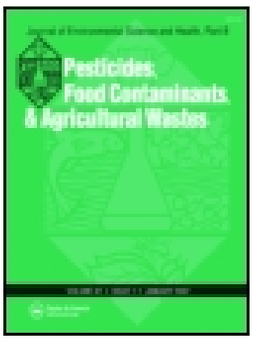

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Resistance in bacteria and indirect beta-lactamase detection in *E. coli* isolated from *Culex pipiens* detected by matrix-assisted laser desorption ionization time of flight mass spectrometry

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ABSTRACT

The aim of this study was detections of antibiotic resistance and resistance mechanism in bacteria isolated from mosquitos (*Culex pipiens*) living near humans. Therefore, antibiotic resistance in bacteria isolated from *Culex pipiens* was investigated by disk diffusion test and MIC E-test in this study. MALDI-TOF mass spectrometry was used for detection of resistant mechanism. In this study, hydrolytic breakdown products after a few hours of incubation of the bacteria isolated from *Culex pipiens* were detected. Results show that enzymatic destruction of ampicillin by beta-lactamases is able to be detected by MALDI-TOF mass spectrometry from wild strains of potential pathogens. The MALDI-TOF mass spectrometry is useful method for routine detection of beta-lactamases resistant mechanism, but overnight incubation of pure culture is necessary. The results are important for proper and fast intervention to limit the spread of beta-lactamase-producing wild bacteria and provide information for appropriate initial therapy of the infections caused by these microbes.

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KEYWORDS
Ampicillin; antibiotic resistance; *Culex pipiens*; MALDI-TOF MS

Introduction

Antibiotic resistance is an increasing problem all over the world in this time.[1] Many kinds of bacterial species are a carrier of resistant genes, which can be transferred from one species to another by several transfer mechanisms, like transduction,[2] conjugation [3] or transformation.[4] Some of these bacteria can be pathogens as genus *Escherichia* sp. and *Acinetobacter* sp., which can be transferred by several vectors.[5,6] One of these vectors can be mosquitoes (*Culex pipiens*). Mosquitoes are vectors for different kinds of bacterial species, which can spread different types of diseases caused by bacteria.[7–10] If these bacteria carry resistant genes, then diseases caused by resistant bacteria are difficult to treat and cost more.[11] There are many methods for detection of antibiotic resistance[12] and identification of potential bacterial pathogens [13] but one method, MALDI TOF Mass Spectrometry, can do both synchronously. Mass spectrometry was developed for exact detection of proteins[14] but it can be used for detection of small molecules, such as antibiotics like ampicillin.[15] Ampicillin is penicillin antibiotic that can be hydrolyzed by enzyme, which is known as beta-lactamase.[16] This enzyme cleavage, the beta-lactams core of penicillin antibiotics and antibiotics becomes ineffective.[17] Beta-lactamase is a large molecule with about 29 kDa [18] and high turnover rate of beta-lactamase is complicating for detection by MALDI TOF MS.[15] Some possibility is there, if effect of beta-lactamase will be measured indirectly. It means that measuring will focus on the breakdown products of ampicillin. Hydrolysis of penicillin antibiotics is commonly known and therefore it is possible to determine enzymatic resistance mechanism in bacteria by MALDI TOF MS too.[15] Therefore, the aim of this study was identification of bacteria isolated from mosquitoes (*Culex pipiens*), detection of antibiotic resistance, and mechanism of resistance in isolated bacterial strains by MALDI TOF mass spectrometry.

Material and methods

Bacterial isolates and antibiotic susceptibility testing

Bacterial isolates were isolated from 42 mosquitoes (*Culex pipiens*), which were collected from western part of Slovakia in 2015. For isolation of bacteria MacConkey agar (Biolife, Italy), 35 ± 2°C during 24 h was used. Recultivation and purification was done on the same agar at the same conditions. Isolated and purified species were identified by using MALDI TOF MS (Bruker Daltonics, Germany, Maldi Biotyper). After identification, bacterial isolates were tested for antibiotic resistance by disk diffusion method and MIC method against antibiotics as: ampicillin* (AMP), piperacillin (PIP), cefuroxime (CXM), cephalexin (CFL), cefepime (FEP), doripenem (DOR), imipenem* (IMI), ertapenem (ETP), meropenem* (MEM), ciprofloxacin* (CIP), levofloxacin* (LEV), norfloxacin (NOR), ofloxacin (OFL), netilmicin (NET), tobramycin (TOB 10), amikacin* (AMI), erythromycin* (ERY), chloramphenicol (CHL), metronidazole* (MET), mupirocin (MUP) and tigecycline* (TGC).

