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Prophylactic application of antibiotics selects extended-spectrum β-lactamase and carbapenemases producing Gram-negative bacteria in the oral cavity

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Significance of the study

It is well known that surgical preoperative prophylaxis with broad spectrum antibiotics can select multidrug-resistant Gram-negative bacteria and change the oral microbiome. This prompted us to analyze oral microbiome before and after prophlylactic dose of antibiotics.

The main finding of the study is that the prophlylactic application of antibiotics is associated with the colonization of oral cavity with Gram-negative bacteria.

Marked diversity of Gram-negative bacteria and resistance mechanisms was found. High resistance rates and acquired extended-spectrum β-lactamases (ESBL) and carbapenemase encoding genes were found among *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates. The dominant resistance mechanisms were the production of ESBLs and carbapenemases.

Abstract

Prophylactic administration of broad-spectrum antibiotics in surgery can change the oral microbiome and induce colonization of oral cavity with Gram-negative bacteria including multidrug (MDR) or extensively- drug resistant (XDR) organisms which can lead to lower respiratory tract infections. The aim of the study was to analyze the Gram-negative isolates obtained from oral cavity of the mechanically ventilated patients in ICUs, after prophylactic application of antibiotics and their resistance mechanisms and to compare them with the isolates obtained from tracheal aspirates from the same patients. The antibiotic susceptibility was determined by broth dilution method. PCR was applied to detect genes encoding β-lactamases. Marked diversity of Gram-negative bacteria and resistance mechanisms was found. High resistance rates and high rate of $bla_{\text{CTX-M}}$ and carbapenemase encoding genes ($bla_{\text{VIM-1}}$, $bla_{\text{OXA-48}}$) were found among *Klebsiella pneumoniae.* *Pseudomonas aeruginosa* was found to harbour bla_{VIM} and in one strain $bla_{\text{PER-1}}$ gene, whereas *Acinetobacter baumannii* produced OXA-23-like and OXA-24/40-like oxacillinases and was XDR in all except one case. All XDR isolates belong to international clonal lineage II (IC II). The main finding of the study is that the prophlylactic application of antibiotics in surgery intensive care units (ICUs) is associated with the colonization of oral cavity and lower respiratory tract with Gram-negative bacteria. The identity of Gram-negative bacteria in oral cavity reflected those found in endotracheal aspirates leading to conclusion that oral swab as non-invasive specimen can predict the colonization of lower respiratory tract with resistant Gram-negative organisms and the risk for development of pneumonia.

Keywords oral cavity, extended-spectrum β-lactamases, carbapenemases, ventilator associated pneumonia, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii*

Introduction

The oral cavity contains a number of different habitats, including the teeth, gingival sulcus, tongue, hard and soft palates, and tonsils, and acts as the tube connecting the outside and the digestive tract and respiratory tract of human body, which provides the appropriate space for the colonization of microorganisms. The microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or oral microbiome (Dewhirst et al. 2010). Over 700 bacterial species may be found in the oral cavity of humans (Paster et al. 2006). Oral microbial dysbiosis is linked to oral inflammation and may contribute to systemic conditions through bacteremia (Han and Wang 2013). Prophylactic administration of broad-spectrum antibiotics in surgery can change the oral microbiome and induce colonization of oral cavity with multidrug-resistant (MDR) or extensively drug-resistant (XDR) Gram-negative bacteria which can lead to lower respiratory tract infections in mechanically ventilated patients (Messika et al. 2018).

Hospital acquired pneumonia is often associated with MDR Gram-negative pathogens, including extended-spectrum β-lactamase (ESBL) and AmpC- positive *Enterobacteriaceae* and carbapenemase producing *Enterobacteriaceae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Dey and Bairy. 2018).

ESBLs hydrolyze penicillins, expanded-spectrum cephalosporins (ESC) and monobactams. They predominantly belong to three major families: TEM, SHV and CTX-M with rare types such as VEB, PER and IBC reported in some geographic regions (Bradford, 2001). Plasmids encoding ESBLs often carry resistance genes for non-β-lactam antibiotics such as aminoglycosides, tetracyclines, sulphonamides, chloramphenicol and fluoroquinolones. (Bonnet, 2004).

AmpC β-lactamases are primarily cephalosporinases encoded by chromosome or plasmid (p-AmpC). P-AmpC β-lactamases are derived from the chromosomally encoded enyzmes in organisms such as *Enterobacter cloacae*, *Citrobacter freundii* and *Morganella morganii*. These enzymes have been detected in *Escherichia coli*, *K. pneumoniae*, *Salmonella* spp and *Proteus mirabilis* (Jacoby, 2009).

β-lactamase mediated resistance to carbapenems in *Enterobacteriaceae* is mostly due to the expression of carbapenemases of class A (KPC), B (metallo-β-lactamases or MBLs of IMP VIM or NDM series) or D (OXA-48) (Canton et al. 2012).

Carbapenemases found in *Acinetobacter* spp. belong to molecular class A (KPC, GES), class B (IMP, VIM, SIM or NDM family) or class D (OXA enzymes) known as CHDL (carbapenem-hydrolyzing class D oxacillinases) (Queenan 2007; Brown and Amyes, 2006).

Acquired resistance in *P. aeruginosa* is rarely due to the production of ESBLs belonging to SHV, GES and PER family and more frequently to the production carbapenemases of class A (KPC, GES), B (VIM, IMP, DIM, NDM, AIM) and D (Livermore 2000; Pagani et al. 2004). Hyperexpression of chromosomal AmpC- β-lactamases, upregulation of efflux systems and decreased outer membrane permeability can contribute to resistance (Pournaras, 2005).

