

Revised Abstract

Background: Extended spectrum β -lactamases (ESBLs) of the TEM, SHV, or CTX-M type and KPC-producers have rapidly emerged worldwide. Fast and reliable detection techniques are essential in order to assist choice of therapy and to limit resistance spread. We used a novel *in vitro* molecular diagnostic test "Check-Points (CP) Check-KPC ESBL™" for the direct identification of ESBL (TEM, SHV and CTX-M) and KPC-producers on a large panel of *Enterobacteriaceae* clinical isolates. Methods: Multicenter worldwide collection of *Escherichia coli*, *Proteus mirabilis*, *Klebsiella* spp and *Enterobacter* spp ESBLs (n=1,126) and non-ESBL (n=59) isolates collected from the SMART program (2008-2009) were tested. Whole DNA extracts were submitted to a ligation reaction with specific probes. Ligated probes were subsequently amplified using a universal set of primers. Amplicons were specifically hybridized to the DNA micro-array and then processed with CP software. Results were confirmed by PCR-sequencing. Results: The CP assay allowed fast and unambiguous detection of all ESBL, non-ESBL and also KPC genes. Further CP assay identifies the point mutation in TEM and SHV genes that confer the ESBL phenotype and allows therefore differentiation between non-ESBL TEM and SHV and their ESBL derivatives. This is particularly useful for *E. coli* and *K. pneumoniae*. Of the 1,126 ESBL-positive isolates tested, 1,080 were detected by the CP array and all ESBL-negative isolates were negative by CP. In this study, the CP array identified 16 TEM, 198 SHV and 943 CTX-M ESBL genes respectively and 27 KPC genes as well. All the ESBL SHV, TEM and CTX-M genes detected by CP were confirmed by PCR. Sequencing data were 96% correlated with the CP results for all the SHV, TEM and CTX-M genes. Conclusion: This novel assay is a high-throughput powerful tool for rapid detection and categorization of ESBLs and KPC-producers. This method offers differentiation of ESBLs from non-ESBLs and definitive results within the same working day. The panel of genotypes detected has been advantageously extended to other genes (AmpC, carbapenemases).

Introduction

Extended-spectrum β -lactamases (ESBLs) are a worldwide public health problem: (7)
➤ they have rapidly emerged worldwide in *Enterobacteriaceae*
➤ they are considered to be one of the most important antibiotic resistance mechanisms

The majority of ESBLs belong to TEM-, SHV- and CTX-M types: (<http://www.lahey.org/studies/>)

- More than 180 TEM-type and 130 SHV-type β -lactamases have been identified worldwide
- The main mutations conferring the ESBL phenotype are found at the following positions:
TEM: 104, 164, 238 and 240
SHV: 238 and 240
- All the CTX-M enzymes are ESBLs: more than 90 CTX-M variants, divided into 5 five groups (CTX-M-1, 2, 9 and 8/25), have been identified (2).

Resistance to carbapenems due to the production of KPC enzymes in *Enterobacteriaceae* is a growing issue as well (3). To date, 11 KPC-variants have been described.

- Optimal detection of ESBLs/KPC is now highly important: (4)
➤ ESBLs detection is primarily based on phenotypic testing and standard molecular tests tend to be used to characterize isolates with these genes. PCR-sequencing is the most widely method used.
- Detection of KPC enzymes is difficult using phenotypic methods

The huge diversity of these enzymes makes their detection truly crucial to routinely monitor their prevalence and worldwide distribution. Fast and reliable molecular techniques which could be used in clinical microbiology laboratories are essential.

Materials & Methods

Bacterial strains:

The Study for Monitoring Antimicrobial Resistance Trends (SMART) is a global longitudinal antimicrobial surveillance study that has been monitoring susceptibility of Gram-negative aerobic pathogens from intra-abdominal infections (IAI) since 2002

In this study, 1,185 recent isolates (2008-2009) from the SMART program were tested.
➤ 1,126 clinical isolates phenotypically identified as ESBL-positive
➤ 59 clinical isolates phenotypically identified as ESBL-negative (negative controls)
ESBL testing was done according to CLSI guidelines (1), looking for a ≥ 3 -doubling dilution decrease in MIC of ceftazidime or cefotaxime in the presence of clavulanic acid.
➤ 7 control strains, representing the ESBL targeted (SHV, TEM, CTX-M1, 2, 9, 8/25) by the Check-Points method and PCR, were used in this study.