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All antibiotic disks were obtained from OXOID, UK and strips indicated with asterisk* were obtained from OXOID, UK and other from HiMedia, India. Antibiotic resistance procedures, usage of antibiotic disks, MIC strips were depended on bacterial strain. Concentration of the strips and content of antibiotic in disks were established and it follows EUCAST.[19] Interpretation of inhibition zones and minimal inhibition concentrations were done by EUCAST.[20]

MALDI TOF MS analysis of ampicillin

For this experiment, commercially available ampicillin trihydrate (Sigma Aldrich, Germany) was used. Different concentrations of ampicillin diluted in 20 mM Tris-HCl buffer, pH 7 (Sigma Aldrich, Germany) were used for detection by MALDI TOF MS. Ampicillin was diluted in pure MS water 99.9% (Sigma Aldrich, Germany) and pure MS ethanol 99.8% (Sigma Aldrich, Germany) for observation. As matrix was used 2.5 mg α-cyano-4-hydroxycinnamic acid (HCCA) and 2.5 mg dihydroxybenzoic acid (DHB) diluted in 250 μL organic solvent (OR). Organic solvent contained: 500 μL of 100% acetonitril, 475 μL of distilled water and 25 μL of 100% tri-fluoracetic acid (all chemicals were obtained from Sigma Aldrich, Germany in MS purity). One microliter of sample (ampicillin in different concentration diluted in Tris-HCl) was applied on the target plate (Bruker Daltonics, Germany, MSP Target) and allowed to dry at a room temperature. Dried sample was covered by matrix (HCCA). Mass spectra were acquired using a Microflex LT mass spectrometer with the flexControl 3.0 software (Bruker Daltonics, Germany) operating in positive linear ion mode between *m/z* 300 and 460. Microflex LT was set using flexControl software in following parameters: ion source 1: 20 kV, ion source 2: 16.7 kV, lens: 7 kV, pulsed ion extraction: 170 ns, detection gain: 3.0x, electronic gain: regular, mode: low range, mass range selection: low range, laser frequency: 60 Hz, digitizer trigger level: 2.500 mV, laser attenuator: 24%, laser attenuator: 30%, laser range: 70–90%. Spectra were measured randomly by 500 laser shots. Initial concentration of ampicillin was 10 mg mL^{-1} reduced by 10% each time. Ampicillin was degraded by 100 mM NaOH and diluted in 20 mM Tris-HCl to final concentration of 10 mM.

Ampicillin hydrolysis assay

Purified bacterial strains isolated from mosquitoes were incubated overnight in Mueller-Hinton broth (Biolife, Italy) to increase density. One mL of high density inoculum, 4 McF°, was pipetted into the Eppendorf tube and centrifuged at the maximum speed (14,000 rpm). Pellet was purified by using physiological solution that was prepared in 1 mL of 20 mM Tris-HCl buffer (pH 7) with 150 mM NaCl, and then centrifuged at the maximum speed for 3 min. The supernatant was discarded. 50 μL of 50% ampicillin diluted in 20 mM Tris-HCl buffer (pH 7) was added to the pellet and mixed by pipette. Afterward, the resulting mixture was incubated at 35 ± 2°C for 3 h. After 3 h of incubation, mixture was centrifuged at the same conditions and 1 μL of supernatant was analyzed by MALDI TOF MS.

Analysis of spectra

For spectra analyzing, flexAnalysis 3.0 software (Bruker Daltonics, Germany) was used. Detection of peaks was done by Centroid detection algorithm with a signal-to-noise threshold of 1, a relative intensity threshold of 0%, a minimum intensity threshold of 0, a peak width of 0.2 *m/z*, a height of 80%, a TopHat baseline subtraction, smoothing with the Savitzky-Golay algorithm, a width of 0.2 *m/z*, and cycle of 1.[21] Theoretical peaks of ampicillin, degradation products, and its sodium salts were compared with our detected masses with a ±0.6 *m/z*.