Clinicians treating patients in ICUs in University Hospital Centre Zagreb experienced huge therapeutic problem with Gram-negative bacteria associated with ventilator-associated pneumoniae (VAP). This initiated a study in collaboration with clinical microbiologist on the oral cavity as possible source of resistant Gram-negative bacteria in mechanically ventilated patients. The aim of the study was to analyze the Gram-negative isolates obtained from oral cavity after prophylactic application of antibiotics in surgery and their resistance mechanisms and to compare them with the isolates obtained from tracheal aspirates from the same patients in order to determine the possible source of lower respiratory tract colonization.

Results and discussion

Bacterial isolates

Bacterial isolates in oral cavity (pre-prophylactic and post-prophylactic oral swab)

The first oral swab taken before antibiotic prophylaxis, contained in the majority of cases, oral microbiome with Gram-negative pathogens in only 22 specimens (9,5%). However, the rate of Gram-negative bacteria rose to 32% (74/230) in second post-prophylactic swab. The rate of isolates was as follows: *P. aeruginosa* 40% (30/74), *A. baumannii* 19% (14/74), *Klebsiella* spp 13% (9 *K. pneumoniae* and 1 *Klebsiella oxytoca*), *E. coli* and *E. cloacae* 5% (4/74), *Stenotrophomonas maltophilia* 4% (3/74), *Citrobacter freundii*, *Klebsiella aerogenes*, *Proteus mirabilis*, and *Serratia marcescens* 2.7% (2/74) and *Burkholderia gladioli* 1.3% (1/74). There were 16% (12/74) MDR organisms, whereas 23% (17/74) were allocated to XDR phenotype. The age range and median of the patients with MDR or XDR strain was 60 (19-87) whereas of those with susceptible strain or sterile specimen was 65 (18-93).

Bacterial isolates in endotracheal aspirates (ETA)

There were 222 ETA analyzed in the study, with total 252 microbial isolates including Gram-positive and Gram-negative bacteria and fungi. The rate of Gram-negative organisms was 103/222 (46%). Out of 222 specimens 110 were sterile (49%). The rate of Gram-negative species was as follows: *P. aeruginosa* 44% (45/103), *A. baumannii* 17% (18/103), *K. pneumoniae* and *S. maltophilia* 10% (10/103), *Haemophilus influenzae* 5% (5/103), *E. coli* 3% (3/103), *E. cloacae* and *P. mirabilis* 2% (2/103), *Citrobacter koseri*, *K. aerogenes*, *C. freundii, S. marcescens, Acinetobacter pittii, Achromobacter xyloxidans, B. gladioli* and *Moraxella catarrhalis* 1% (1/103).

Pathogens associated with ventilator associated pneumonia (VAP)

VAP was diagnosed in 59 patients with 35 having early onset pneumoniae and 24 late onset pneumonia. The causative agents in early onset pneumonia were following: *P. aeruginosa* 25% (9/35), *A. baumannii* 11% (4/35), *K. pneumoniae* 6% (2/35) and *K. aerogenes* 3% (1/35).

Among Gram-positive typical hospital pathogens methicillin-resistant *S. aureus* (MRSA) was found in 6% (2/35) and *Enterococcus faecium* in 3% (1/35). Interestingly, typical community acquired pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* were found in only 1 isolate (3%), respectively. Four ETAs (11%) were sterile. Late onset pneumonia was characterized with predominance of hospital acquired pathogens with *P. aeruginosa* as dominant pathogen found 42% (10/24) of the specimens, followed by *S. maltophilia* 20% (5/24) and *A. baumannii* 12% (3/24). MRSA as causative agent of late-onset pneumonia was found in only one sample 4% (1/24). *P. mirabilis* and *C. freundii* were found only in a single specimen (4%), respectively.

Concordance in the identity of Gram-negative isolates in 2nd oral swab and ETA was 51% (38/74) with four cases in which two isolates were found in ETAs and only one in oral swab.

Antibiotic susceptibility, resistance gene content and plasmids of Gram-negative bacteria from oral cavity

Klebsiella pneumoniae

The isolates were uniformly resistant to amoxicillin due to intrinsic resistance. Moderate resistance rates of 50% (5/10) were observed for amoxycilin/clavulanic acid, piperacillin/tazobactam and cefazoline, and whereas high resistance rate was detected for ciprofloxacin 70% (7/10). There was uniform susceptibility to imipenem, meropenem and colistin, but reduced susceptibility to ertapenem was observed in two isolates. Two isolates tested phenotypically positive for ESBLs and two for carbapenemases exhibiting positive Hodge and CIM test. Three isolates were classified as MDR. Two of them exhibited resistance to expanded-spectrum cephalosporins (ESC) (Table 1). Cefotaxime resistance was transferred to *E. coli* recipient from both strains. Resistance to tetracycline and gentamicin was cotransferred from both and to sulphamethoxazole/trimetroprim from one strain. Ertapenem resistance was transferred from two resistant strains, but the transconjugants did

not harbour additional resistance determinants. Two ESBL isolates and their respective transconjugants were positive for *bla*$_{\text{CTX-M-15}}$ genes. *bla*$_{\text{CTX-M-15}}$ genes were preceded by IS*Ecp* insertion sequence. Two isolates tested positive for *bla*$_{\text{OXA-48}}$ genes with IS*1999* upstream of the gene. All isolates harboured intrinsic *bla*$_{\text{SHV-1}}$ gene. FIIs plasmid was found in two ESBL positive organisms whereas L plasmid was associated with OXA-48 carbapenemase.

