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Evaluation of MBT STAR-Cepha and MBT STAR-Carba kits for the detection of extended-spectrum β-lactamases and carbapenemase producing microorganisms using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

Rapid and simple detection of extended-spectrum β-lactamase (ESBL) and carbapenemase is essential for antimicrobial treatment and infection control. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based MBT STAR-Cepha and MBT STAR-Carba kits have been developed with simplified MBT STAR-BL operations. However, the utility of these kits has not been fully examined in clinical microbiology laboratories. In this study, we evaluated the utility of MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kits to detect ESBL and carbapenemase-producing bacteria, and compared it with the conventional broth microdilution test and PCR amplification assay. We found that the MBT STAR-Cepha kit efficiently enabled to distinguish resistant strains of third-generation cephalosporin susceptibility phenotypes and non-SHV-type ESBL producers. In the receiver operating characteristic analysis, the area under the receiver operating characteristic curve (AUC) for detecting third-generation cephalosporin resistance using the MBT STAR-Cepha kit was 0.97-1.00, but the AUC for detecting ESBL producers was 0.64. In addition, we showed that the MBT STAR-Carba kit enable the accurate detection of antimicrobial resistance by IMP-type carbapenemase producers. The AUC for detecting carbapenemase producers was 1.00. The results suggested that the target bacterial strains, antimicrobial susceptibility phenotypes, and resistance genes were important for the utility of the MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kits in bacterial routine diagnostics.

Keywords:

matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MBT STAR-Cepha kit, MBT STAR-Carba kit, extended-spectrum β-lactamase, carbapenemase

Abbreviations:

AUC: area under the receiver operating characteristic curve

CLSI: Clinical and Laboratory Standards Institute

CTRX: ceftriaxone

CTX: cefotaxime

ESBL: extended-spectrum β-lactamase

EUCAST: European Committee on Antimicrobial Susceptibility Testing

IPM: imipenem

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MEPM: meropenem

ROC: receiver operating characteristic

1. INTRODUCTION

Extended-spectrum β-lactamase (ESBL) and carbapenemase are enzymes that confer resistance to most β-lactam antibiotics and are exclusively found in gram-negative bacteria. ESBL or carbapenemase producers have represented serious clinical problems associated with high mortality and increased healthcare costs (Schwaber, 2006; Gasink, 2009; Liu, 2015). In addition, the prevalence of resistant bacteria with these enzymes has been increasing because the plasmid encoding ESBL or carbapenemase gene can be transferred between bacteria within the same species or between different species via conjugation or transformation (Queenan, 2007; Vaidya, 2011). In clinical fields, rapid determination of effective antibiotic treatment improves clinical outcome (Barenfanger, 1999; Buehler, 2016). Therefore, assays that are rapid and sensitive in detecting ESBL or carbapenemase producers are very important for infection control and essential. Detection of ESBL producers is based on cultured methods using the synergy between a third-generation cephalosporin and a β-lactamase inhibitor (Drieux, 2008), while the detection of carbapenemase producers has been based on culture-based phenotypic methods such as the modified Hodge test and carbapenem inactivation method among gram-negative organisms in clinical laboratories (Tamma, 2018). However, these culture-based phenotypic assays for detecting ESBL and carbapenemase are time-consuming and not easier to determine antibiotic resistant bacteria due to subjective visual judgment.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is routinely used to identify bacterial species (Wieser, 2012). Although this methodology can rapidly identify pathogenic microbes, prediction of antibiotic susceptibility in microbes has not been accurate. Recently, Bruker Daltonics developed the automated MBT STAR-BL software module (Billerica, MA, USA) for the rapid detection of the presence of β-lactamase activity by monitoring the specific mass shift derived from the

hydrolysis products of antibiotics (Doern, 2016). The methodology has been developed to provide high sensitivity and specificity for the detection of ESBL activity using MALDI-TOF MS and reagents (Oviaño, 2017; Lee, 2018; Ota, 2019). The methodology is significantly faster than culture-based conventional methods (Oviaño, 2017; Lee, 2018; Ota, 2019). Nevertheless, the operation of this MALDI-TOF MS-based MBT STAR-BL assay still requires the preparation of various reagents and antibiotics before each measurement. Thus, Bruker Daltonics simplified the MALDI-TOF MS-based MBT STAR-BL operations as MBT STAR-Cepha and MBT STAR-Carba kits (Bruker Daltonics), which allow the assays to be optimized using benchmark antibiotics. Furthermore, these kit assays reduce the measurement time when compared to the conventional MALDI-TOF MS-based MBT STAR-BL assay from 2 h to 30 min. The kit assays are expected to contribute to antimicrobial treatment and infection control. Only few studies have reported on the utility of the MBT STAR-Carba kit for the detection of resistant bacteria with carbapenemase (Rapp, 2018; Anantharajah, 2019), and no research using the MBT STAR-Cepha kit for the detection of ESBL producers have been reported in clinical microbiology laboratories.

In the present study, we evaluated the utility of MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kits for the detection of ESBL and carbapenemase-producing bacteria using MALDI-TOF MS. We found that the MBT STAR-Cepha kit efficiently enabled to distinguish resistant strains of third-generation cephalosporin susceptibility phenotypes and non-SHV-type ESBL producers. In addition, the MBT STAR-Carba kit enable the accurate detection of antimicrobial resistance by IMP-type carbapenemase producers. The results suggested that the target bacterial species, antimicrobial susceptibility phenotypes, and resistance genes were important for the utility of the those kits in bacterial routine diagnostics.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

We analyzed 32 *Escherichia coli*, 31 *Klebsiella pneumoniae*, 16 *Klebsiella oxytoca*, 15 *Proteus mirabilis*, 33 *Pseudomonas* sp., and 15 *Acinetobacter* sp. that were isolated from clinical specimens obtained from patients at the Hamamatsu University School of Medicine, Shizuoka, Japan (Table 1 and Table 2). The sources of the samples we used were 54 blood, 25 urine, 21 sputum, 9 stool, 7 pus, 7 drainage, and 19 others from inpatients with various infectious disease in this study. These isolates were stored at – 80 ℃ until this investigation, and identified using the Microflex LT system and MALDI Biotyper Compass software 4.1.100 (Bruker Daltonics). All identifications were reported with the following score values: ≥ 2.00 indicated species identification, and 1.70-1.99 indicated identification at the genus level. A clinical *E. coli* isolate expressing $bla_{\text{CTX-M}}$ group 9 and *E. coli* ATCC 25922 was used as ESBL-positive and -negative control strains, respectively. *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used as carbapenemase-positive and -negative control strains, respectively.

2.2. Conventional broth microdilution test

Bacterial isolates were cultured overnight on blood agar plates. The 96-well MicroScan panels (Beckman Coulter, Brea, CA, USA) were inoculated with each isolate to yield an appropriate density of 5×10^5 CFU/mL and incubated in the MicroScan WalkAway® 96 Plus system (Beckmann Coulter) at 35 °C. The minimum inhibitory concentrations were read at 16-20 h. The “susceptible”, “intermediate”, and “resistant” categories were based on the Clinical and Laboratory Standards Institute (CLSI) document M100-S26 (Wayne, 2016). The “susceptible”, “susceptible, increased exposure”, and “resistant” categories were based on the

European Committee on Antimicrobial Susceptibility Testing (EUCAST) document (EUCAST, 2021).

2.3. Genotype characterization using PCR detection assay

Each bacterial isolate was plated on blood agar and incubated at 36 °C overnight. DNA extraction was performed using the Cica Geneus DNA extraction reagent (Kanto Chemical Co. Inc., Tokyo, Japan) according to the manufacturer's instructions. PCR amplification of ESBL genes was performed with TaKaRa Ex Taq® (Takara Bio, Shiga, Japan) in *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* isolates by using specific primers for bla_{SHV}/bla_{TEM}/$bla_{\text{OXA-1}}$-like genes, and $bla_{\text{CTX-M}}$ genes, including the phylogenetic groups 1, 2, and 9 (Dallenne, 2010) (Table 3). Carbapenemase genotypes were characterized in *Pseudomonas* sp. and *Acinetobacter* sp. isolates by the use of Cica Geneus® Carbapenemase Genotype Detection KIT 2 (Kanto Chemical Co., Inc.), which can detect 6 carbapenemase genotypes (IMP-1, VIM, KPC, NDM, OXA-48, and GES groups) and distinguish the IMP-6 gene from the IMP-1 group.

2.4. Detection of extended-spectrum β-lactamases and carbapenemase with MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kits

We performed MALDI-TOF MS-based MBT STAR-Cepha kit testing in *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* for detecting ESBL-producing isolates, and MBT STAR-Carba kit testing in *Pseudomonas* sp. and *Acinetobacter* sp. for detecting carbapenemase producers. Briefly, the MBT STAR-BL incubation buffer was added to the MBT STAR-BL antibiotic reagent. One to five bacterial colonies from overnight cultures (concentration of over 10^9 CFU/mL per a bacterial colony) were suspended in the mixed reagent. We

also managed the mucoid colony variants in the same manner. After incubation at 35 °C for 30 min with shaking, the reaction mixture was centrifuged and 1 μL of the supernatant was loaded onto the MALDI target. The dried spots were overlaid with 1 μL of MBT STAR-BL matrix solution. After drying, mass spectrometric analysis was performed on the Microflex LT system with the MALDI-TOF MS-based MBT STAR-BL software module. This software automatically calculated the normalized logRQ value as an interpretation of β-lactamase activity based on the antibiotic hydrolysis intensity compared with a negative and positive control strain. On using a MALDI-TOF MS-based MBT STAR-Cepha kit, a logRQ value > 0.22 indicated positive ESBL activity, while a logRQ value < 0.08 indicated a negative result. A logRQ value between 0.08 and 0.22 indicated an unclear result. On using a MALDI-TOF MS-based MBT STAR-Carba kit, a logRQ value > 0.40 indicated positive carbapenemase activity, a logRQ value < 0.20 indicated a negative result, and a logRQ value between 0.20 and 0.40 indicated an unclear result. We compared the performance of the MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kits with the conventional broth microdilution test and PCR amplification assay for detecting ESBL- and carbapenemase-producing microorganisms.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism ver. 7.03 (GraphPad Software, San Diego, CA, USA) and JMP 13.2.0 (SAS Institute Inc., Cary, NC, USA). We defined the optimal cutoff value and area under the curve (AUC) for detecting ESBL and carbapenemase producers using receiver operating characteristic (ROC) curve analysis.

3. RESULTS

3.1. Strain characterization

Table 1 shows the characteristics of *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* isolates. The cefotaxime (CTX) and ceftriaxone (CTRX) susceptibility in these strains was classified by the broth microdilution test. There were 32 CTX-sensitive / 62 CTX-resistant isolates and 32 CTRX-sensitive / 1 CTRX-intermediate / 61 CTRX-resistant isolates based on CLSI categories (32 CTX-sensitive / 62 CTX-resistant isolates and 32 CTRX-sensitive / 1 CTRX-susceptible, increased exposure / 61 CTRX-resistant isolates based on EUCAST categories). We performed a PCR detection assay to detect the ESBL-encoding genes. Sixty of the 94 isolates had at least one ESBL-encoding gene, 9 were TEM- and CTX-M-type, 6 were SHV- and CTX-M-type, 6 were TEM-type, 15 were SHV-type, 19 were CTX-M-type, and 5 were other-type ESBLs. These genes could not be detected in the remaining 34 isolates. The characteristics of *Pseudomonas* sp. and *Acinetobacter* sp. isolates are shown in Table 2. Imipenem (IPM) and meropenem (MEPM) susceptibility was classified using the broth microdilution test, and there were 12 IPM-sensitive / 36 IPM-resistant isolates and 13 MEPM-sensitive / 1 MEPM-intermediate / 34 MEPM-resistant isolates based on CLSI categories (Based on EUCAST categories, there were 3 IPM-susceptible, increased exposure / 30 IPM-resistant and 4 MEPM-sensitive / 5 MEPM-susceptible, increased exposure / 24 MEPM-resistant *Pseudomonas* isolates, and there were 9 IPM-sensitive / 6 IPM-resistant and 9 MEPM-sensitive / 1 MEPM-susceptible, increased exposure / 5 MEPM-resistant *Acinetobacter* isolates). Fourteen of the 48 strains (8 *Pseudomonas* sp. and 6 *Acinetobacter* sp.) had carbapenemase-encoding genes; 11 were IMP-6 and 3 were IMP-1. These genes were not detected in 34 strains.

