calculated by the SpectraCell RA software. Above this value, duplicate test results from one isolate would be indicated as being not identical. The middle cutoff value, 0.99955, was the minimal cutoff value calculated by the software for this collection. Below this value, unique isolates may be considered as identical. To evaluate the effect on sensitivity and specificity, we also chose a cutoff value below the ones recommended by the software; 0.99935. Table 3.2.1 shows the effects of the different cutoff values. At the cutoff value of 0.99975, the sensitivity was very low, with a value of 9.4%, but the specificity was good with a value of 95.6%. At the cutoff value of 0.99955, both sensitivity and specificity were poor, with values of 43.9% and 69.8%, respectively. When we chose a cutoff value of 0.99935, the sensitivity rose to 66.7% and the specificity dropped to 34.4%. Figure 3.2.2 illustrates the MLST branched tree, with the SpectraCell RA clusters in colors for the different cutoff values. It shows that some isolates in SpectraCell RA clusters are far from related when using MLST and that many isolates in MLST clusters were not detected with SpectraCell RA.

Table 3.2.1 Results of SpectraCell RA.

Cutoff value SpectraCell RA	Number of clusters	Clusters with human and meat isolates	True positive results	True negative results	False positive results	False negative results	Sensitivity	Specificity
		Number (Percentage)	Number	Number	Number	Number	Percentage	Percentage
0.99975	6	0 (0.0)	10	43	2	96	9.4	95.6
0.99955	26	5 (19.2)	43	37	16	55	43.9	69.8
0.99935	36	14 (38.9)	58	22	42	29	66.7	34.4

Of the 74 MLST types, 10 clusters contained isolates from both human and meat samples. When using SpectraCell RA, the number of clusters containing isolates from both human and meat samples depended on the cutoff used, as shown in Table 3.2.1. When a cutoff value of 0.99975 was used, none of the 6 clusters contained both human and meat isolates. At a cutoff of 0.99955, 5 out of 26 clusters (19.2%) contained isolates of both human and meat origin; and at a cutoff of 0.99935, 14 out of 36 clusters (38.9%), contained isolates of both origins.

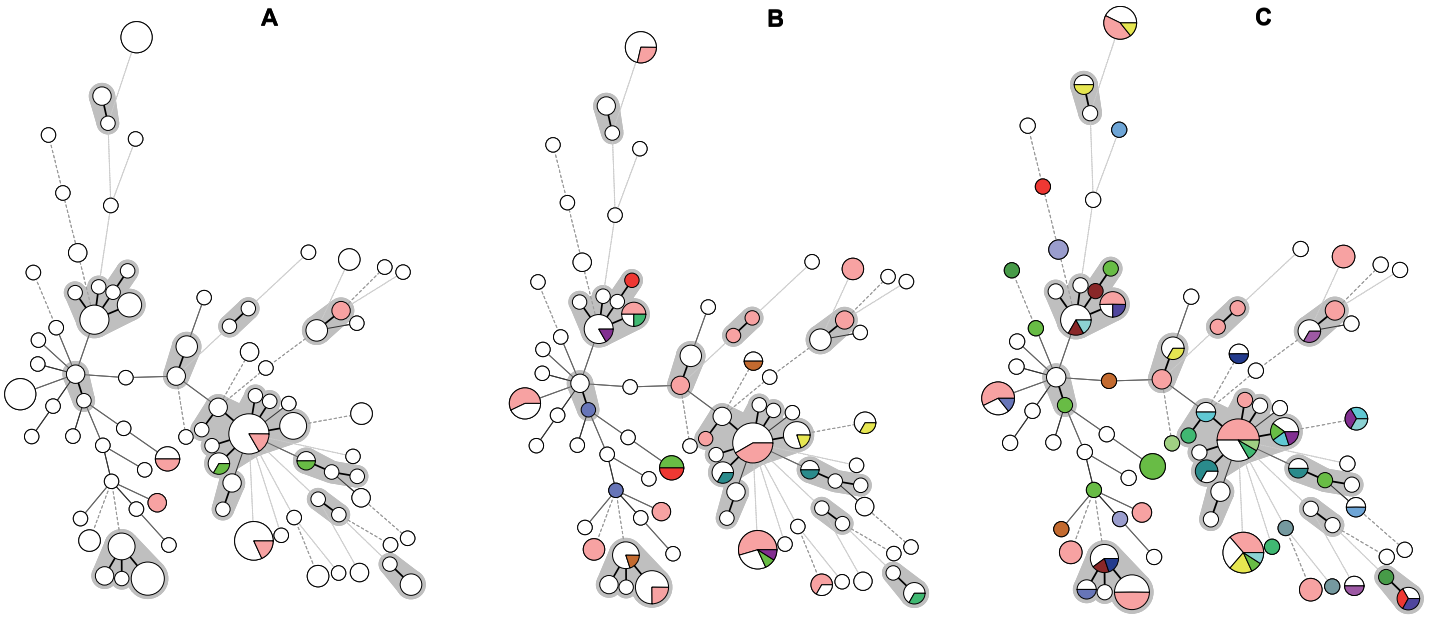

Figure 3.2.2. MLST branched tree with SpectraCell RA clusters.
A: cutoff value 0.99975; B: cutoff value 0.99955; C: cutoff value 0.99935
All SpectraCell RA clusters with isolates in one MLST cluster have the same colour ( ); all other clusters have a separate colour per cluster.

Discussion

Our study evaluates the performance of SpectraCell RA for typing with a perspective on population epidemiology. The study was performed using a well-defined collection of ESBL-producing *E. coli* isolates obtained from meat, human rectal swabs, and clinical blood cultures.[5] Using MLST as gold standard, the performance of SpectraCell RA was evaluated for 3 different cutoff values: 0.99975, 0.99955, and 0.99935. Depending on the cutoff value, the sensitivity was mediocre to unacceptably low with values of 9.4%, 43.9%, and 66.7%, respectively. The specificities were 95.6%, 69.8%, and 34.4%, respectively. MLST is a well-established typing method for this kind of purpose. Since the results of SpectraCell RA are poorly comparable with the results of MLST, we cannot validate SpectraCell RA for typing with the purpose of population epidemiology. Furthermore, our results highlight the danger in using phenotypic data to infer genetic relationships for outbreak analysis. After all, the low specificity shows that not only the resolution is different but also the results themselves are not comparable.

The performance of SpectraCell RA was previously studied for Gram-positive cocci,[6,7] showing a high reproducibility of the SpectraCell RA results in comparison with pulsed-field gel electrophoresis (PFGE). The performance of SpectraCell RA for typing of *Enterobacteriaceae* has only recently been evaluated.[8] In this study, SpectraCell RA was compared with several other typing methods, including PFGE. Three collections of ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates from clinical outbreaks were used. This study showed that the results of SpectraCell RA were in concordance with those of PFGE, and the authors concluded that SpectraCell RA is suitable for typing on a larger scale. We presume that the contradiction between this study and ours is caused by the difference in reference method and the difference in isolates used. Both PFGE and SpectraCell RA have a high resolution and are therefore perfect for discovering direct transmission. MLST, with its low resolution, is perfect to discover relationships at the population level. The isolates of our collection do not have a direct relationship, and therefore a typing method with a low resolution is the proper method to use.

In conclusion, our study shows that SpectraCell RA is not suitable for typing of ESBL-producing *E. coli* with an objective on population epidemiology.

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