Material & Methods(cont.)

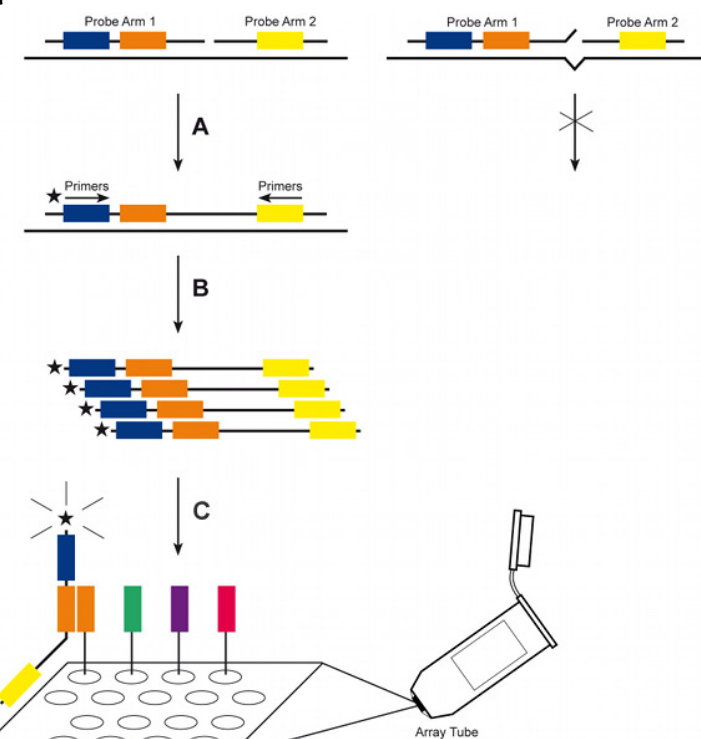
DNA sample preparation:

Whole DNAs were extracted with the QIAGEN QiaAmp DNA mini kit using the QIAcube instrument (12 samples an hour)



DNA micro-array method:

Figure 1: Principle of the CP method:



A-Ligation Detection Reaction

Probe contains:
Probe Arm 1: A specific sequence at the 5' with universal primers (blue box) linked to a unique ZIP code (orange box)
Probe Arm 2: A specific sequence at the 3' with universal primers (yellow box)

Probes are hybridized to target DNA and only in case of a perfect match, the probes are joined by a ligase. Critical mismatches in the target sequence will cause ligation to fail, leaving the probes apart.

B-Polymerase Chain reaction (PCR)

Successful ligation products are amplified by PCR using a single pair of universal primers annealing to complementary sequences included in the probes (blue and yellow boxes)

C-Hybridization and Detection

Unique ZIP codes (orange box) assigned to each probe will be specifically captured by complementary oligonucleotides spotted on the microarray and will be detected using a biotin label incorporated in one of the PCR primers.

The final results are obtained using a specific reader (ATRO3) and software:



PCR-sequencing:

Primers used for detection and sequencing of the blaCTX-M, blaSHV, blaTEM and blaKPC were described previously (5, 6, 8, 9)
➤ Both strands of the PCR products were sequenced using an Applied Biosystems sequencer (ABI 3730)
➤ Sequences were analyzed using the SeqScape software

Material & Methods(cont.)