3.2. Evaluation of the MALDI-TOF MS-based MBT STAR-Cepha kit assay

We performed the MALDI-TOF MS-based MBT STAR-Cepha kit assay for detecting CTX-resistance based on CLSI, CTRX resistance based on CLSI, and ESBL producers. The distribution of the normalized logRQ values of the strains examined is shown in Table 1 and Figure 1. Thirty-one of the 32 CTX-sensitive strains showed negative ESBL activity, and 59 of the 62 CTX-resistant strains accurately identified positive results. One of the 32 CTX-sensitive and 3 of the 62 CTX-resistant isolates indicated unclear results (Fig. 1A and Table 4). In the receiver operating characteristic (ROC) analysis, susceptibility to CTX using the MALDI-TOF MS-based MBT STAR-Cepha kit assay perfectly matched with the results of the conventional broth microdilution test based on CLSI, excluding some isolates that showed unclear results in logRQ values with the MBT STAR-Cepha kit. The area under the curve (AUC) for detecting CTX resistance based on CLSI using the assay was 1.00 (Fig. 1B). In susceptibility to CTRX using the MALDI-TOF MS-based MBT STAR-Cepha kit assay, the results of the conventional broth microdilution test based on CLSI and AUC for detecting CTRX-resistant and intermediate strains based on CLSI were matched and similar to CTX assay (Supplementary Fig. 1A and 1B). Additionally, we evaluated the kit assay for detecting CTX and CTRX susceptibility based on EUCAST. The results of the conventional broth microdilution test and AUC for detecting CTX and CTRX-resistance based on EUCAST were comparable to those based on CLSI (Supplementary Fig. 2). Next, we evaluated the detection of ESBL producers in this assay. Forty-eight of the 60 ESBL producers correctly showed positive results, and 22 of the 34 ESBL non-producers were negative (Fig. 1C). Nine ESBL producers and 11 ESBL non-producers were misclassified, and 3 ESBL producers and 1 ESBL non-producer indicated unclear results. The AUC for detecting ESBL producers in all strains was 0.64 (Fig. 1D). The MALDI-TOF MS-based MBT STAR-Cepha kit assay to detect ESBL producers did not give high accuracy in the present study. Consequently, we investigated the differences between strains and drug resistance genes. In ESBL non-producers, the

non-*K. oxytoca* isolates were accurately classified, and all the strains showed negative results. Eleven of the 16 ESBL non-producing *K. oxytoca* strains showed positive logRQ results with the MALDI-TOF MS-based MBT STAR-Cepha kit assay (Fig. 1E, Table 1). In ESBL producers, we compared the normalized logRQ values in isolates with various ESBL-encoding genes. Isolates with TEM, CTX-M, and other ESBL genes were approximately classified based on positive logRQ results, while 9 of the 16 isolates with SHV-type ESBL genes showed negative logRQ results and were not identified in this assay (Fig. 1F).

3.3. Evaluation of the MALDI-TOF MS-based MBT STAR-Carba kit assay

We performed the MALDI-TOF MS-based MBT STAR-Carba kit assay for detecting IPM resistance based on CLSI and carbapenemase producers. The distribution of the normalized logRQ values of the strains examined is shown in Table 2 and Figure 2. All 12 IPM-sensitive strains completely showed negative logRQ results, and 14 of the 36 IPM-resistant strains showed positive results (Fig. 2A). In the ROC analysis for detecting IPM-resistance, the AUC was 0.58 (Fig. 2B). In susceptibility to MEPM based on CLSI using the MALDI-TOF MS-based MBT STAR-Carba kit assay, 13 MEPM-sensitive strains completely showed negative logRQ results, 1 MEPM-intermediate strain was negative, and 14 of the 34 MEPM-resistant strains showed positive results (Supplementary Fig. 3A). In the ROC analysis for detecting MEPM-resistance based on CLSI, the AUC was 0.56 (Supplementary Fig. 3B). Additionally, we investigated the kit assay for detecting IPM and MEPM susceptibility based on EUCAST. The results of the conventional broth microdilution test and AUC for detecting IPM and MEPM-resistance based on EUCAST were similar to those based on CLSI (Supplementary Fig. 4). Next, we assessed the detection of carbapenemase producers in this

assay. (Fig. 2C). In the ROC analysis, the AUC for detecting carbapenemase producers in this assay was 1.00 (Fig. 2D).

4. DISCUSSION

In this study, we evaluated novel diagnostic methods for detecting β-lactamase activity using MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit assays. Our results indicate that the rapid and simple assay can detect antimicrobial resistance by producing ESBL and IMP-type carbapenemase. In addition, this diagnostic method can fetch results within an hour, thus being much faster than conventional growth-based assays. Therefore, the MALDI TOF-MS-based β-lactamase detection assay is very useful for both adequate antibiotic selection and infection control of resistant bacteria.

To detect ESBL activity, some reports have shown the utility of MALDI-TOF MS using the MBT STAR-BL module assay with CTX as a third-generation cephalosporin. Kawamoto *et al.* reported that a MALDI-TOF MS-based CTX hydrolysis assay enabled the rapid prediction of resistance (Kawamoto, 2019). The sensitivity and specificity for detecting ESBL producers in *E. coli* and *K. pneumoniae* were 100 % and 91.5%, respectively (Kawamoto, 2019). On the contrary, we have previously reported a CTX hydrolysis assay with the MALDI-TOF MS-based MBT STAR-BL module and the sensitivity and specificity to detect ESBL producers in *Enterobacteriaceae* were 86.7% and 68.1%, respectively (Ota, 2019). In the present study, the MALDI-TOF MS-based MBT STAR-BL assay for detecting ESBL producers did not have high sensitivity and specificity because some ESBL non-producing *K. oxytoca* isolates resulted in false-positive results, and some SHV-type ESBL producers showed false-negatives. These results are consistent with the previous findings of the limited ability of MALDI-TOF MS-based MBT STAR-BL module assay to detect ESBL producers (Ota, 2019). The isolates that showed unclear results in logRQ values with the MBT STAR-Cepha kit were performed by culture-based ESBL detection assay using ESBL disk diffusion test and presented those characteristics in Table 4. The isolates were 3 *E. coli* and 1 *K. oxytoca*. Each *E. coli* had ESBL gene; 2 were SHV-type and another was TEM-type.

Previous reports have shown that SHV-type or TEM-type ESBL had less hydrolyzing activity against CTX than CTX-M-type ESBL (Rossolini, 2008), and the isolates carrying TEM-type ESBL gene did not have positive β-lactamase activity in the MALDI-TOF MS-based MBT STAR-BL assay (Oviaño, 2017). Our previous research has similarly reported that the logRQ values of SHV-type strains was significantly lower than those of CTX-M-type strains in the MALDI-TOF MS-based MBT STAR-BL approach (Ota, 2019). Also, we previously found that the MALDI-TOF MS-based MBT STAR-BL assay could not distinguish between ESBL producers and non-producers in *Klebsiella* spp. because the logRQ values of *Klebsiella* strains hyper-producing K-1 type β-lactamase changed by the MALDI-TOF MS-based MBT STAR-BL assay using different β-lactamase inhibitors. Thus, we suggested that the genotypes of ESBL and bacterial strains might affect the unclear results in logRQ values with the MBT STAR-Cepha kit. On the other hand, the MALDI-TOF MS-based MBT STAR-Cepha kit testing detected CTX and CTRX resistance based on CLSI and EUCAST with almost perfect sensitivity and specificity, except for isolates that showed an unclear result. Oviaño *et al.* also reported high sensitivity and specificity in the detection of β-lactam resistance by measuring the hydrolysis of ceftriaxone with the MBT STAR-BL module (Oviaño, 2017). Therefore, the MALDI-TOF MS-based MBT STAR-Cepha kit assay is considered to be useful for detecting third-generation cephalosporin resistance phenotype by the production of ESBLs; however, the target bacterial species needs to be given careful consideration.

The MALDI-TOF MS-based MBT STAR-Carba kit assay uses IPM as a benchmark antibiotic. In the present study, IPM hydrolysis analysis with this assay revealed negative β-lactamase activity in IPM-sensitive isolates and carbapenemase-non-producing IPM-resistant isolates. Previous reports have reported that the MALDI-TOF MS assay is not capable of detecting carbapenem resistance due to non-enzymatic mechanisms (Anantharajah, 2019). The common mechanisms of carbapenem resistance include overexpression of efflux

pumps and loss of porins, but not enzymes in non-fermenting gram-negative bacteria (Codjoe, 2018). In addition, the MALDI-TOF MS-based MBT STAR-Carba kit assay detects β-lactamase activity by monitoring the mass peaks of the hydrolysis products of antibiotics. Because the kit assay is based on enzymatic reaction, we cannot accurately detect carbapenem resistant isolated with non-enzymatic resistance mechanisms by using this assay. On the other hand, the MBT STAR-Carba kit testing accurately detected *Pseudomonas* sp. and *Acinetobacter* sp. strains producing carbapenemase in our study. This result is consistent with previous reports by Rapp *et al*. and Anantharajah *et al*. (Rapp, 2018; Anantharajah, 2019). Those findings indicate that this assay could be useful to rapidly detect carbapenemase producers and select the appropriate antibiotic therapy.

The emergence of antimicrobial resistance inducing β-lactamases such as ESBL and carbapenemase has a significant impact on mortality rates and hospital costs due to infectious diseases (Schwaber, 2006; Gasink, 2009; Liu, 2015). Although, there have not been many rapid, standardized methods to detect ESBL or carbapenemase, the rapid and prompt assays to detect β-lactamase producers are essential in clinical microbiology laboratories. Recently, the ESBL NDP test has been developed for a rapid identification of ESBL in *Enterobacteriaceae* (Nordmann, 2012). Similarly, the Carba NP test based on the direct detection of carbapenem hydrolysis has been developed to detect carbapenemase producers (Nordmann, 2012). These simple tests can be used to rapidly detect ESBL and carbapenemase activity in clinical microbiology laboratories. In particular, CLSI recommends the use of the Carba NP test for the detection of carbapenemase producers (Wayne, 2016). However, the Carba NP test as well as the ESBL NDP test must be manually performed for all operations, and the test result depends on subjective visual judgment (Nordmann, 2012; Wayne, 2016). While, the MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit assays has been developed to automate and standardize the prompt detection of β-lactamase activity. In the

present study, we showed that the novel MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit assays are reliable for detecting non-SHV-type ESBL producers in *Enterobacteriaceae* and IMP-type carbapenemase producers in *Pseudomonas* sp. and *Acinetobacter* sp. isolates, respectively. Additionally, the hands-on time and detection by the MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit testing are equivalent to those obtained by the ESBL NDP test and the Carba NP test (Nordmann, 2012; Rapp, 2018). Thus, the MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit assays have the potential to be used on a daily basis for the detection of β-lactamase-producing bacteria in clinical microbiology laboratories.