Enterobacter cloacae

E. cloacae showed variable resistance phenotypes. Two isolates were resistant to cefotaxime, ceftriaxone and cefepime whereas one demonstrated resistance to carbapenems (Table 1). One isolate exhibited positive combined disk test with clavulanic acid indicating production of an ESBL whereas the other exhibited positive Hodge and CIM test and inhibitor based test with EDTA, being suspicious for MBL. Ertapenem and cefotaxime resistance were transferable to *E. coli* recipient strain, but the resistance markers to non-β-lactam antibiotics were not cotransferred. PCR and sequencing identified VIM-1 and CTX-M-15 in one isolate, respectively, as shown in Table 1. *bla*$_{\text{VIM}}$ gene was carried by A/C plasmid and *bla*$_{\text{CTX-M-15}}$ by FIA plasmid.

Escherichia coli

Three *E. coli* isolates were susceptible to all tested antibiotics except of amoxicillin whereas one strain showed resistance to ESC and cefepime and positive inhibitor based test with clavulanic acid (Table 1). Cefotaxime resistance was transferred to *E. coli* recipient strain. One MDR isolate positive phenotypically for ESBL was shown to possess *bla*$_{\text{CTX-M-15}}$ gene while *bla*$_{\text{TEM-1}}$ gene was identified by PCR in three amoxicillin resistant strains (Table 1). ESBL positive strain and the respective transconjugant were found to harbour FIA plasmid.

Table 1. Antibiotic susceptibilites and β-lactamase content of *Enterobacteriaceae*

								MIC (µgml^{-1})													
No	**Strain**	**Department**	**category**	**ESBL**	**AMP-C**	**Hodge**	**CIM**	**AMX**	**AMC**	**TZP**	**CAZ**	**CTX**	**CRO**	**FEP**	**IPM**	**MEM**	**ERT**	**GM**	**CIP**	**COL**	**BL**
Klebsiella spp																					
1	*K. pneumoniae*	AIK	S	-	-	-	-	>128	4	0.5	2	0.06	0.06	0.12	0.06	0.06	0,12	0.25	>128	0.06	SHV-11
2	*K. pneumoniae*	AIK	S	-	-	-	-	>128	2	8	0.12	0.12	0.12	0.06	0.06	0.12	0.5	0.25	>128	0.06	SHV-11
3	*K. pneumoniae*	AKA	MDR	-	-	+	+	>128	>128	>128	0.5	0.5	1	0.25	1	1	4	0.5	>128	0.06	SHV-11, OXA-48
4	*K oxytoca*	AIK	S	-	-	-	-	>128	64	64	0.12	0.25	0.12	0.06	0.12	0.25	0.25	0.12	>128	0.25	SHV-1
5	*K. pneumoniae*	AKA	S	-	-	+	+	>128	>128	>128	0.5	0.5	1	0.25	1	2	4	0.25	0.25	0.06	SHV-11 OXA-48
6	*K. pneumoniae*	AIK	S	-	-	-	-	>128	8	2	0.25	0.12	0.12	0.06	0.12	0.25	0.5	0.25	0.06	0.06	SHV-11
7	*K. pneumoniae*	AIN	S	-	-	-	-	>128	4	8	0.12	0.06	0.12	0.06	0.06	0.25	1	0.5	0.06	0.12	SHV-11
8	*K. pneumoniae* ESBL	AIK	MDR	+	-	-	-	>128	>128	>128	64	>128	>128	32	0.12	0.06	1	32	>128	1	CTX-M-15
9	*K. pneumoniae*	AKA	S	-	-	-	-	>128	2	16	0.5	1	1	0.12	0.06	0,12	0.5	32	>128	0.25	SHV-1
10	*K. pneumoniae* ESBL	AIK	MDR	+	-	-	-	>128	>128	>128	64	>128	>128	64	0.12	1	1	>128	>128	0.25	CTX-M-15
E. cloacae																					
1	*E. cloacae*	AIN	MDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	32	8	16	2	1	2	AmpC, VIM
2	*E. cloacae ESBL*	AKA	MDR	+	+	-	-	>128	>128	>128	>128	>128	>128	>128	1	0.5	1	0.25	>128	0.5	CTX-M-15 -1
3	*E. cloacae*	AIK	S	-	+	-	-	>128	>128	32	4	2	4	1	0.5	0.12	1	8	0.25	0.06	AmpC
4	*E. cloacae*	AIN	S	-	+	-	-	>128	>128	64	1	2	2	0.5	1	0.5	0.5	0.5	8	0.12	AmpC
K. aerogenes																					
3	*Klebsiella aerogenes*	AIN	S	-	-	-	-	>128	>128	16	1	0.5	1	0.25	0.12	0.06	0.5	0.25	0.06	0.12	ampC
4	*Klebsiella aerogenes*	AIK	S	-	-	-	-	>128	>128	8	0.5	0.5	0.25	0.25	0.25	0.06	0.5	0.25	0.06	0.06	ampC
Escherichia coli																					
1	*E. coli*	AIK	S	-	-	-	-	>128	2	2	0.12	0.25	0.25	0.06	0.25	0.06	1	0.25	1	0.06	TEM-1
2	*E. coli*	AIN	MDR	+	-	-	-	>128	8	16	32	>128	>128	16	0.5	0.12	1	1	2	0.25	CTX-M-15, TEM-1