Calibration of MALDI TOF MS

Calibration of MALDI TOF MS in this experiment was done by using ampicillin and its degradation products and sodium salts from artificial hydrolysis of ampicillin by NaOH.

Results

Identified bacteria and its resistance

In this work, four species that grew on MacConkey agar were identified, such as: *Staphylococcus hominis* (5 isolates), *Escherichia coli* (42 isolates), *Pantoea agglomerans* (40 isolates) and *Acinetobacter pittii* (42 isolates). After antibiotic susceptibility testing, resistance in these bacterial strains against ampicillin (*Escherichia coli*), doripenem (*A. pittii, Pantoea agglomerans*), erythromycin (*S. hominis*) and piperacillin (*P. agglomerans*) were detected. All antibiotic tests showed inhibition zones around the disks expect *Escherichia coli* with ampicillin. In this case, no inhibition zone was created. Therefore, *E. coli* for MALDI TOF MS analysis was selected. The following table (Table 1) shows results from susceptibility testing by disk diffusion method.

MIC distribution and antimicrobial susceptibility of bacteria isolated from *Culex pipiens* are summarized in Table 2. Minimal inhibition susceptibility testing shows that five isolates of *Acinetobacter pittii* and two isolates of *Pantoea agglomarans* were resistant against doripenem. *Pantoea agglomerans* was resistant against piperacillin and cefuroxime. Probably, *Staphylococcus hominis* was secondary contamination or transferred from human. In this

Table 1. Antibiotic susceptibility testing of isolates from *Culex pipiens*.

		Susceptibility testing	
Bacterial strain	No. of isolates	Resistant[No. of isolates]	Sensitive
Acinetobacter pittii	42	DOR[5]	TOB, NET, LEV, AMI, CIP, MEM, IMI
Pantoea agglomerans	40	DOR[2], PIP[3], CXM[1]	AMI, CHL, AMP, OFL, NOR, IMI, LEV, MEM, FEP, CFL, ETP, CIP
Escherichia coli	42	AMP[12]	PIP, FEP, CXM, CFL, DOR, ETP, IMI, MEM, CIP, NOR, AMI, NET, TOB, LEV, OFL
Staphylococcus hominis	5	ERY[1]	LEV, NET, AMI, MUP, TGC, TOB, CIP, CHL

Legend: DOR: doripenem, TOB: tobramycin, NET: netilmicin, LEV: levofloxacin, AMI: amikacin, CIP: ciprofloxacin, MEM: meropenem, IMI: imipenem, CHL: chloramphenicol, FEP: cefepime, CFL: cephalexin, ETP: ertapenem, PIP: piperacillin, NOR: norfloxacin, OFL: ofloxacin, MUP: mupirocin, TGC: tigecycline, ERY: erythromycin, AMP: ampicillin, CXM: cefuroxime.

Table 2. MIC distribution and antimicrobial susceptibilities of bacteria isolated from *Culex pipiens*.