A limitation of the study is the small sample size, and we analyzed *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* for the detection of ESBL producers using MALDI-TOF MS-based MBT STAR-Cepha kit assay based on CLSI and EUCAST recommendations (Wayne, 2016; EUCAST, 2021) in this study. The detection of ESBL producers in other *Enterobacteriaceae* such as *Citrobacter* sp., *Enterobacter* sp., and *Serratia* sp., have also been reported (Paterson, 2005); however, CLSI and EUCAST exclude those species from the target bacteria of ESBL producers since it is difficult to detect ESBL in strains in which the gene encoding class C β-lactamase is present on the chromosome, and not on the plasmid. In addition, we analyzed limited bacterial species and carbapenemase genotypes using the MALDI-TOF MS-based MBT STAR-Carba kit assay in this study. The frequency of detection of carbapenemase producers is low in Japan. In particular, the Japan Nosocomial Infections Surveillance 2018 report stated that the rates of IMP resistance according to CLSI 2012 breakpoints were 0.5% for *E. coli*, 1.0% for *K. pneumoniae*, 16.2% for *P. aeruginosa*, and 2.0% for *Acinetobacter* sp.. Moreover, non-IMP-type carbapenemase producers are extremely rare in Japan (Ohno, 2017; Osawa, 2019), although various types of carbapenemase-producing isolates have increased worldwide (Bonomo, 2018). We haven't

detected them in our microbiology laboratory during the study. Therefore, it will be necessary to confirm the validity of the assay with a greater number of bacterial species or other-type carbapenemase producers in future studies.

In conclusion, MALDI-TOF MS-based MBT STAR-Cepha and MBT STAR-Carba kit testing enable the accurate detection of antimicrobial resistance by non-SHV-type ESBL and IMP-type carbapenemase producers, respectively. These simple and rapid kit assays have the potential to be used on a daily basis for the detection of ESBL and carbapenemase-producing bacteria in clinical microbiology laboratories.

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Figure Legends

Fig. 1. Evaluation of the MALDI-TOF MS-based MBT STAR-Cepha kit assay. Normalized logRQ values > 0.22 indicate positive ESBL activity, while the normalized logRQ value < 0.08 indicates a negative result. A normalized logRQ value between 0.08 and 0.22 indicates an unclear result. Ninety-four clinical isolates (32 CTX sensitive (S) based on CLSI, 62 CTX resistance (R) based on CLSI, 34 ESBL negative, 60 ESBL positive) were used to calculate the normalized logRQ values (A, C). ROC curves to detect CTX resistance (B) and ESBL producers (D) for this assay, excluding isolates which showed an unclear result. A comparison of the logRQ values between 16 *Klebsiella oxytoca*, and 18 non-*Klebsiella oxytoca* strains in ESBL non-producers (E). A comparison of the logRQ values among ESBL-encoding genes (15 SHV, 6 TEM, 19 CTX-M, and 20 others) in ESBL producers (F).

Fig. 2. Evaluation of the MALDI-TOF MS-based MBT STAR-Carba kit assay. Normalized logRQ values > 0.40 indicate positive carbapenemase activity, while the normalized logRQ value < 0.20 indicates a negative result. A normalized logRQ value between 0.20 and 0.40 indicates an unclear result. Forty-eight clinical isolates (12 IPM sensitive (S) based on CLSI, 36 IPM resistant (R) based on CLSI, 34 carbapenemase negative, 14 carbapenemase positive) were used to calculate the normalized logRQ values (A, C). ROC curves to detect IPM resistant (B) and carbapenemase producers (D) in this assay.

Supplementary Figure Legends

Supplementary Fig. 1. Evaluation of the MALDI-TOF MS-based MBT STAR-Cepha kit assay. Normalized logRQ values > 0.22 indicate positive ESBL activity, while the normalized logRQ value < 0.08 indicates a negative result. A normalized logRQ value between 0.08 and 0.22 indicates an unclear result. Ninety-four clinical isolates (32 CTRX sensitive, 1 CTRX intermediate, 61 CTRX resistant based on CLSI) were used to calculate the normalized logRQ values (A). ROC curve to detect CTRX resistance for this assay, excluding isolates which showed an unclear result (B).

Supplementary Fig. 2. Evaluation of the MALDI-TOF MS-based MBT STAR-Cepha kit assay. Normalized logRQ values > 0.22 indicate positive ESBL activity, while the normalized logRQ value < 0.08 indicates a negative result. A normalized logRQ value between 0.08 and 0.22 indicates an unclear result. Ninety-four clinical isolates (32 CTX sensitive (S), 62 CTX resistant (R), 32 CTRX sensitive (S), 1 CTRX susceptible, increased exposure (I), 61 CTRX resistant (R) based on EUCAST) were used to calculate the normalized logRQ values (A, C). ROC curves to detect CTX resistance (B) and CTRX resistance (D) for this assay, excluding isolates which showed an unclear result.

Supplementary Fig. 3. Evaluation of the MALDI-TOF MS-based MBT STAR-Carba kit assay. Normalized logRQ values > 0.40 indicate positive carbapenemase activity, while the normalized logRQ value < 0.20 indicates a negative result. A normalized logRQ value between 0.20 and 0.40 indicates an unclear result. Forty-eight clinical isolates (13 MEPM sensitive, 1 MEPM intermediate, 34 MEPM resistant based on CLSI) were used to calculate the normalized logRQ values (A). ROC curve to detect MEPM resistance in this assay (B).

Supplementary Fig. 4. Evaluation of the MALDI-TOF MS-based MBT STAR-Carba kit assay. Normalized logRQ values > 0.40 indicate positive carbapenemase activity, while the normalized logRQ value < 0.20 indicates a negative result. A normalized logRQ value between 0.20 and 0.40 indicates an unclear result. Forty-eight clinical isolates (9 IPM sensitive (S), 3 IPM susceptible, increased exposure (I), 36 IPM resistant (R), 13 MEPM sensitive (S), 6 MEPM susceptible, increased exposure (I), 29 MEPM resistant (R) based on EUCAST) were used to calculate the normalized logRQ values (A, C). ROC curves to detect IPM resistance (B) and MEPM resistance (D) in this assay.

Table 1. The distribution of bacterial identification, cefotaxime (CTX) and ceftriaxone (CTRX) susceptibility, ESBL-encoding genes, and the normalized logRQ with the MBT STAR-Cepha kit in *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *P. mirabilis* isolates.

Bacterial identification with MALDI Biotyper		Specimen	Susceptibility to CTX (μg/mL)		Susceptibility to CTRX (μg/mL)		PCR detection of ESBL-encoding genes	logRQ values with MBT STAR-Cepha kit
Escherichia coli	2.17	urine	>2	R*	>2	R	TEM, CTX-M-9	3.14
Escherichia coli	2.40	sputum	>2	R	>2	R	TEM, CTX-M-9	0.56
Escherichia coli	2.38	ascites	>2	R	>2	R	TEM, CTX-M-9	1.17
Escherichia coli	2.28	drainage	>2	R	>2	R	TEM, CTX-M-9	2.15
Escherichia coli	2.24	bile	>2	R	>2	R	OXA-1, CTX-M-1	0.33
Klebsiella pneumoniae	2.37	bile	>2	R	>2	R	TEM, SHV, OXA-1, CTX-M-1	0.82
Klebsiella pneumoniae	2.36	sputum	>2	R	>2	R	TEM, SHV, CTX-M-1	2.37
Klebsiella pneumoniae	2.32	drainage	>2	R	>2	R	TEM, SHV, CTX-M-1	1.94
Klebsiella pneumoniae	2.50	stool	>2	R	>2	R	TEM, CTX-M-1	0.54
Klebsiella pneumoniae	2.35	urine	>2	R	>2	R	TEM, CTX-M-1	0.53
Klebsiella pneumoniae	2.46	pleural effusion	>2	R	>2	R	TEM, CTX-M-1	1.16
Klebsiella pneumoniae	2.51	blood	>2	R	>2	R	TEM, CTX-M-1	2.35
Klebsiella pneumoniae	2.39	blood	>2	R	>2	R	TEM, CTX-M-1	2.23
Klebsiella pneumoniae	2.40	urine	>2	R	>2	R	SHV、CTX-M-2	1.24
Klebsiella pneumoniae	2.43	stool	>2	R	>2	R	SHV, CTX-M-9	3.19
Klebsiella pneumoniae	2.32	exudate	>2	R	>2	R	SHV, CTX-M-9	1.35
Klebsiella pneumoniae	2.42	pleural effusion	>2	R	>2	R	SHV, CTX-M-1	0.57
Klebsiella pneumoniae	2.38	sputum	>2	R	>2	R	SHV, CTX-M-1	1.29
Klebsiella pneumoniae	2.45	pus	>2	R	>2	R	SHV, CTX-M-1	0.94
Klebsiella pneumoniae	2.22	urine	>2	R	>2	R	OXA-1, CTX-M-9	1.34
Escherichia coli	2.16	blood	>2	R	>2	R	TEM	0.65
Escherichia coli	2.31	blood	>2	R	>2	R	TEM	0.09
Escherichia coli	2.10	blood	>2	R	>2	R	TEM	0.36
Escherichia coli	2.38	stool	>2	R	>2	R	TEM	0.23
Proteus mirabilis	2.31	sputum	>2	R	>2	R	TEM	1.52
Proteus mirabilis	2.33	pus	>2	R	>2	R	TEM	2.11
Escherichia coli	2.29	urine	>2	R	>2	R	SHV	0.18
Escherichia coli	2.20	blood	>2	R	>2	R	SHV	0.09
Klebsiella pneumoniae	2.39	blood	>2	R	>2	R	SHV	0.80
Klebsiella pneumoniae	2.40	stool	>2	R	>2	R	SHV	0.30
Klebsiella pneumoniae	2.49	urine	>2	R	>2	R	SHV	1.94
Klebsiella pneumoniae	2.28	urine	>2	R	>2	R	SHV	0.34