3		*E. coli*	AIN	S	-	-	-	-	>128	2	16	0.25	0.12	0.12	0.06	0.25	0.06	0.25	0.5	0.12	0.06	TEM-1
4	170ć	*E. coli*	AIK	S	-	-	-	-	>128	4	8	0.5	0.25	0.12	0.25	0.12	0.06	2	2	0.06	0.06	TEM-1
Serratia marcescens																						
1		*S. marcescens*	AIN	S	-	+	-	-	>128	>128	32	1	0.5	0.5	1	0.12	0.5	0.5	1	0.06	0.12	AmpC
2		*S. marcescens*	AKA	S	-	+	-	-	>128	64	16	0.5	0.12	0.12	0.5	0.5	0.5	0.25	1	0.12	0.12	AmpC
Proteus mirabilis																						
1		*P. mirabilis*	AIK	S	-	-	-	-	4	1	8	0.12	0.25	0.25	0.12	0.06	0.06	0.5	0.25	0.06	0.06	
2		*P. mirabilis*	AIN	S	-	-	-	-	16	0.5	16	0.5	0.12	0.12	0.5	1	0.06	0.06	8	8	0.06	TEM-1
Citrobacter spp																						
1		*C. freundii*	AIK	S	-	+	-	-	>128	>128	2	0.12	0.25	0.25	0.06	0.12	0.06	1	0.5	0.06	0.06	AmpC
2		*C. freundii*	AIK	S	-	+	-	-	>128	>128	4	0.5	1	1	0.12	0.06	0.06	0.5	1	0,12	0.06	AmpC

MIC-minimum inhibitory concentration; AMX-amoxycillin; AMC-amoxycillin/clavulanic acid; TZP-piperacillin/tazobactam; CAZ-ceftazidime; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; ERT-ertapenem; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases;Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC beta-lactamases; BL-beta-lactamase content; CIM-carbapenem inactivation method, AIK-surgery ICU; AKA-cardiosurgery ICU; AIN-neurosurgery ICU; S-susceptible; MDR-multidrug-resistant

Proteus mirabilis

One isolate was found to be resistant to ampicillin due to TEM-1 broad spectrum β-lactamase.

Klebsiella aerogenes, Citrobacter freundii, Serratia marcescens

Only intrinsic resistance was detected among above species (Table 1).

Pseudomonas aeruginosa

P.aeruginosa exhibited high resistance rates to meropenem and imipenem 63% (19/30) and moderate to gentamicin 53% (16/30), ciprofloxacin 47% (14/30), ceftazidime 40% (12/30) and cefepime and piperacillin/tazobactam 30% (9/30). Amikacin preserved good activity with only 10% (3) resistant strains as shown in Table 2. There was no resistance to colistin and ceftolozane/tazobactam. Among 30 isolates seven were classified as MDR and five as XDR. Five isolates demonstrated positive Hodge, CIM and inhibitor based test for MBLs. Four isolates yielded PCR product with VIM specific primers and one with PER-1 specific primers as shown in Table 2. MBL

Table 2. Antibiotic susceptibility, phenotypic tests, resistance genes content, and clinical data of *P. aeruginosa* isolates.

								MIC (minimum inhibitory concentration) µgml-1								
Isolate number	Department	Category	ESBL	AMP-C	Hodge	CIM	EDTA	TZP	CAZ	FEP	IMI	MEM	GM	AMI	CIP	COL	BL
1.	AIK	S	-	-	-	-	-	1	0.5	1	16	32	16	4	32	0.5	
2	AIK	S	-	-	-	-	-	2	32	2	16	64	32	4	0.25	2	
3.	AIK	MDR	-	-	-	-	-	2	2	1	32	32	32	8	>128	2	
4	AIK	XDR	-	-	+	+	+	>128	>128	>128	>128	>128	64	>128	>128	2	VIM-2
5	AIK	S	-	-	-	-	-	4	0.5	1	2	2	1	8	>128	2	
6	AIN	XDR	-	-	+	+	+	>128	>128	>128	>128	>128	64	64	>128	2	VIM-2
7	AKA	S	-	+	-	-	-	64	32	16	4	2	0.5	4	0.5	2	PER-1
8	AKA	S	-	+	-	-	-	>128	32	32	2	4	1	8	0.25	2	AmpC
9	AIN	S	-	-	-	-	-	4	1	1	1	4	1	8	0.25	0.5	
10	AKA	S	-	-	-	-	-	1	1	1	1	4	1	4	0.25	0.5	
11	AIK	MDR	-	-	-	-	-	1	2	0.5	16	32	>128	2	64	2	
12	AIN	S	-	-	-	-	-	4	1	1	1	4	2	44	0.5	2	
13	AIK	XDR	-	-	+	+	+	>128	>128	>128	>128	>128	>128	16	>128	2	VIM-2
14	AIK	XDR	-	-	+	+	+	>128	>128	>128	>128	>128	>128	16	>128	1	VIM-2
15	AIK	MDR	-	-	-	-	-	8	1	0.5	16	32	32	4	>128	2	
16	AIK	S	-	-	-	-	-	4	2	0.5	1	0.25	0.25	16	>128	2	
17	AIK	S	-	-	-	-	-	2	1	1	16	16	32	16	>128	1	
18	AIN	S	-	-	-	-	-	1	2	2	2	0.25	0.5	4	0.5	1	
19	AIN	S	-	-	-	-	-	4	1	0.5	16	32	1	8	0.25	2	
20	AIK	MDR	-	-	-	-	-	8	2	2	32	32	64	16	>128	1	
21	AIN	S	-	-	-	-	-	2	1	0.5	1	0.25	0.5	16	0.25	2	
22	AKA	MDR	-	-	-	-	-	64	32	64	32	32	>128	64	8	2	AmpC
23	AIN	MDR	-	-	-	-	-	32	32	16	16	32	0.5	16	8	2	AmpC
24	AIN	S	-	-	-	-	-	>128	64	64	16	32	0.5	0.25	2	1	AmpC
25	AIK	XDR	-	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	32	2	VIM-2

26	AKA	S	-	-	-	-	-	16	2	16	16	32	0.25	2	8	0.5	AmpC
27	AKA	S	-	-	-	-	-	8	2	2	2	0.25	0.12	0.5	8	2	
28	AKA	S	-	-	-	-	-	16	16	2	16	32	0.25	2	8	0.5	AmpC
29	AKA	S	-	-	-	-	-	16	4	1	32	16	0.25	1	4	1	AmpC
30	AIK	S	-	-	-	-	-	8	2	1	16	64	4	16	8	1	

MIC-minimum inhibitory concentration; CAZ-ceftazidime; FEP-cefepime; IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum β-lactamases; Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC β-lactamases; CIM-carbapenem inactivation method; BL-β-lactamase content; AIK-surgery ICU; AKA-cardiosurgery ICU; AIN-neurosurgery ICU; S-susceptible; VIM-Verona-integron-associated metallo-β -lactamase; MDR-multidrug-resistant; XDR-extensively drug resistant

production was associated with high level resistance to carbapenems and PER-1 with resistance to ceftazidime and cefepime.