		Concentration of antibiotics in strips in μg mL^{-1}																		
Antibiotics	Bacterial strain	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
Doripenem	*Acinetobacter pittii*					4	2	5	11	10	3	2	**4**	**1**						0.25	2
	Pantoea agglomerans					2	6	10	7	8	3	2	**2**							0.125	1
	Escherichia coli			2	11	23	4	2												0.032	0.064
Meropenem	*Acinetobacter pittii*							1	2	7	15	12	5							1	2
	Pantoea agglomerans			2	8	19	9	2												0.032	0.064
	Escherichia coli			4	16	13	7	1	1											0.016	0.064
Imipenem	*Acinetobacter pittii*					1	6	14	15	5	1									0.125	0.25
	Pantoea agglomerans						1	3	11	20	5									0.25	0.5
	Escherichia coli			4	3	6	5	16	4	3	1									0.064	0.25
Ertapenem	*Pantoea agglomerans*		1	3	18	8	5	5												0.016	0.064
	Escherichia coli		3	16	12	7	4													0.008	0.032
Ampicillin	*Pantoea agglomerans*											2	20	18						4	8
	Escherichia coli									1	5	8	10	6	**4**	**5**	**2**	**1**		4	32
Piperacillin	*Pantoea agglomerans*									4	7	9	12	5			**1**	**2**		2	8
	Escherichia coli								1	5	9	13	10	4						2	4
Cefalexin	*Pantoea agglomerans*											2	8	17	13					8	16
	Escherichia coli											4	15	21	2					4	8
Cefepime	*Pantoea agglomerans*						4	29	3		2	2								0.125	0.5
	Escherichia coli			1	4	9	11	8	6	3										0.064	0.25
Cefuroxime	*Pantoea agglomerans*									2	3	6	18	10	**1**					4	8
	Escherichia coli									1	5	12	16	8						2	4
Netilmicin	*Acinetobacter pittii*					3	7	21	9	2										0.125	0.25
	Escherichia coli						3	3	15	18	3									0.25	0.5
	Staphylococcus hominis				1	1	2	1												0.032	0.064
Tobramycin	*Acinetobacter pittii*							2	2	7	19	12								1	1
	Escherichia coli							2	13	23	4									0.5	0.5
	Staphylococcus hominis					1	2		1	1										0.064	0.25
Amikacin	*Acinetobacter pittii*									4	7	19	9	3						2	4
	Escherichia coli								1	2	7	16	12	4						2	4
	Pantoea agglomerans									5	9	15	11							1	4
	Staphylococcus hominis									1	1	3								1	2
Ciprofloxacin	*Acinetobacter pittii*			2	3	7	9	15	5	1										0.064	0.25
	Escherichia coli	1	6	12	17	4	2													0.008	0.032
	Staphylococcus hominis						1	4												0.125	0.125
Levofloxacin	*Acinetobacter pittii*				3	5	12	14	8											0.064	0.25
	Escherichia coli				1	23	16	2												0.032	0.064
	Pantoea agglomerans			1	5	13	20	1												0.032	0.064
	Staphylococcus hominis					1	1	3												0.064	0.125
Norfloxacin	*Escherichia coli*				2	3	21	14	2											0.064	0.125
	Pantoea agglomerans				1	8	15	16												0.064	0.125
Ofloxacin	*Escherichia coli*				1	11	18	8	4											0.064	0.125
	Pantoea agglomerans				2	4	21	10	3											0.064	0.125
Erythromycin	*Staphylococcus hominis*						1	2	1					**1**						0.125	0.25
Tigecycline	*Staphylococcus hominis*				1	1	2	1												0.032	0.064
Chloramphenicol	*Pantoea agglomerans*										1	4	19	16						4	8
	Staphylococcus hominis											1	3	1						4	8
Mupirocin	*Staphylococcus hominis*						1	1	3											0.125	0.25

Bolded values show the numbers of resistant bacteria isolated in this experiment findings according EUCAST.[20]

study, five strains were found and one isolate was resistant against erythromycin only. The most resistant isolate was *Escherichia coli*, which was resistant against ampicillin. For MALDI-TOF MS analysis, the resistant and susceptible *E. coli* was used.

Analysis of ampicillin by MALDI-TOF mass spectrometry

Matrix HCCA and DHB were used in this experiment. The spectra of pure HCCA matrix were identified in 189 *m*/*z* and 378 *m*/*z* and it was obtained by using 10 mg mL^{-1} of α-hydroxy-4-cinnamic acid (HCCA) in pure distilled H_2O for mass spectrometry. The spectra of pure DHB matrix were identified in 155 *m*/*z* and 309 *m*/*z* and obtained by using 10 mg mL^{-1} of dihydroxybenzoic acid (DHB) in pure distilled H_2O for mass spectrometry. Also native pure ampicillin (348.236 *m*/*z*) and its sodium salt (370.832 *m*/*z*) were observed (Fig. 1A). The minimal concentration of ampicillin that can be observed by MALDI-TOF MS was determined at 20 μg mL^{-1}. Hrabák et al. [21] observed meropenem in native form and its hydrolyzed products with using MALDI-TOF MS. They dissolved different matrix, 2.5-dihydroxybenzoic acid (DHB) in 50% ethanol and they observed meropenem and its sodium salts. Also, they determined the lowest limiting concentration for meropenem detection (19.173 mg L^{-1}) by MALDI-TOF MS. Theoretically, the modification of ampicillin molecule produced by beta-lactamases is similar to the one produced by hydrolysis of NaOH. NaOH alkaline hydrolysis produced several following products: hydrolyzed ampicillin (366.754 *m*/*z*), hydrolyzed ampicillin sodium salt (389.380 *m*/*z*) and hydrolyzed ampicillin disodium salt (412.251 *m*/*z*). Equally, hydrolyzed ampicillin subject to spontaneous decarboxylation and its produced hydrolyzed decarboxylated ampicillin (323.909 *m*/*z*) and hydrolyzed decarboxylated ampicillin sodium salt (344.359 *m*/*z*) (Fig. 1). Sparbier et al.[15] observed