Klebsiella pneumoniae	2.37	blood	≦1	S	≦1	S	SHV	-0.38
Klebsiella pneumoniae	2.36	blood	≦1	S	≦1	S	SHV	-0.50
Klebsiella pneumoniae	2.33	blood	≦1	S	≦1	S	SHV	-0.35
Klebsiella pneumoniae	2.39	blood	≦1	S	≦1	S	SHV	-0.32
Klebsiella pneumoniae	2.31	blood	≦1	S	≦1	S	SHV	-0.40
Klebsiella pneumoniae	2.57	blood	≦1	S	≦1	S	SHV	-0.41
Klebsiella pneumoniae	2.30	blood	≦1	S	≦1	S	SHV	-0.22
Klebsiella pneumoniae	2.19	blood	≦1	S	≦1	S	SHV	-0.31
Klebsiella pneumoniae	2.31	blood	≦1	S	≦1	S	SHV	-0.30
Escherichia coli	2.28	sputum	>2	R	>2	R	CTX-M-1, CTX-M-9	0.48
Escherichia coli	2.30	blood	>2	R	>2	R	CTX-M-1	1.76
Escherichia coli	2.21	urine	>2	R	>2	R	CTX-M-2	0.96
Escherichia coli	2.13	blood	>2	R	>2	R	CTX-M-2	1.20
Escherichia coli	2.46	urine	>2	R	>2	R	CTX-M-9	0.27
Escherichia coli	2.38	sputum	>2	R	>2	R	CTX-M-9	1.15
Escherichia coli	2.19	stool	>2	R	>2	R	CTX-M-9	0.61
Escherichia coli	2.43	urine	>2	R	>2	R	CTX-M-9	1.08
Escherichia coli	2.26	bile	>2	R	>2	R	CTX-M-9	1.27
Escherichia coli	2.34	urine	>2	R	>2	R	CTX-M-9	2.29
Escherichia coli	2.19	stool	>2	R	>2	R	CTX-M-9	2.08
Proteus mirabilis	2.11	urine	>2	R	>2	R	CTX-M-2	2.63
Proteus mirabilis	2.52	urine	>2	R	>2	R	CTX-M-2	3.49
Proteus mirabilis	2.30	urine	>2	R	>2	R	CTX-M-2	2.62
Proteus mirabilis	2.41	urine	>2	R	>2	R	CTX-M-2	2.21
Proteus mirabilis	2.42	urine	>2	R	>2	R	CTX-M-2	2.23
Proteus mirabilis	2.32	exudate	>2	R	>2	R	CTX-M-2	2.15
Proteus mirabilis	2.46	blood	>2	R	>2	R	CTX-M-2	2.13
Proteus mirabilis	2.34	blood	>2	R	>2	R	CTX-M-9	0.89
Klebsiella oxytoca	2.30	skin	>2	R	2	I	-	2.58
Klebsiella oxytoca	2.08	bile	>2	R	>2	R	-	2.55
Klebsiella oxytoca	2.22	drainage	>2	R	>2	R	-	2.47
Klebsiella oxytoca	2.38	stool	>2	R	>2	R	-	2.62
Klebsiella oxytoca	2.22	urine	>2	R	>2	R	-	1.91
Klebsiella oxytoca	2.34	urine	>2	R	>2	R	-	2.50
Klebsiella oxytoca	2.44	urine	>2	R	>2	R	-	1.33
Klebsiella oxytoca	2.28	pleural effusion	>2	R	>2	R	-	2.54
Klebsiella oxytoca	2.36	drainage	>2	R	>2	R	-	2.11
Klebsiella oxytoca	2.15	pus	>2	R	>2	R	-	0.70

Klebsiella oxytoca	2.31	stool	>2	R	>2	R	-	2.04
Escherichia coli	2.15	blood	≦1	S	≦1	S	-	-0.02
Escherichia coli	2.28	blood	≦1	S	≦1	S	-	-0.12
Escherichia coli	2.41	blood	≦1	S	≦1	S	-	0.04
Escherichia coli	2.19	blood	≦1	S	≦1	S	-	0.00
Escherichia coli	2.37	blood	≦1	S	≦1	S	-	0.00
Escherichia coli	2.31	blood	≦1	S	≦1	S	-	-0.23
Escherichia coli	2.32	blood	≦1	S	≦1	S	-	-0.03
Escherichia coli	2.11	blood	≦1	S	≦1	S	-	-0.16
Escherichia coli	2.30	blood	≦1	S	≦1	S	-	-0.27
Escherichia coli	2.34	blood	≦1	S	≦1	S	-	-0.12
Klebsiella pneumoniae	2.52	blood	≦1	S	≦1	S	-	-0.11
Klebsiella pneumoniae	2.36	blood	≦1	S	≦1	S	-	-0.06
Klebsiella pneumoniae	2.40	blood	≦1	S	≦1	S	-	-0.18
Klebsiella oxytoca	2.33	blood	≦1	S	≦1	S	-	-0.21
Klebsiella oxytoca	2.27	blood	≦1	S	≦1	S	-	0.00
Klebsiella oxytoca	2.12	blood	≦1	S	≦1	S	-	-0.02
Klebsiella oxytoca	2.34	blood	≦1	S	≦1	S	-	0.09
Klebsiella oxytoca	2.20	blood	≦1	S	≦1	S	-	-0.46
Proteus mirabilis	2.39	blood	≦1	S	≦1	S	-	-0.12
Proteus mirabilis	2.38	blood	≦1	S	≦1	S	-	-0.08
Proteus mirabilis	2.22	blood	≦1	S	≦1	S	-	-0.16
Proteus mirabilis	2.39	blood	≦1	S	≦1	S	-	0.01
Proteus mirabilis	2.24	blood	≦1	S	≦1	S	-	-0.22

*”Susceptible (S)”, “Intermediate (I)”, and “Resistant (R)” were based on CLSI document (Wayne, 2016).

Table 2. The distribution of bacterial identification, imipenem (IPM) and meropenem (MEPM) susceptibility, carbapenemase-encoding gene, and the normalized logRQ with the MBT STAR-Carba kit in *Pseudomonas* sp. and *Acinetobacter* sp. isolates.

Bacterial identification with MALDI Biotyper		specimen	Susceptibility to IPM (μg/mL)		Susceptibility to MEPM (μg/mL)		PCR detection of carbapenemase gene	logRQ values with the MBT STAR-Carba kit
Pseudomonas putida	2.29	urine	>8	R*	>8	R	IMP-6	0.91
Pseudomonas putida	2.32	urine	>8	R	>8	R	IMP-6	1.18
Pseudomonas putida	2.30	urine	>8	R	>8	R	IMP-6	1.27
Pseudomonas sp.	1.94	blood	>8	R	>8	R	IMP-6	1.08
Pseudomonas monteilii	2.06	urine	>8	R	>8	R	IMP-6	1.21
Pseudomonas sp.	1.95	sputum	>8	R	>8	R	IMP-6	1.16
Pseudomonas sp.	1.84	urine	>8	R	>8	R	IMP-6	1.07
Pseudomonas monteilii	2.02	stool	>8	R	>8	R	IMP-6	1.15
Acinetobacter nosocomialis	2.04	sputum	>8	R	>8	R	IMP-6	1.00
Acinetobacter sp.	1.98	sputum	>8	R	>8	R	IMP-6	0.54
Acinetobacter baumannii	2.34	urine	>8	R	>8	R	IMP-6	1.11
Acinetobacter ursingii	2.22	throat swab	>8	R	8	R	IMP-1 group	1.04
Acinetobacter junii	2.01	blood	>8	R	>8	R	IMP-1 group	1.18
Acinetobacter baumannii	2.15	sputum	>8	R	>8	R	IMP-1 group	1.06
Pseudomonas aeruginosa	2.49	sputum	>8	R	>8	R	-	-0.15
Pseudomonas aeruginosa	2.27	drainage	>8	R	>8	R	-	-0.08
Pseudomoas aeruginosa	2.51	nasal cavity	>8	R	8	R	-	-0.09
Pseudomoas aeruginosa	2.30	pus	>8	R	8	R	-	-0.13
Pseudomoas aeruginosa	2.32	skin	>8	R	>8	R	-	-0.04
Pseudomoas aeruginosa	2.37	tissue	8	R	>8	R	-	-0.13
Pseudomoas aeruginosa	2.29	sputum	>8	R	>8	R	-	-0.14
Pseudomoas aeruginosa	2.10	drainage	>8	R	>8	R	-	-0.16
Pseudomoas aeruginosa	2.21	pus	>8	R	>8	R	-	-0.24
Pseudomoas aeruginosa	2.36	sputum	>8	R	>8	R	-	-0.09
Pseudomoas aeruginosa	2.30	blood	>8	R	>8	R	-	-0.29
Pseudomoas aeruginosa	2.44	drainage	>8	R	>8	R	-	-0.08
Pseudomoas aeruginosa	2.21	blood	>8	R	>8	R	-	-0.15
Pseudomoas aeruginosa	2.38	pus	>8	R	>8	R	-	-0.07
Pseudomoas aeruginosa	2.38	blood	>8	R	8	R	-	-0.10
Pseudomoas aeruginosa	2.27	sputum	>8	R	>8	R	-	-0.15
Pseudomoas aeruginosa	2.16	sputum	>8	R	>8	R	-	-0.09
Pseudomoas aeruginosa	2.33	pus	>8	R	>8	R	-	-0.23
Pseudomoas aeruginosa	2.41	tissue	>8	R	8	R	-	-0.29
Pseudomoas aeruginosa	2.29	sputum	>8	R	>8	R	-	-0.04
Pseudomonas aeruginosa	2.33	blood	8	R	≦0.5	S	-	-0.08
Pseudomonas aeruginosa	2.32	sputum	8	R	4	I	-	-0.04
Pseudomonas aeruginosa	2.19	blood	2	S	≦0.5	S	-	-0.12
Pseudomonas aeruginosa	2.25	blood	2	S	≦0.5	S	-	-0.01
Pseudomonas aeruginosa	2.40	exudate	2	S	≦0.5	S	-	0.14
Acinetobacter baumannii	2.34	blood	≦0.5	S	≦0.5	S	-	0.06
Acinetobacter baumannii	2.27	blood	≦0.5	S	≦0.5	S	-	0.04

Acinetobacter baumannii	2.30	sputum	≦0.5	S	≦0.5	S	-	0.00
Acinetobacter ursingii	2.32	blood	≦0.5	S	≦0.5	S	-	0.07
Acinetobacter baumannii	2.26	exudate	≦0.5	S	≦0.5	S	-	0.01
Acinetobacter sp.	1.70	sputum	≦0.5	S	≦0.5	S	-	0.12
Acinetobacter sp.	1.76	sputum	≦0.5	S	≦0.5	S	-	0.02
Acinetobacter baumannii	2.48	sputum	≦0.5	S	≦0.5	S	-	0.03
Acinetobacter baumannii	2.19	otorrhea	≦0.5	S	≦0.5	S	-	-0.04

*"Susceptible (S)", "Intermediate (I)", and "Resistant (R)" were based on CLSI document (Wayne, 2016).

1 **Table 3. The primers used for ESBL genotype characterization by PCR.**

Primer name	Sequence (5’ - 3’)	Reference
MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	Dallenne, 2010
MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	Dallenne, 2010
MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	Dallenne, 2010
MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC	Dallenne, 2010
MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	Dallenne, 2010
MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	Dallenne, 2010
MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA	Dallenne, 2010
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	Dallenne, 2010
MultiCTXMGp2_for	CGTTAACGGCACGATGAC	Dallenne, 2010
MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT	Dallenne, 2010
MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	Dallenne, 2010
MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG	Dallenne, 2010
CTX-Mg8/25_for	AACRCRCAGACGCTCTAC	Dallenne, 2010
CTX-Mg8/25_rev	TCGAGCCGGAASGTGTYAT	Dallenne, 2010

2

Table 4. The distribution of cefotaxime (CTX) and ceftriaxone (CTRX) susceptibility, ESBL-encoding genes, the normalized logRQ, and the result of ESBL disk diffusion test in isolates that showed unclear results in logRQ values with the MBT STAR-Cepha kit.

Isolate	Specimen	Susceptibil-ity to CTX		Susceptibil-ity to CTRX		PCR detection of ESBL-encoding genes	logRQ values with MBT STAR-Cepha kit	ESBL disk diffusion test
Escherichia coli	blood	>2	R*	>2	R	TEM	0.09	positive
Escherichia coli	urine	>2	R	>2	R	SHV	0.18	negative
Escherichia coli	blood	>2	R	>2	R	SHV	0.09	negative
Klebsiella oxytoca	blood	≦1	S	≦1	S	-	0.09	negative

*"Susceptible (S)", "Intermediate (I)", and "Resistant (R)" were based on CLSI document (Wayne, 2016).