Acinetobacter baumannii

All except one strain (93%) were resistant to imipenem, meropenem, piperacillin/tazobactam, ceftazidime, cefepime, gentamicin and ciprofloxacin (Table 3). The resistance rate to ampicillin/sulbactam was 36% (5/14). No resistance to colistin was observed. Only one strain was sensitive, whereas all other were classified as XDR and tested positive in Hodge and CIM test indicating carbapenemase production. Except of intrinsic *bla*$_{\text{OXA-51}}$ gene three isolates harboured *bla*$_{\text{OXA-23-like}}$ and ten *bla*$_{\text{OXA-24- like}}$ genes (Table 3). One *A. bauumanii* strain, negative for CHDL, was found to belong to SG 2 (sequence group) corresponding to IC I (international clonal lineage) whereas the rest of the strains positive for CHDL were allocated to SG (sequence group) 1 corresponding to IC (international clonal lineage) II. PBRT (PCR-based replicon typing) was negative for any of the plasmid incompatibility groups reported in *A. baumannii* so far, except of the three strains with OXA-23 being positive for Inc group 6 encoding aci6-replicase gene originally found on plasmid pACICU2.

Stenotrophomonas maltophilia

Susceptibility to sulphmethoxazole/trimethoprim and levofloxacin was confirmed by disk-diffusion test in all isolates.

Burkholderia gladioli

B. gladioli was resistant to all tested antibiotics.

Isolates from endotracheal aspirates

Thirty-eight isolates from ETA of the same identity as those in oral cavity, had, according to routine disk-diffusion test, same antibiotic susceptibility pattern as respective isolates from oral swab, but the molecular analysis of resistance determinants was not done. However, immunochromatographic O.K.N.V test identified same carbapenemases in Enterobacteriaceae as those detected by PCR in the respective isolates from oral cavity.

The main finding of the study is that the prophlylactic application of antibiotics in surgery ICU-s is associated with the colonization of oral cavity and lower respiratory tract with Gram-

Table 3. Antibiotic susceptibility, phenotypic tests and resistance genes content of *A. baumanii* isolates.

							MIC (µgml^{-1})										
No	**depatment**	**Category**	**ESBL**	**Hodge**	**CIM**	**EDTA**	**TZP**	**CAZ**	**CTX**	**CRO**	**FEP**	**IPMI**	**MEM**	**GM**	**CIP**	**SAM**	**COL**	**BL**
1	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	1	OXA-24-like
2	AIN	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	4	1	OXA-24-like
3	AIN	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	4	1	OXA-24-like
4	AIN	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	1	OXA-24-like
5	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	2	OXA-24-like
6	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	8	2	OXA-24-like
7	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	16	2	OXA-24-like
8	AIK	S	-	-	-	-	8	8	>128	>128	8	2	1	0.5	0.12	4	0.5	
9	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	16	1	OXA-23-like
10	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	1	OXA-23-like
11	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	0.5	OXA-23-like
12	AKA	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	16	2	OXA-24-like
13	AIN	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	64	1	OXA-24-like
14	AIK	XDR	-	+	+	+	>128	>128	>128	>128	>128	>128	>128	>128	>128	32	1	OXA-24-like

Ceftazidime-CAZ; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; TZP-piperacillin/tazobactam; SAM-ampicilliin/sulbactam; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; ; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases;Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC β-lactamases; EDTA-inhibior based test with EDTA for detection of MBLs; BL-β-lactamase content; CIM-carbapenem inactivation method; ICU-intensive care unit; AIK-surgery ICU; AIN-neurosurgry ICU; AKA-cardiosurgery ICU; AIN-neurosurgery ICU; S-susceptible; MDR-multidrug-resistant; XDR-extensively drug resistant
all isolates harboured intrinsic *bla*$_{\text{OXA-51}}$-like gene, the isolate 8 harboured only *bla*$_{\text{OXA-51}}$-like gene without having acquired CHDL

negative bacteria. The identity of Gram-negative bacteria in oral cavity reflected those found in ETAs in half of the patients, leading to conclusion that oral swab which is non-invasive specimen can predict the colonization of lower respiratory tract with resistant Gram-negative organisms and the risk for development of pneumonia in selected patient population receiving perioperative antibiotic prophylaxis and could be used as screening method. Dynamic evolution of the oral microbiota in relation to antibiotic prophylaxis was observed. Antibiotic resistance patterns of the oral isolates was identical to those found in ETAs indicating that oral cavity was the source of lower respiratory tract colonization. The second goal was to analyze resistance mechanisms of Gram-negative bacterial isolates from oral cavity after prophylactic antibiotic administration. A high diversity of bacterial species, resistance mechanisms and plasmid types was reported. The study showed that oral cavity can pose a significant reservoir of MDR Gram-negative bacteria in mechanically- ventilated patients. The age of the patient was not identified as the risk factor for the acquisition of the MDR or XDR isolates since there was no difference in the age of the patients between the two groups of the patients: with and without resistant isolates.