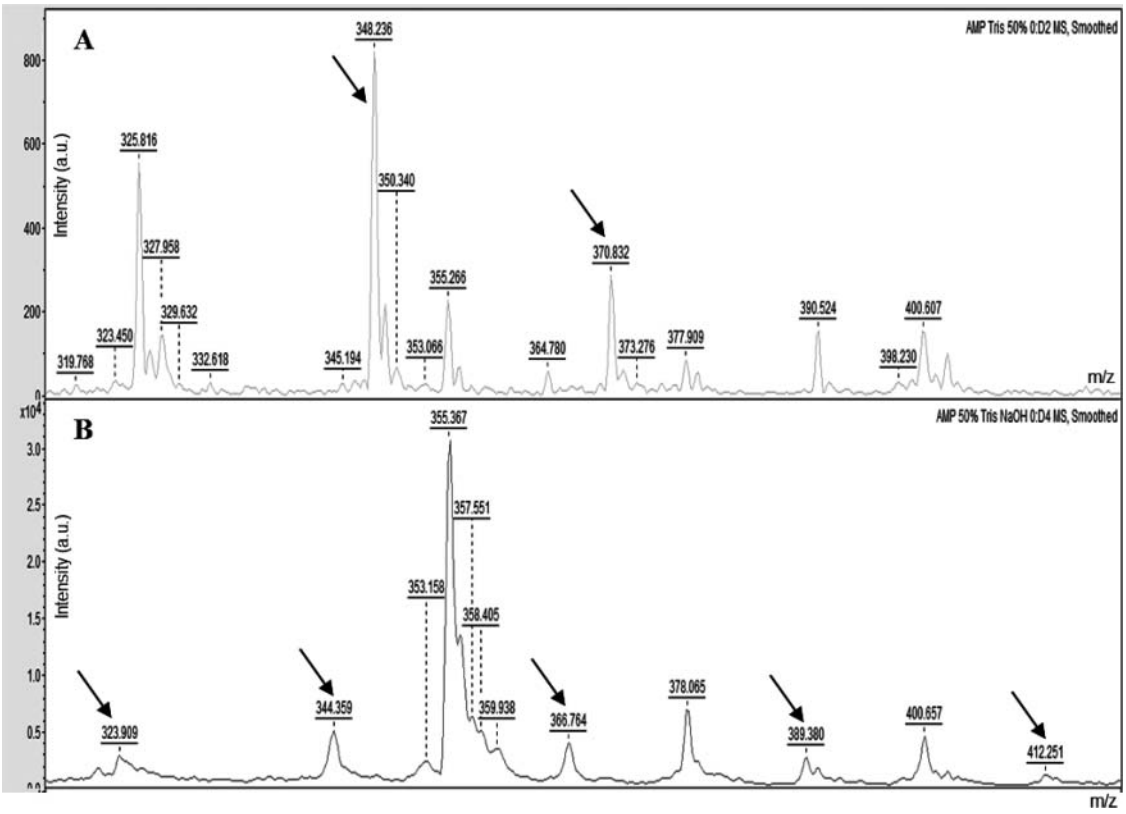

Figure 1. Mass spectra of ampicillin (detected mass 348.236 *m/z*) and its sodium salt (370.832 *m/z*) (A) and ampicillin with disrupted amide bond (366.754 *m/z*) by alkaline hydrolysis (NaOH), its sodium salts (monosodium salt 389.380 *m/z*, disodium salt 412.251 *m/z*), spontaneous decarboxyled ampicillin (323.909 *m/z*) and decarboxyled ampicillin sodium salt (344.359 *m/z*) (B). Tested peaks are indicated with arrows.

different type of antibiotics, which can be hydrolyzed by NaOH and beta-lactamases and determined following mass spectra for ampicillin and its sodium salt and hydrolyzed products: ampicillin + H (350.1 *m/z*), ampicillin sodium salt (372.1 *m/z*), ampicillin disodium salt (394.1 *m/z*), hydrolyzed ampicillin (367.9 *m/z*), hydrolyzed ampicillin sodium salt (389.9 *m/z*), hydrolyzed ampicillin disodium salt (411.9 *m/z*) and decarboxylated hydrolyzed ampicillin (324.0 *m/z*). In contrast with their results, decarboxylated hydrolyzed ampicillin sodium salt (344.359 *m/z*) was observed too.