Figure 1

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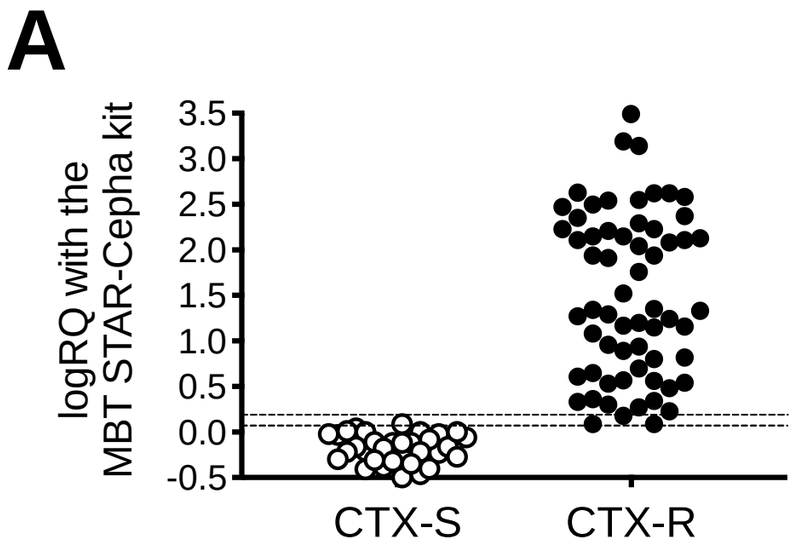

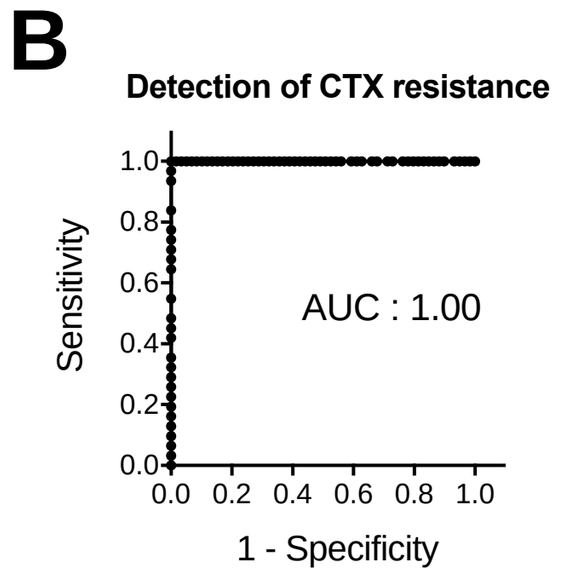

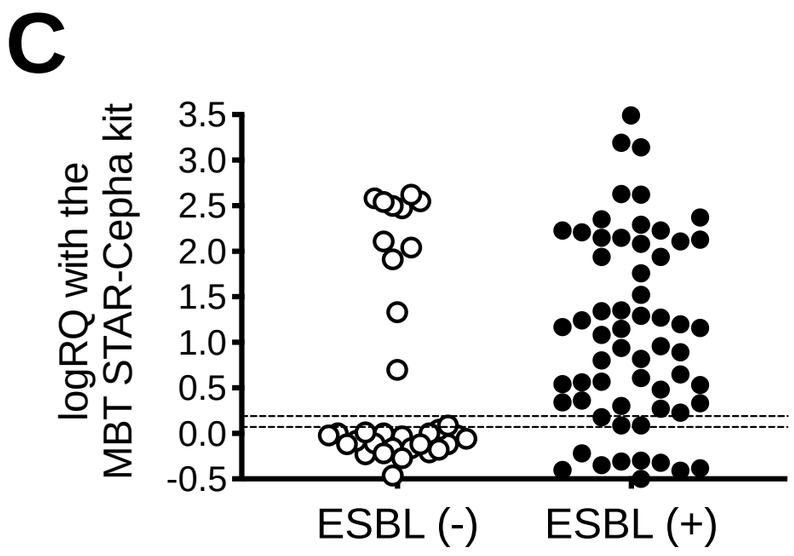

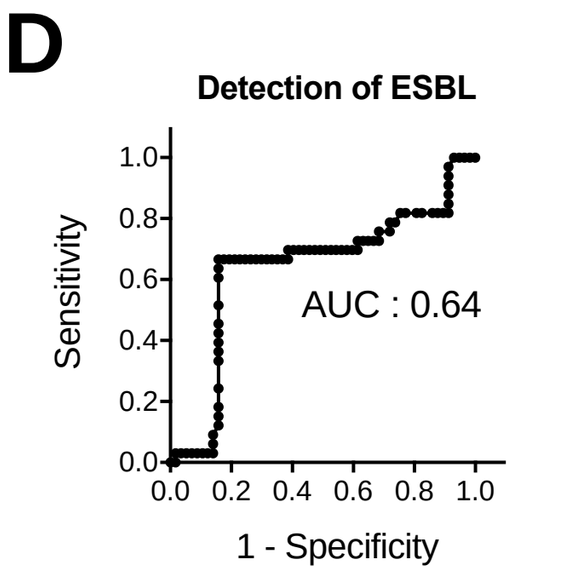

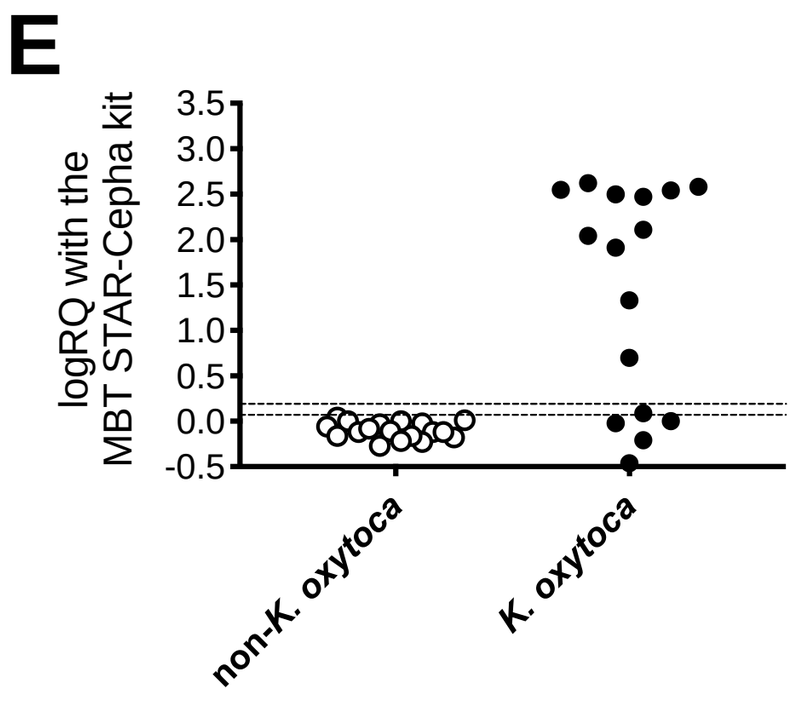

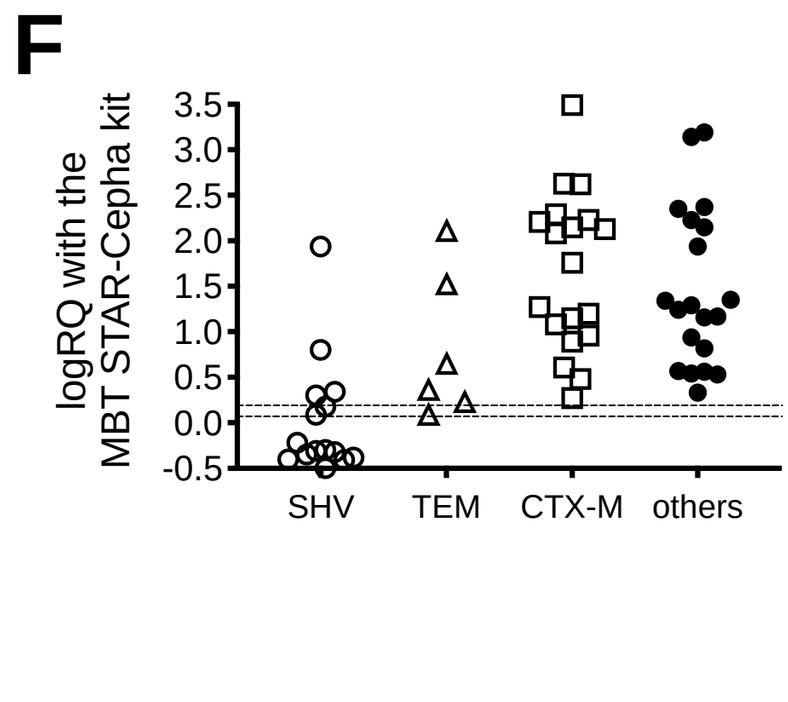

Figure 2

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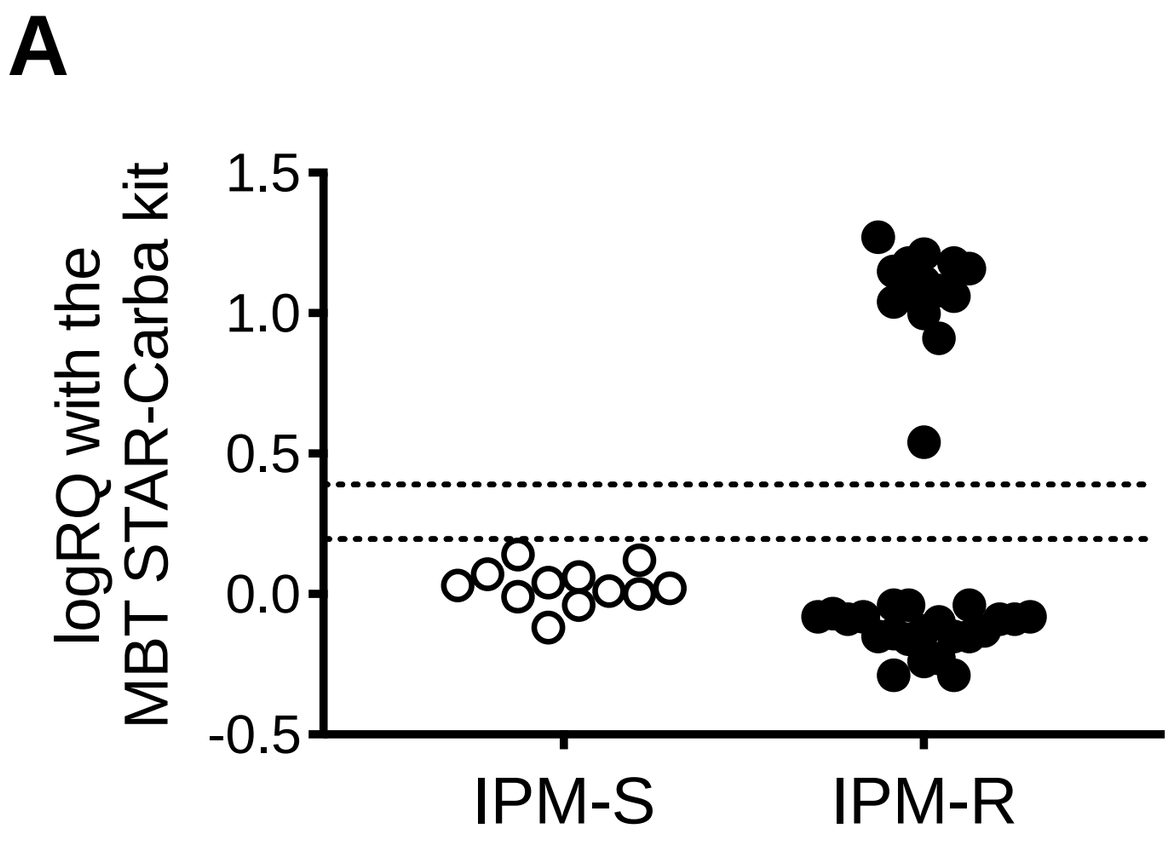