As recently showed by Sommerstein et al (2019) even in antibiotic-naive patients, causative pathogen gained access to oropharynx and outgrew commensals in the microbiome early during mechanical ventilation, even after 2 days. Their point was that that surgical prophylaxis, or/and antibiotics, or/and hospital environment change ecological community of commensals. Thus the microbiome was changed even without antibiotics administration due to the hospital stay and exposure to the typical hospital pathogens. It was considered for a long time that lower respiratory tract is sterile. However, introduction of new molecular tehniques identified high diversity of microbial populations, including *Prevotella* spp, *Streptococcus* spp and *Veilonella* spp in the lungs of healthy persons, which are now considered as respiratory microbiota and are biomarkers of respiratory health (Fromentin et al. 2021). Mechanical

ventilation and stay in ICU was found to be associated with the loss of microbial diversity and shift to predominance of one species, particularly *P. aeruginosa* and enrichment with gut microbiota. This alteration in respiratory microbiota in ventilated patients was not linked to antibiotic usage (Fromentin et al. 2021). Previous studies found that change of respiratory microbiome affected the emergence of acute respiratory distress syndroma (ARDS) and systemic inflammatory response (SIRS). Disruption of the lung microbiota community was found to be due to the overgrowth of the opportunistic pathogens in patients developing VAP compared to the critically ill patients without VAP (Tsitklis et al. 2021).

Regarding present study, it is difficult to explain whether the predominance of Gram-negative bacteria in post-prophylactic oral was due to antibiotic prophylaxix or the consequence of stay in ICU and mechanical ventilation. Further studies are necessary to clarify this issue.

P. aeruginosa was found to harbour VIM-2 which was identified among *P. aeruginosa* from Croatia in the previous studies including isolates from the same hospital wards as in the present study (Bošnjak et al. 2010; Sardelić et al. 2012). It conferred on the producing isolates high level of resistance to carbapenems, cefepime and ESC. There was sporadic occurrence of PER-1, ESBL widespread in *P. aeruginosa* in Turkey (Kolayli, 2005) and Italy (Pagani et al. 2004). In Poland (Empel et al. 2007) and Italy (Luzzaro et al. 2001) it was associated with nosocomial outbreaks. It conferred on the producing isolate high level of resistance to ceftazidime and cefepime, but spared carbapenems. Interestingly, phenotypic ESBL testing yielded positive result only after addition of PBA, ampC inhibitor, on the cephalosporin disks. The isolates resistant only to carbapenems, but susceptible to ESC did not harbour any acquired β-lactamase and probably had upregulation of efflux pumps or porin loss, but the clarification of resistance mechanisms was beyond this study.

Enterobacteriaceae phenotypically positive for ESBLwere found to possess group 1 of CTX-M β-lactamases which is in concordance with the previous studies. Carbapenem resistance or

reduced susceptibility was associated with the production of OXA-48 in *K. pneumoniae* and VIM-1 in *E. cloacae* and *C. freundii*. The first studies on carbapenem resistance in Croatia reported high prevalence of VIM-1 among *E. cloacae* (Zujić-Atalić et al. 2014; Bedenić et al. 2016a), but recently epidemic spread of OXA-48 was noticed in *K. pneumoniae* (Bedenić et al. 2018). In contrast to previous study, one of the OXA-48 producing *K. pneumoniae* strain in the present study showed full susceptibility to all tested antibiotics except ertapenem and penicillins alone or combinations with inhibitors and was categorized as susceptible, in spite of carbapenemase production. *S. marcescens*, *P. mirabilis* and *K. aerogenes* had only intrinsic resistance mechanisms. Previous studies showed that urinary isolates of *P. mirabilis* in Croatia often harbour CMY AmpC β-lactamases (Bedenić et al. 2016b) confering resistance to ESC, but the isolates from oral cavity showed full susceptibility to antimicrobial agents.

A. baumannii was the most resistant species with all except one strain being XDR and positive for CHDL. OXA-24-like was the dominant CHDL among tested isolates. This type of CHDL was previously reported to be the dominant type of CHDL in different geographic regions of Croatia, including hospitals (Franolić-Kukina et al. 2011; Vranić-Ladavac et al. 2014) and nursing homes (Bedenić et al. 2015) and were also found in environmental samples in Croatia (Goić-Barišić et al. 2016; Šeruga-Musić et al. 2017). This type is also predominant in the neighbouring countries Serbia (Dortet et al. 2015) and Bosnia and Herzegovina (Petrović et al. 2018). Other studies found OXA-23 to be predominant among *A. baumannii* in VAP patients (Mohammadi et al. 2017), but in our study it was outnumbered by OXA-24-like.

Since VIM-2 positive *P. aeruginosa* (Bošnjak, et al. 2010), OXA-24-like positive *A. baumannii* (Franolić-Kukina et al. 2011) and VIM-1 producing *E. cloacae* (Bedenić et al. 2016) were previously reported from the same hospital wards of the University Hospital