Ampicillin hydrolysis assay

Ampicillin hydrolysis assay was validated by 42 strains. Twelve isolated strains of *Escherichia coli* from *Culex pipiens* were producers of beta-lactamases. Thirty strains of *Escherichia coli* did not produce any beta-lactamases. The both groups were divided according to the EUCAST breakpoint table.[20] The non-beta-lactamases producing bacteria (*E. coli*) were incubated for 3 h in buffered ampicillin solution. Peaks representing ampicillin and its sodium salt were detected as ampicillin (348.363 *m/z*) and its sodium salt (370.983 *m/z*). The peaks of beta-lactamase hydrolysis were not observed for all (30 isolates) *Escherichia coli* strains isolated from *Culex pipiens* (Fig. 2). Using software flexControl to detect the peaks, the peak corresponding to intact ampicillin was not presented in all (12 isolates of *E. coli*) beta-lactamase producing isolates isolated from *Culex pipiens*. In beta-lactamase-producing isolates, the observed peaks corresponded to 366 *m/z* (hydrolyzed ampicillin), 389 *m/z* (hydrolyzed ampicillin sodium salt), 412 *m/z* (hydrolyzed ampicillin disodium salt), 324 *m/z* (spontaneous decarboxylated ampicillin with disrupted amide bond) and 344 *m/z* (spontaneous decarboxylated ampicillin sodium salt with disrupted amide bond) in all present isolates of *Escherichia coli* isolated from *Culex pipiens*.

Discussion

Routine diagnostics of bacteria in clinical microbiology laboratories has been developed long time ago what resulted into MALDI-TOF mass spectrometry.[22,23] Primary investment is high but routine usage of MALDI-TOF MS is very quick and cheap.[24,22] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently become a standard method in many microbiological laboratories for identification of human bacterial pathogens. Identification of cultivated organisms by MALDI-TOF MS is based on the generation of mass spectra obtained from colony material, which are compared to the spectra of known species in a reference library.[25,26] MALDI-TOF mass spectrometry has been widely used for the analysis of biomolecules with high molecular weights.[27–29] For the analysis of small molecules with MALDI-TOF mass spectrometry, various types of solid matrices have been reported, which could ionize analytes without producing the mass peaks of matrix molecules. Previously, metal nanoparticles,[30–33] semiconductor nanowires,[34,35] two-dimensional nanostructures [36] and carbon