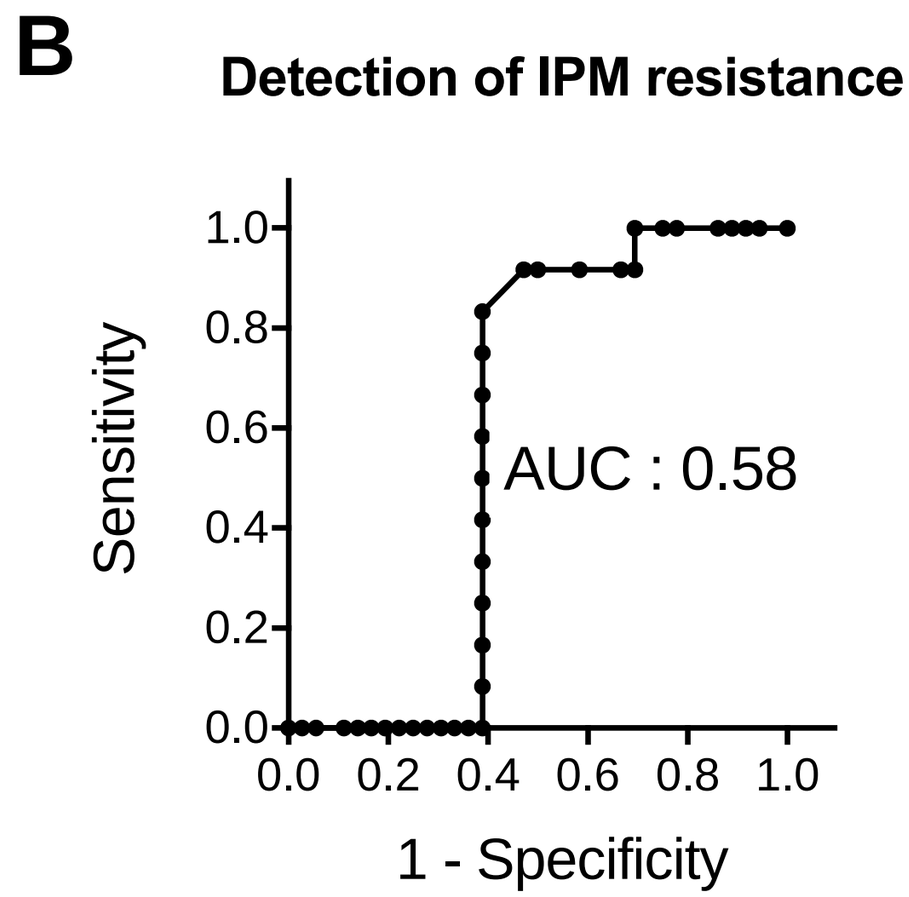

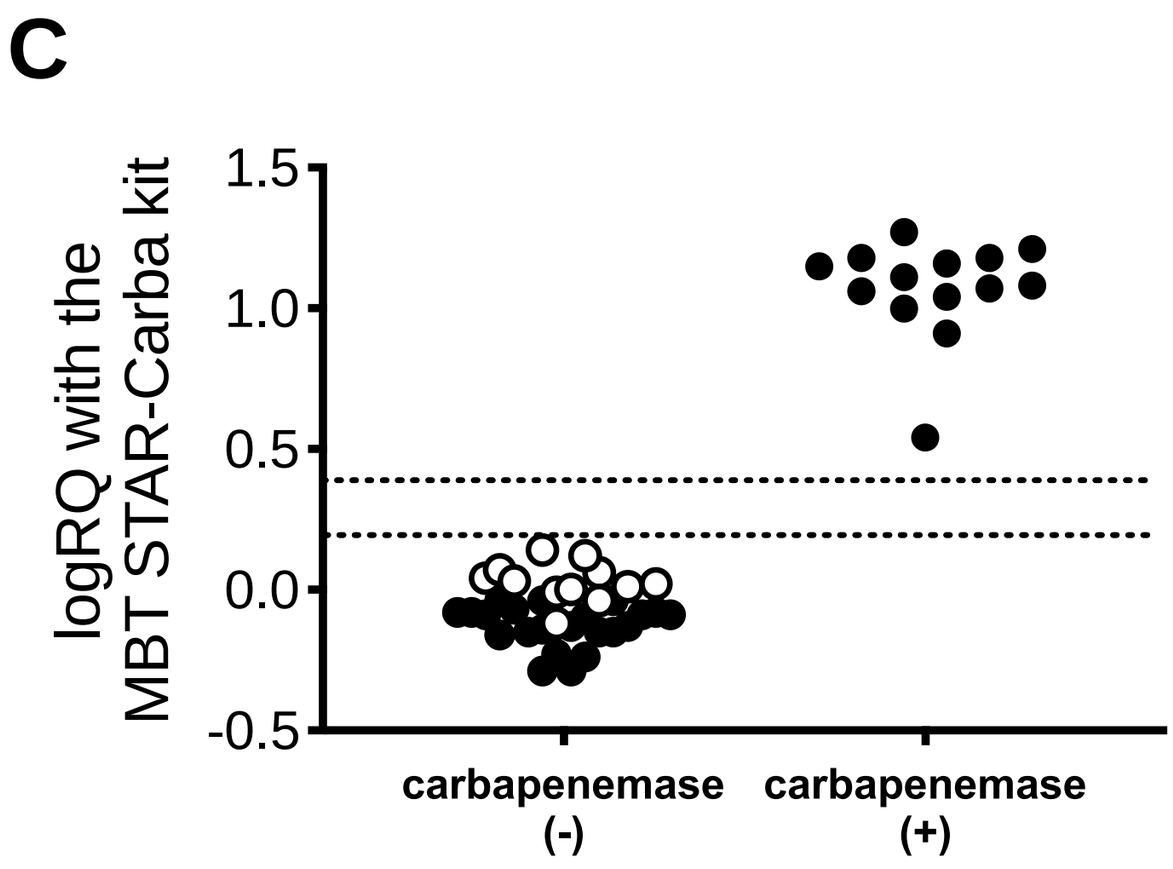

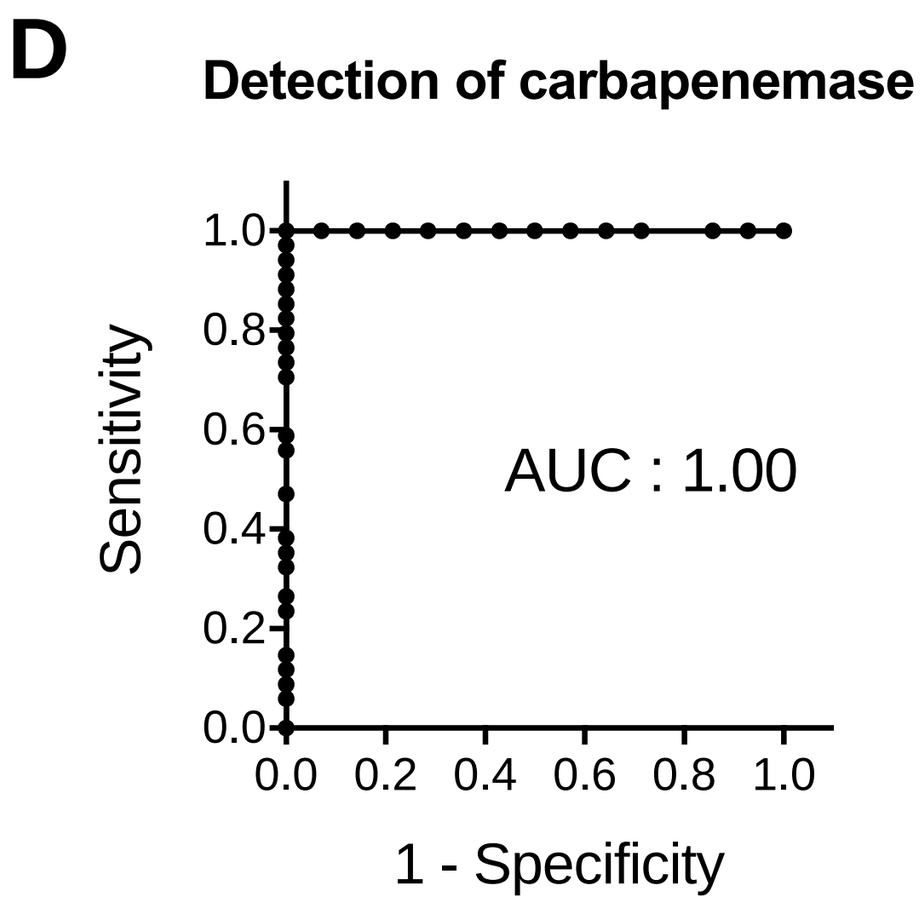

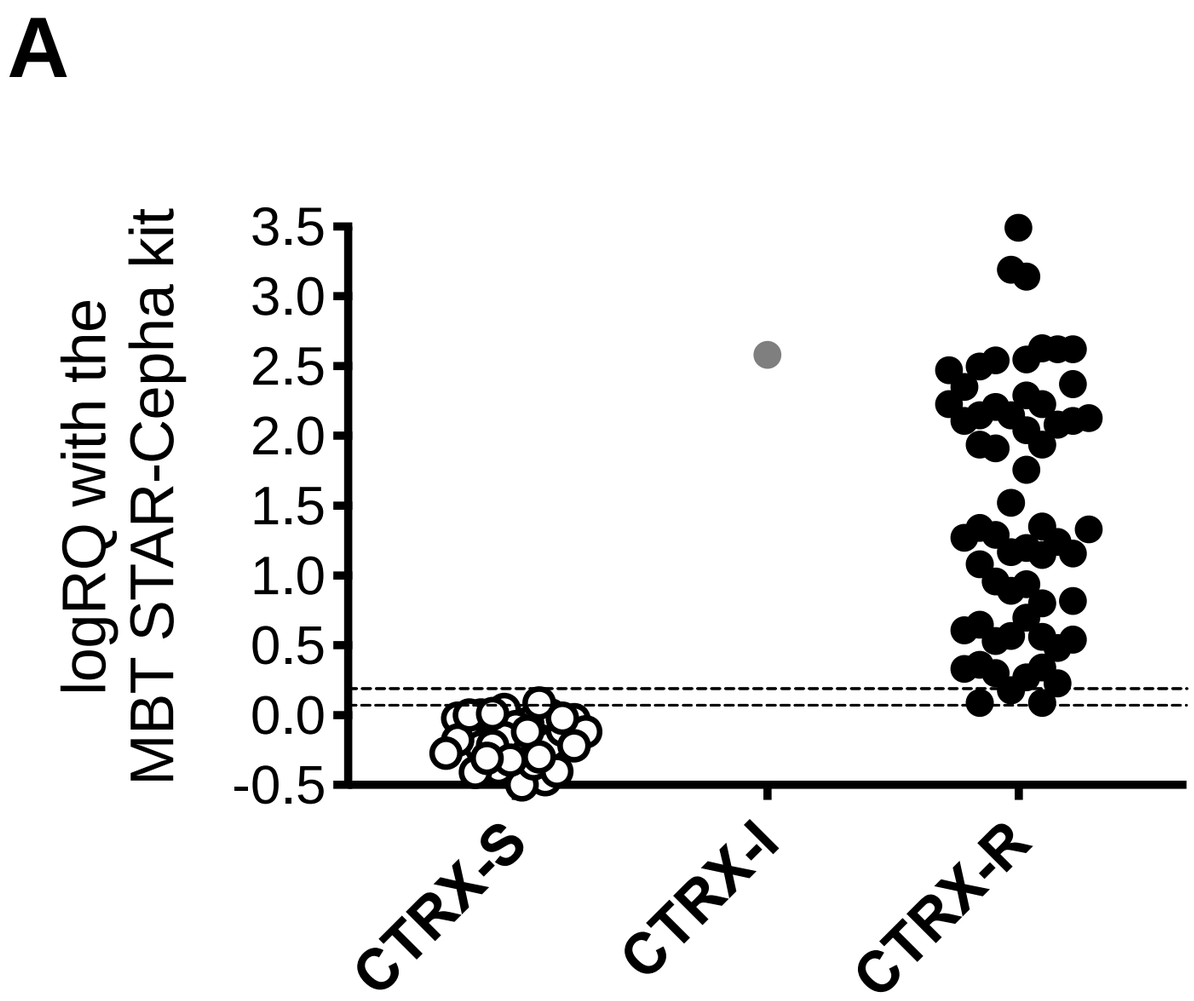