Center Zagreb, it is possible that these isolates remained unnoticed in the hospital environment, particulary on the equipment for the mechanical ventilation, which served as a source of patient colonization. An interesting conclusion of the study is that the appearence of MDR Gram-negative bacteria, particularly those producing ESBLs and carbapenemases, in oral cavity, reflects the distribution of those resistance determinants in clinically relevant specimens, indicating that the colonization of upper respiratory tract may be the source of serious infections such as hospital pneumonia. The rates of particular resistance determinants were in line with the local epidemiology. The scientific bibliography on the role of oral flora in the development of pneumonia is relatively scarce. Other studies found *P. aeruginosa* to be a transient flora in the oral cavity particularly in children (Botzenhart et al. 1985; Kusahara et al. 2012). However, the molecular characterization of the Gram-negative bacteria was not done. The clinical trials have found that oropharyngeal decontamination decreases the incidence of VAP (Pugin et al. 1991). The previous studies found that colonization of dental plaque with respiratory pathogens is important in the aetiology of VAP in the light of the well known facts that oral hygiene is deteriorated during mechanical ventilation. Typically, early onset pneumonia is suppposed to be caused by community-acquired pathogens like *H. influenzae* or *S. pneumoniae*, but suprisingly, a lot of patiens with early-onset pneumonia had typical hospital pathogens such as *P. aeruginosa* and *A. baumannii* in ETAs. This could be explained by the fact that these patients were hospitalized in other hospital wards like surgery or medical ward before being admitted in ICU. The similar observation was reported by other authors (Dey and Bairy, 2007). The rate of MDR Gram-negative bacteria in our study was lower compared to other studies analyzing VAP (Dey and Bairy, 2007; Tedja et al. 2014). Data on the prevalence of MDR bacteria in oral cavity are not available in bibliographical referfences to enable comparison. Previous studies analyzed microbiome in the oral cavity of the patients suffering from periodontal diseases, but resistance genes except of bla_{TEM} , bla_{CfxA}

and *erm* genes were not found (Almeida et al. 2020; Arredondo at al 2020). The dominant species harbouring resistance determinants in subgingival samples were *Streptococcus* spp and *Prevotella* spp, bacteria belonging to normal microbiota of the oral cavity. It seems that hospital stay and antibiotic consumption are the driving force for selection of ESBL and carbapenemase producing Gram-negative bacteria.

The limitation of the study is the low number of isolates originating from only one hospital center and the lack of molecular analysis of the isolates from ETAs. Moreover, genotyping was not performed to determine if there is clonal dissemination within the hospital wards. The strength of the study is the profound molecular analysis of the strains from oral cavity and their resistance mechanisms. This is the first study on molecular analysis of MDR Gram-negative bacteria in oral cavity of mechanically ventilated patients

Material and methods

Patients

In total 225 mechanically ventilated patients receiving preoperative antibiotic prophylaxis were included in the study. The total number of post-prophylactic swabs was 230 because two patients with prolonged stay in the hospital had multiple samples taken in the period of >3 months. The patients with elective surgical procedures were admitted to the hospital one day before operation while urgent patient were received in the ICU immediately after the surgical procedure. Antibiotic prophylaxis was started in ICU. The antibiotics were not administered before.

Sample collection

The study was approved by the Ethical Committee of the University Hospital Centre Zagreb (Ethical permission number 02/21 AG). The patients signed the informed consent form before

being put in a medically induced coma. The staff explained the procedures being done for research purpose to the patients included in the study. The samples were collected from three intensive care units (ICU): surgery ICU (AIK), neurosurgery ICU (AIN) and cardiosurgery ICU (AKA) of the University Hospital Centre Zagreb from January to December 2019.

The oral swab was taken before and on the 5th day after prophylactic administration of antibiotics by an educated bachelor of nursing as follows the tongue was pressed with a sterile wooden spatula which was lightly rotated and the stick removed from the original sterile 15 cm long cotton pad (swab), manufactured by Nerbe plus, with pressure to wipe the oral cavity. The timing of sample taking was chosen based on the duration of the antibiotic prophylaxis with the time span from 1 to 4 days, depending on the surgical procedure. The swab was stored in sterile original packaging and was immediately taken to the laboratory, if not possible, the swab was stored in the refrigerator until the delivery to the laboratory (maximum 2 h). The first preprophylactic oral swab was seeded on blood agar in the frames of routine, diagnostic bacteriological analysis and the second, post-prophylactic, taken for research purpose, on selective medium for Gram-negative bacteria MacConcey agar (Copan, Zagreb). Endotracheal aspirates were taken by an educated bachelor of nursing as follows: sterile suction catheter with mucosal extractor, manufactured by Covidien, Argyle is inserted through the endotracheal tube at a distance of 25 cm, after aspiration the catheter is removed through the endotracheal tube. Saline was injected with a sterile syringe to flush the exudate into a sterile container. The sample was immediately taken to the laboratory and subjected to Gram and methylene blue staining. Ten µl of the sample was seeded on blood agar for quantitative analysis. Only organisms with $>10^5$ CFU/ml were further analyzed. Bacteria were identified by conventional biochemical tests and the MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry) (Bruker, Illinois, USA).The growth of microorganisms below the breakpoint was considered contamination or colonization. The

diagnosis of pneumonia was based on X ray, C- reactive- protein (CRP) and polymorphonuclear count. Early-onset pneumonia was defined as pneumonia developing within the first 4 days on mechanical ventilation whereas late onset pneumonia developed after 4 days on mechanical ventilation (Dey and Bairy, 2007).

The isolates from oral cavity were subjected to detailed antimicrobial susceptibility testing and analysis of resistance genes and were compared with those obtained from ETA during routine diagnostic. The isolates from ETAs were analyzed according to standard routine microbiological procedures. Carbapenemases were identified in Enterobacteriaceae by immunochromatographic O.K. N. V. test.