Interpretation of Check-ESBL KPC results

Table 1a: Example of correlation of Check-ESBL scores and the SHV genes

CP codes	ESBL SHV types	238G	G238S	G238A	240E	E240K
22	Combination of wt with one of SHV-2, 2a, 3, 20, 21, 30, 34, 39, 86		+			+
26	Combination of wt with one of SHV-13, 29, 102	+			+	
50	Combination of wt with one of SHV-31, 91, 97, 115	+				+
54	Combination of wt with one of SHV-4, 5, 7, 9, 10, 12, 15, 22, 23, 45, 46, 55, 64, 66, 90, 105	+	+			+

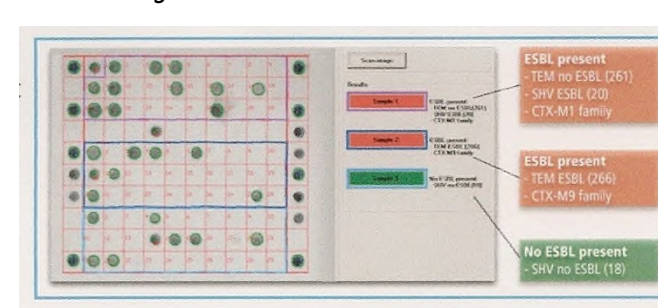
Table 1b: Example of correlation of Check-ESBL scores and the TEM genes

CP codes	ESBL TEM types	104E	E104K	164R	D164G	E164K	R164V	238G	G238S
263	Combination of wt with one of TEM-17, 26, 96, 106, 111, 124, 141		+	+					+
269	Combination of wt with one of TEM-7, 10, 12, 14, 16, 86, 102, 114, 125, 136, 137, 158	+							+
519	Combination of wt with one of TEM-4, 15, 21, 22, 50, 52, 66, 68, 80, 90, 94, 113, 121, 130, 139	+	+						+
773	Combination of wt with one of TEM-10, 20, 25, 42, 47, 48, 49, 64, 71, 72, 91, 101, 111, 120	+	+						+

Figure 2: ESBL-KPC array layout

Gene	Probe	Probe	Probe	Probe	Probe	Probe	Probe	Probe	Probe
TEM-1	TEM-1	TEM-1	TEM-1	TEM-1	TEM-1	TEM-1	TEM-1	TEM-1	TEM-1
SHV-1	SHV-1	SHV-1	SHV-1	SHV-1	SHV-1	SHV-1	SHV-1	SHV-1	SHV-1
CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1	CTX-M1

Figure 3: Check-KPC ESBL software



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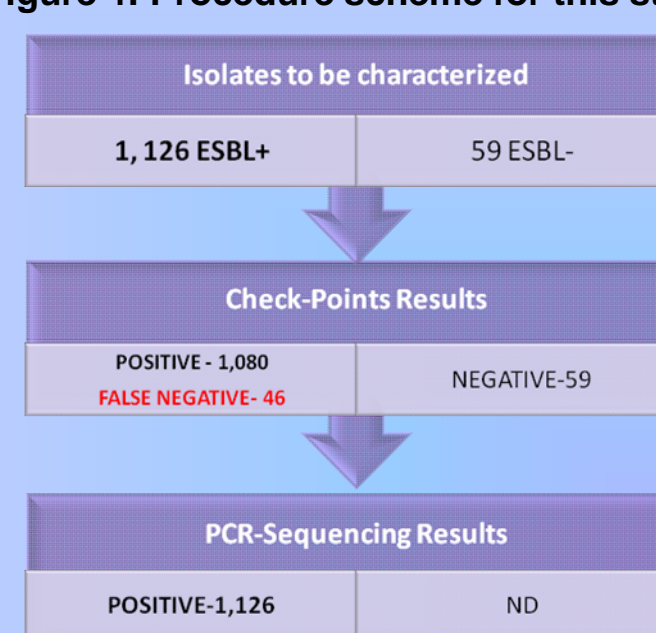
Acknowledgments

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Results

Check-Points combined to PCR-sequencing:

Figure 4: Procedure scheme for this study



- The 46 negative results by CP were due to CTX-M1 "weak signals" not read by the CP software but which could be read by the experimenter. Recently, the CP software has been updated and the CTX-M1 probe has been improved.
- All KPC detected by Check-Points have been confirmed by PCR-sequencing (27 isolates)

Discrepancies between Check-Points results (codes) and sequences:

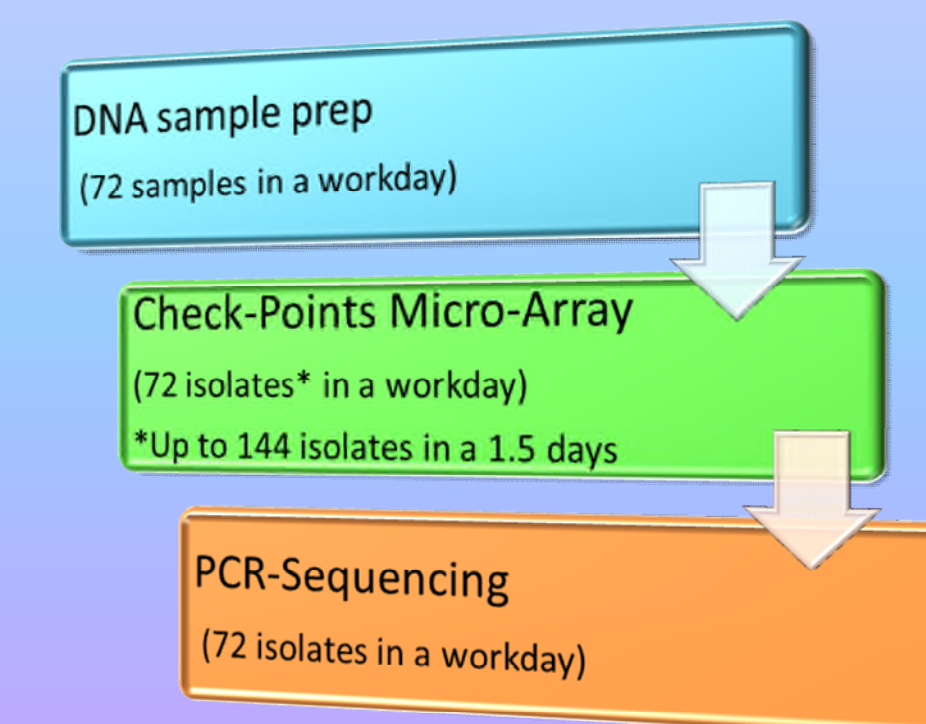
Table 2: SHV genes

Number of isolates	Check-Points	Sequencing	Comments
10	Unknown codes	SHV-12 or wt+SHV-12	"smear" signals read incorrectly by CP software
6	22, 26, 50 or 54	SHV-1 or SHV-11	Only wt sequence
18	50	SHV-12, 5 or wt+SHV-12, 5	"Weak signal" not read by CP software
2	22	SHV-2A	No wt sequence
1	26	SHV-28	"Background spot"

Table 3: TEM genes

Number of Isolates	Check-Points	Sequencing	Comments
4	263, 269, 773	TEM-1	Only wt sequence
1	263	wt+TEM-52	Weak signal not read by CP software
4	519	TEM-52, 92	No wt sequence

Figure 8: Time to response for complete characterization of 72 isolates



- 3 working days for complete characterization of 72 isolates

Conclusions

Check-Points array :

- Is a powerful tool for the detection of ESBL-phenotype and identification of:
- TEM types
- SHV types
- CTX-M group 1, 2, 9 and 8/25