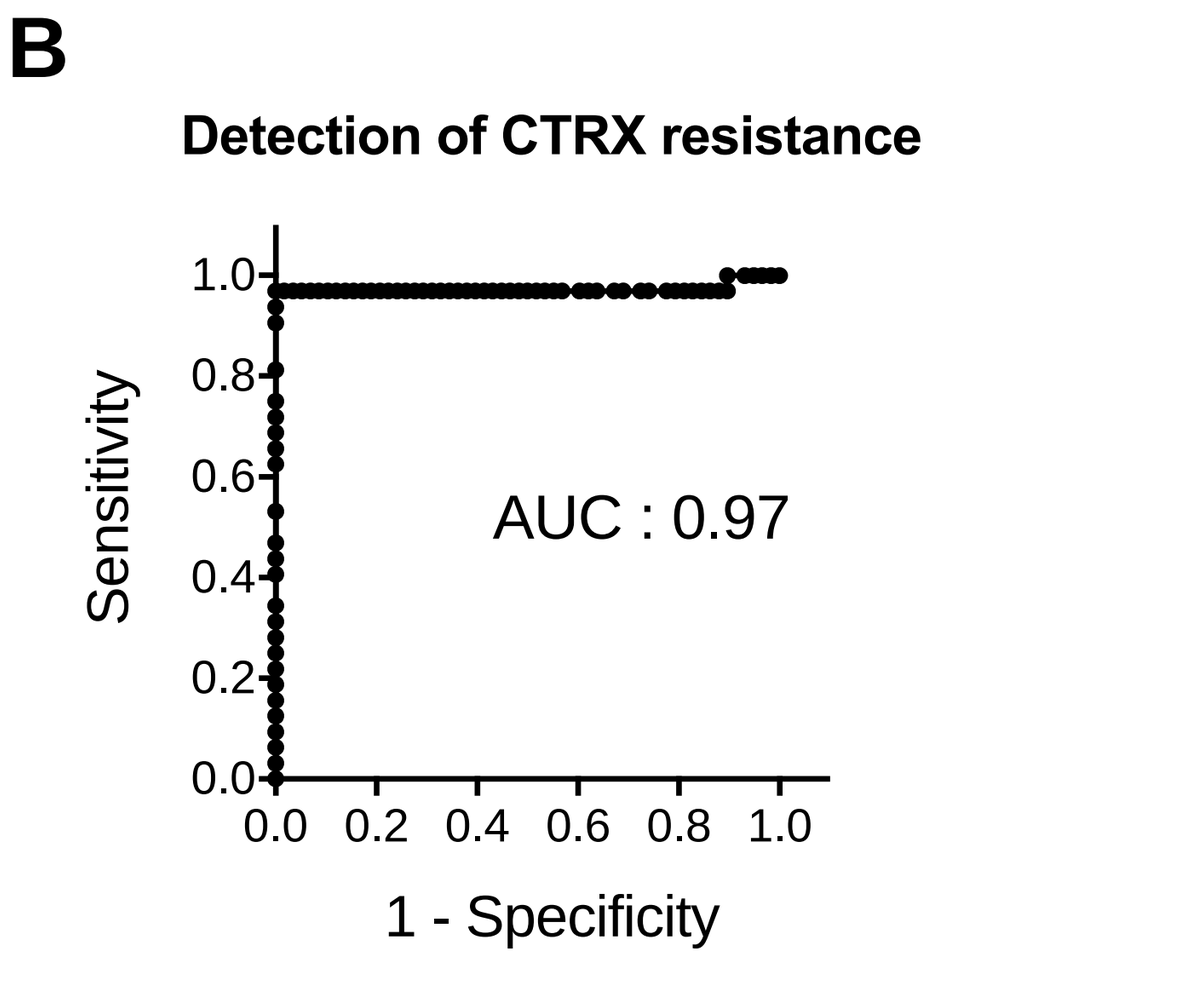

Supplementary Figure 2

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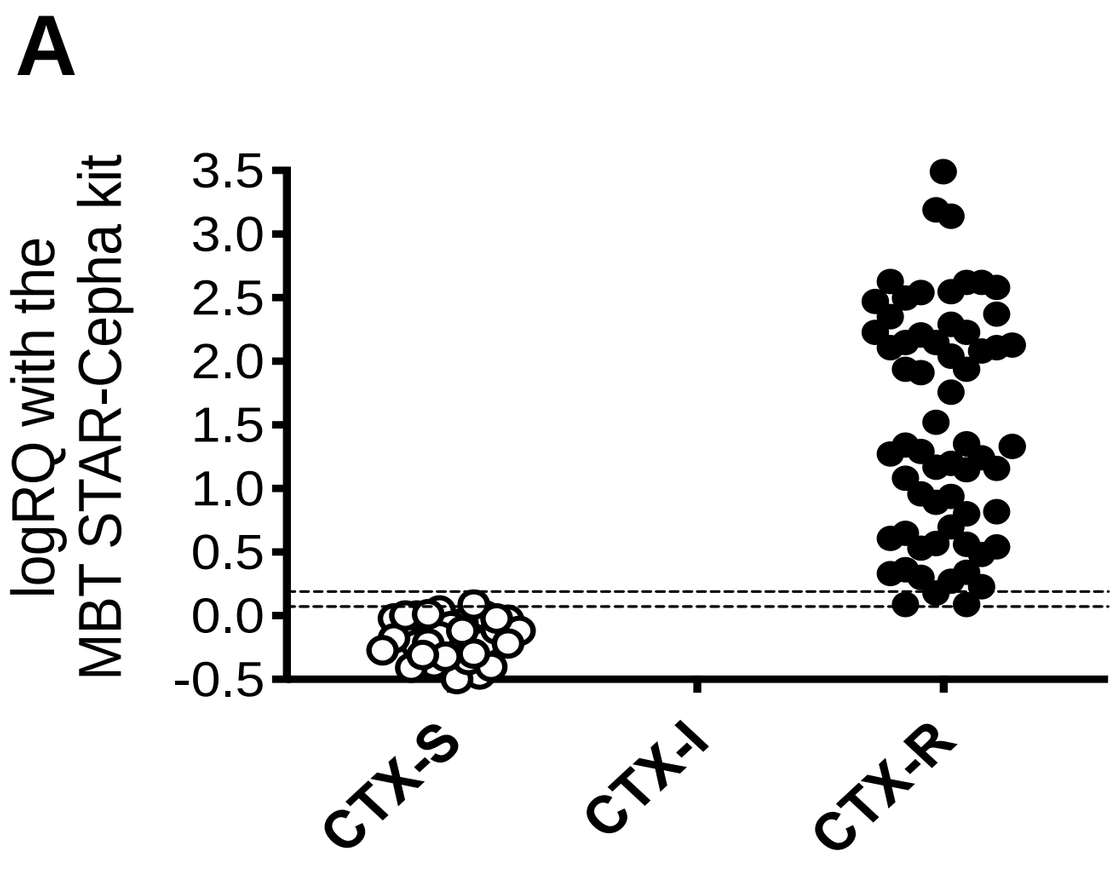