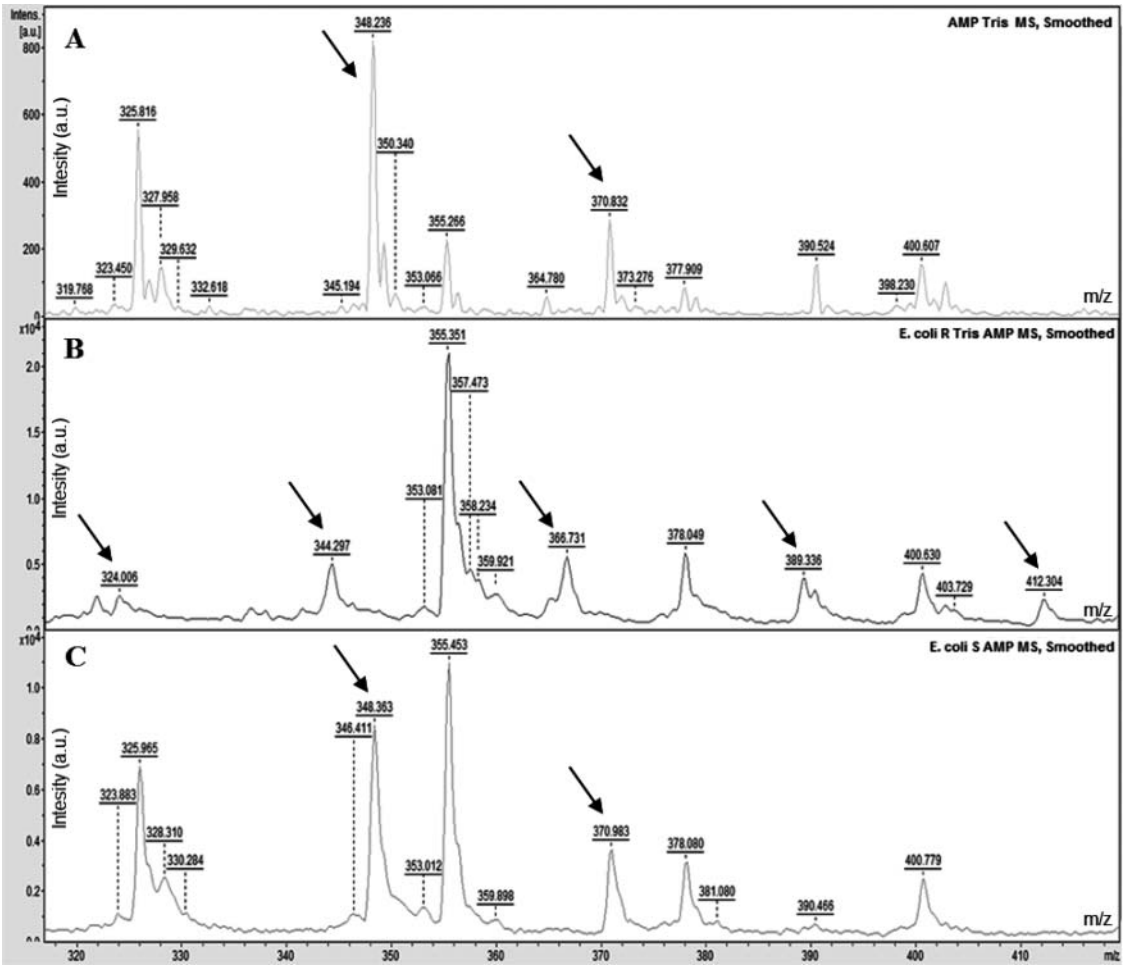

Figure 2. Mass spectra of ampicillin hydrolysis assay. (A) Ampicillin and its sodium salt, (B) beta-lactamase producing *Escherichia coli* with degradation products and (C) non-beta-lactamase producing *Escherichia coli* without degradation products. Corresponding peaks are indicated with arrows.

nanotubes [37] have been reported to be used as solid matrices in MALDI-TOF mass spectrometry. Therefore, in this study ampicillin resistance by disk diffusion method in wild bacteria isolated from *Culex pipiens* and detected ampicillin were determined. Also, its sodium salt and hydrolyzed products, sodium salts hydrolyzed by alkaline and beta-lactamases, which are produced by ampicillin resistant bacteria isolated from these mosquitoes were observed. In this study, *Escherichia coli* that is potential human pathogen and carrier of ampicillin resistance was identified. Many authors have confirmed that *E. coli* cause nosocomial infections [38–40] and bacteremia.[41] Ampicillin resistance by disk diffusion method and MIC E-tests was determined. Detection of resistance mechanism of samples was observed by using MALDI-TOF MS. Our results showed that HCCA matrix can be used to visualize ampicillin, its sodium salt and hydrolyzed ampicillin and its sodium salts mass spectra. Visualization of ampicillin was positive in the all tested solvents (ethanol, water and Tris-HCl buffer solution). Tested DHB matrix and its mass spectra had significant noise background. Therefore, interpretation of the results was very difficult. Conversely, Hrabák et al.[21] used DHB matrix for successful visualization of carbapenems antibiotics as meropenem. Before the main experiment, where beta-lactamase hydrolysis was tested, alkaline hydrolysis by NaOH was used as calibration. These calibration results showed that alkaline hydrolysis can be useful method for mass spectrum analysis. Therefore, MALDI-TOF mass spectrometry may be used for detection of ampicillin, its sodium salt, hydrolyzed ampicillin and its sodium salts with using HCCA matrix and resistant mechanism based on enzymatic destruction by beta-lactamases can be detected indirectly. More authors demonstrated that MALDI-TOF mass spectrometry may be useful equipment for detection of small molecules as antibiotics. Also, there is hope that MALDI-TOF mass spectrometry will be suitable method not only for research but also for routine microbiological diagnosis.

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