- identifies ESBL from non-ESBL TEM and SHV

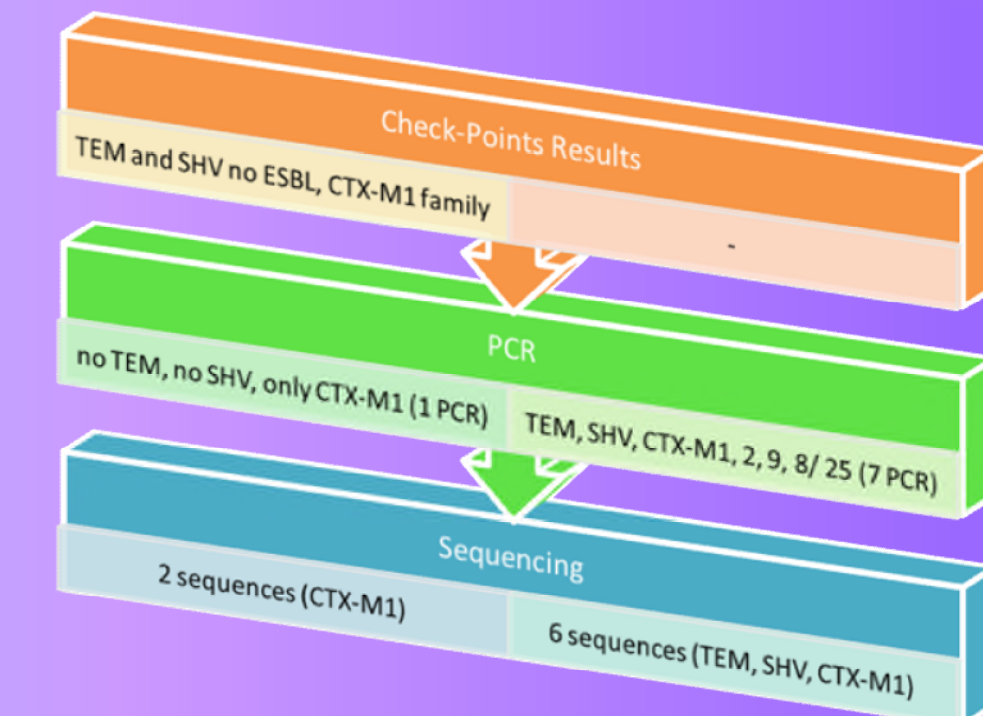
- is more adapted for batch testing in clinical lab equipped for molecular biology

- can be combined with PCR-sequencing, for fast, accurate and complete characterization of ESBLs

- is truly a promising tool for the detection of ESBL (SHV, TEM, CTX-M) but also carbapenemases and AmpC enzymes.

Advantages of Check-ESBL KPC combined with PCR-sequencing

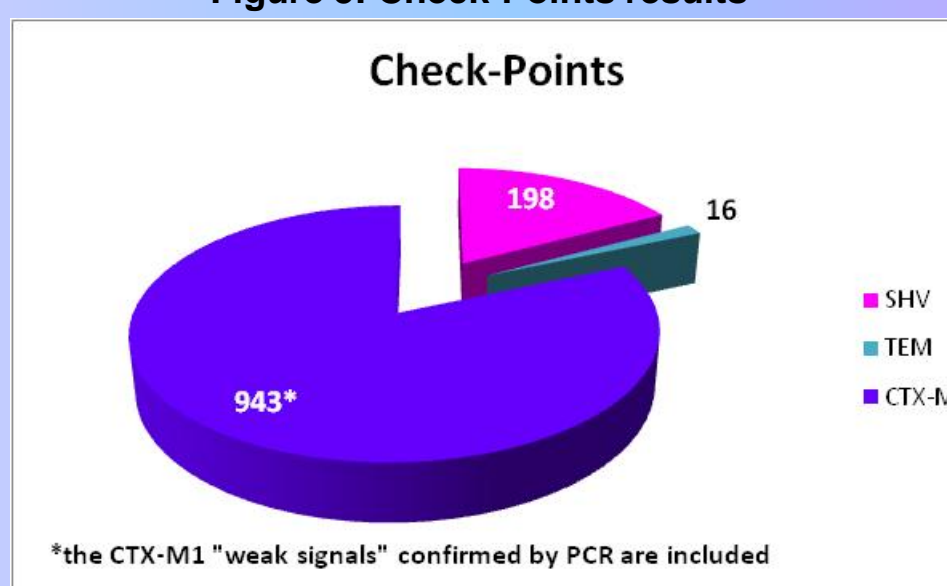
Figure 7: Example for an isolate carrying only the CTX-M1 enzyme:



- The Check-Points array results allowed avoiding sequencing of the non-ESBL enzymes.

Confirmation of the Check-Points results by PCR-sequencing

Figure 5: Check-Points results



Correlation : 96%

Figure 6: Sequencing results