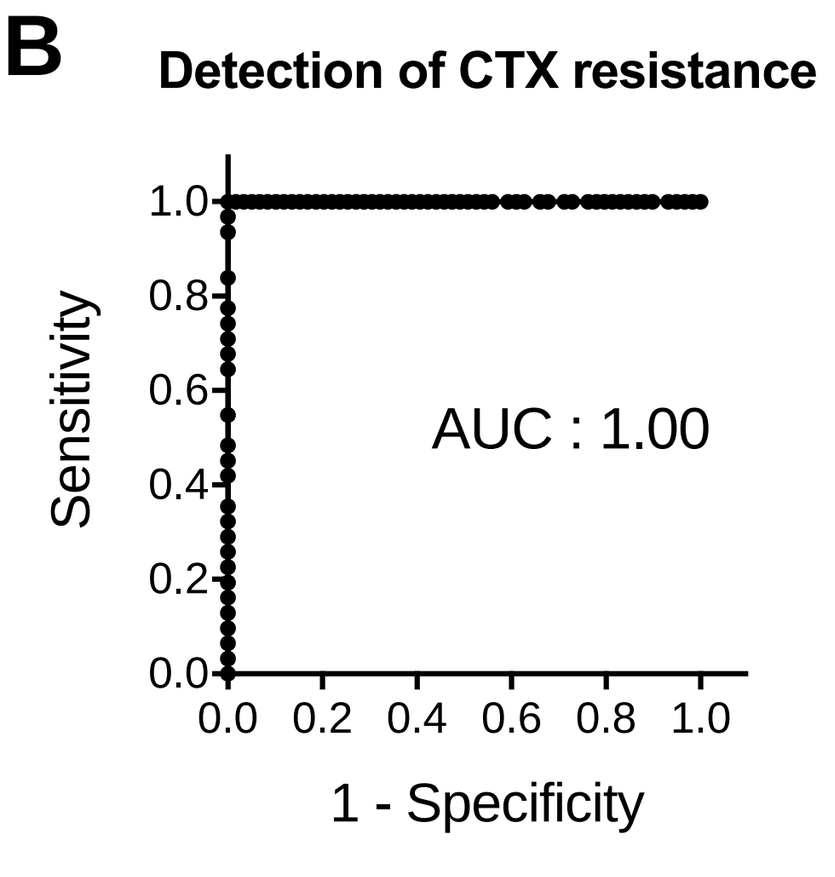

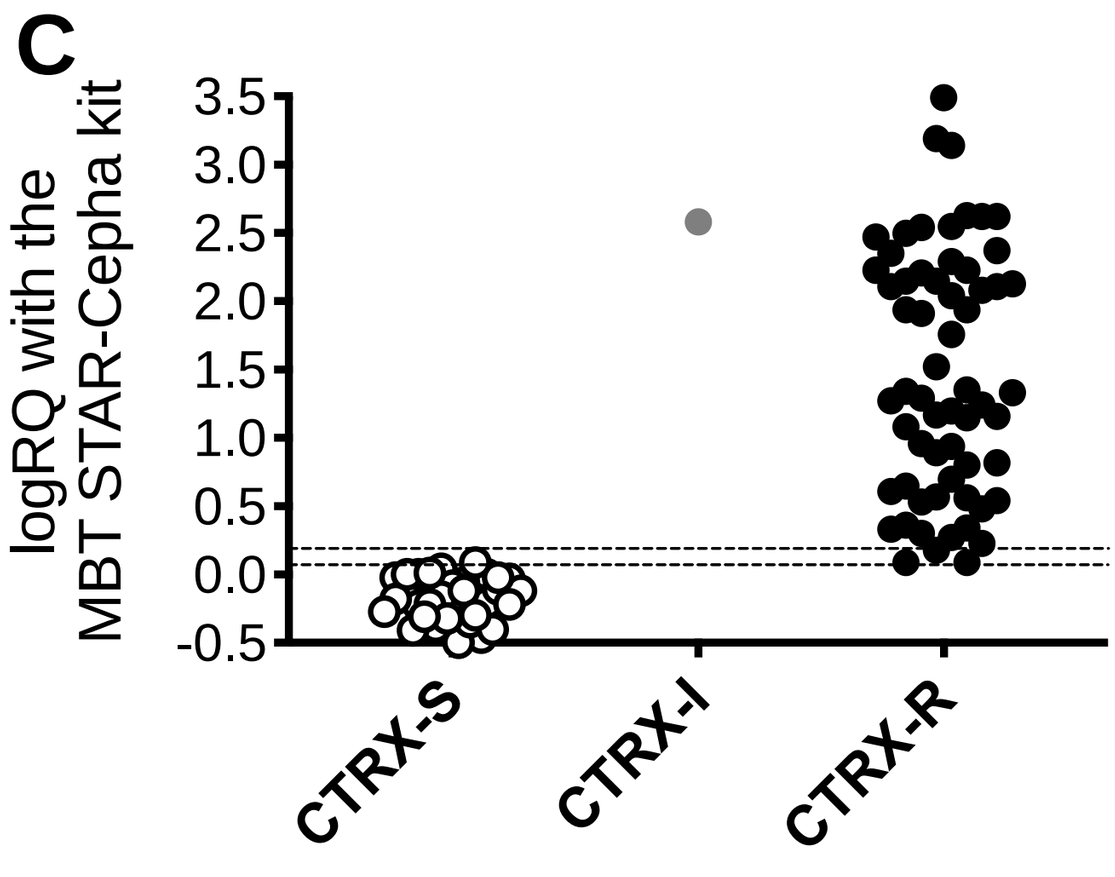

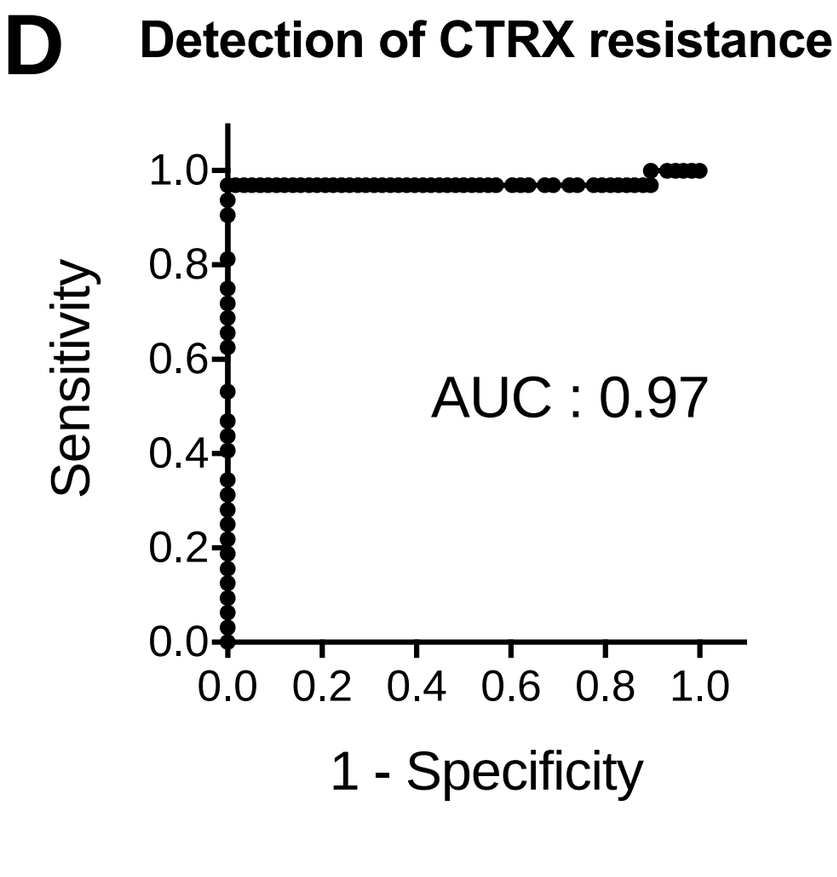

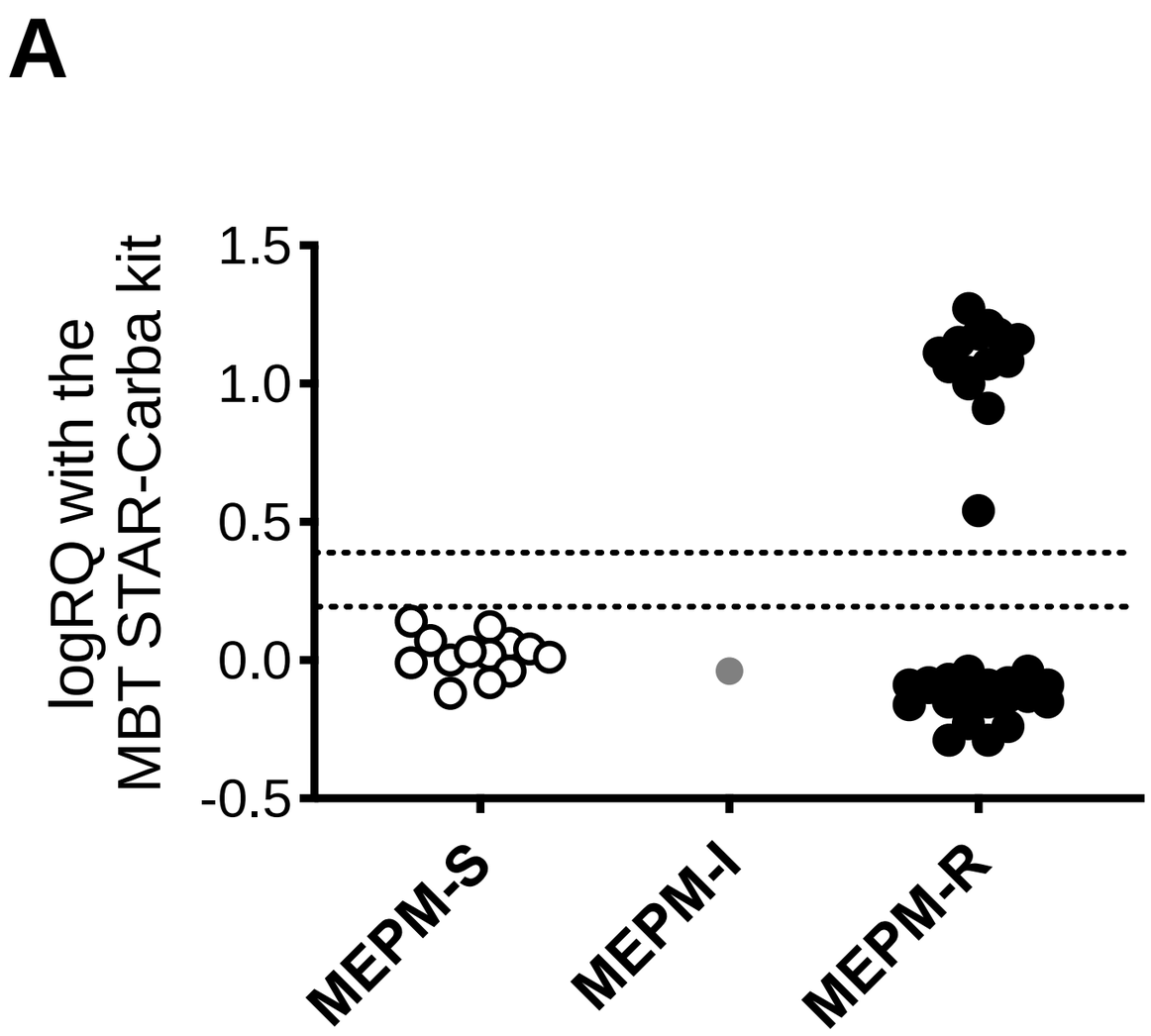

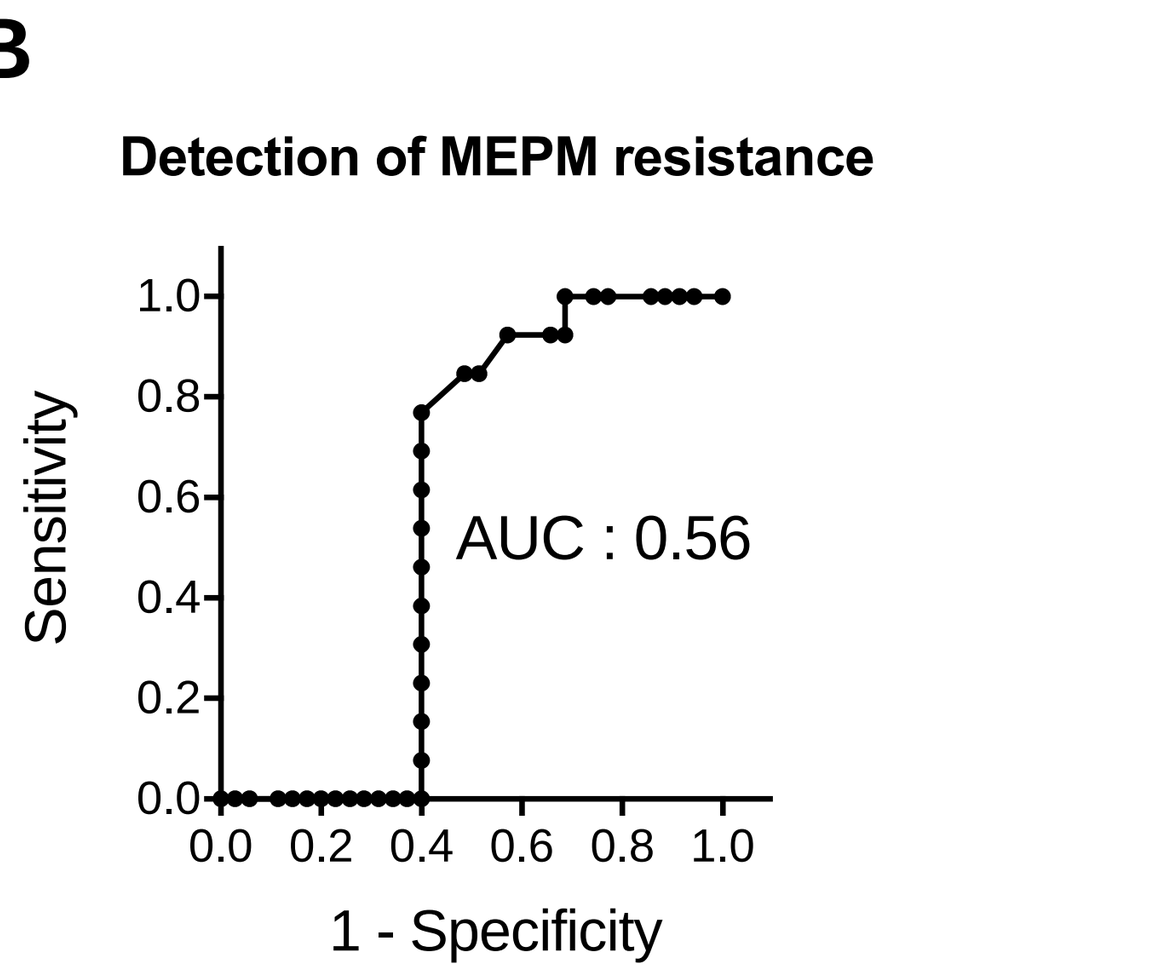

Supplementary Figure 4

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