Antibiotics used in perioperative prophylaxis

There were 21 different antibiotics, or a combination of two or three of these agents used in perioperative prophylaxis. Patients received prophylaxis with antibiotics commonly used in intensive care units such as cefazolin, cefuroxime, metronidazole, piperacillin in combination with tazobactam or cloxacillin and antibiotic therapy was changed if necessary, after the antibiogram result. Antibiotic choice depended on the clinical picture and the type of procedure to be applied to the and whether it was primarily “clean” procedure such as skull or heart surgery or procedures that are at risk of contamination such with intestinal flora such as abdominal surgery. According to the protocols applied in the surgical ICU’s in our hospital the patients received antibiotics during 24 h. The first dosis was applied one hour before the operation for both elective and urgent surgical procedures. Cardiosurgical and neurosurgical patients received only one dosis of antibiotic whereas general surgical patient received two or three dosis depending on the procedure. The patients were received in the ICUs immediately after elective and urgent operations. The prophylaxix is AIK is based on administration of cefazoline and metronidazole or ciprofloxacin and metronidazol, in AIN ceftriaxone whereas in AKA cefuroxime is used. The choice of the antibiotic was inline with the Croatian National

Guidelines (Francetić et al. 2010) with some specific recommendations depending on the hospital, the type of patients and procedures in each ICU. Ceftriaxone is generally not recommended for prophylaxis according to Croatian guidelines but it is used in neurosurgery ICU because of its high activity against Gram-negative pathogens associated with postoperative meningitis and high concentrations in cerebrospinal fluid. Vancomycin is used only for the patients who recently stayed in the hospital or came from nursing home and had MRSA isolate before. Similary, meropenem is used in case when patients had ESBL positive organism within the last three months. Fifty-nine patients who developed VAP during mechanical ventilation and stay in ICU were treated with antibiotics according to the causative agent and susceptibility testing results.

Antimicrobial susceptibility testing

The antimicrobial susceptibility to a wide range of antibiotics including amoxicillin alone and combined with clavulanic acid, piperacillin/tazobactam, ampicillin/sulbactam, cefuroxime, cefazolin, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, ertapenem, gentamicin, ciprofloxacin and colistin was determined by disk-diffusion and broth microdilution method in 96 well microtitar plates and interpreted according to CLSI breakpoints (CLSI. 2016). The tested range of antibiotic concentrations was 0.06 to 128 μg/ml. The concentration of antibiotics in the disks (BBL) were 30 μg except for gentamicin, ertapenem, imipenem and meropenem 10 μg and ciprofloxacin 5 μg. The isolates were classified as multidrug-resistant (MDR), extensively drug resistant (XDR) or pan-drug resistant (PDR) (Magiorakis et al. 2012).

Phenotypic tests for detection of ESBLs, plasmid-mediated AmpC β-lactamases and carbapenemases

Double disk synergy test (DDST) (Jarlier et al., 1988) and CLSI combined disk test with addition of clavulanic acid were performed to detect ESBLs (CLSI. 2016). Plasmid-mediated AmpC β-lactamases were detected by combined disk test using cephalosporin disks combined

with PBA (3-aminophenylboronic acid (Coudron 2005). Modified Hodge test (MHT) (Lee et al. 2003) and carbapenem inactivation method (CIM) (Van der Zwaluw et al. 2015) were used to screen for the production of carbapenemases. Additionally the isolates were tested by combined disk tests with imipenem and meropenem alone and combined with 3-aminophenylboronic acid (PBA), 0.1 mol l^{-1} EDTA or both to screen for KPC, MBLs, or simultaneous production of KPC and MBL, respectively (Pasteran et al. 2009; Lee e al. 2003). Disk antagonism test with cefoxitin as inducing agent was used to detect the inducibility of the β-lactamases. The antagonism was detected by blunting of the oxymino cephalosporin inhibition zone surrounding the cefoxitin disk (CLSI, 2016).

Transfer of resistance

The mating experiments were done by mixing equal volumes of each test strain and *E. coli* J65 resistant to sodium azide (Elwell and Falkow 1986). β-lactam resistant *E. coli* transconjugants were selected on combined plates containing ertapenem (1 mgL^{-1}) or cefotaxime (2 mgL^{-1}) to inhibit the growth of recipient strain and sodium azide (100 mgL^{-1}) to supress the donor strains.

Molecular detection of resistance genes

The total DNA was extracted by boiling method. The genes conferring resistance to β-lactams including broad spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{PER-1}$) (Nuesch-Inderbinen et al. 1996; Arlet et al. 1995; Woodford et al. 2004; Pagani et al. 2004; Woodford et al. 2006), plasmid-mediated AmpC β-lactamases (Perez-Perez and Hanson 2002), class A carbapenemases (*bla*$_{KPC}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{NMC}$,), class B (*bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$), carbapenem hydrolyzing oxacillinases (*bla*$_{OXA-48}$) (Poirel, 2011) and fluoroquinolones (*qnr*A, *qnr*B, *qnr*S) (Robiscek et al. 2006) were determined by PCR using protocols and conditions as described previously. PCR assays with primers 5'-CS and 3'-CS combined with forward and reverse primers for *bla*$_{VIM}$ was done to determine the location of

bla$_{VIM}$ gene within class 1 integron (Jeong et al. 2003). Reference strains producing SHV-1, SHV-2, TEM-1, CTX-M-3, CTX-M-15, IMP-1, VIM-1, KPC-2 and OXA-48 were used as positive control. Amplicons of the selected strains were purified with Qiagen DNA purification kit (Inel, Zagreb, Croatia) and sequenced in Eurofins sequencing services (Graz, Austria).

Genetic context of *bla*$_{CTX-M}$ genes was determined by PCR mapping with forward primer for IS*Ecp1* and IS*26* combined with primer MA-3 (universal reverse primer for *bla*$_{CTX-M}$ genes) (Saladin et al. 2002). The flanking regions of *bla*$_{OXA-48}$ genes were analyzed with forward primer for IS*1999* combined with reverse primer for *bla*$_{OXA-48}$ (Gianni et al. 2012).

Characterization of plasmids

Plasmids were extracted with Qiagen Mini kit from enterobacterial isolates and *A. baumannii* according to the manufacturer's instructions. PCR-based replicon typing (PBRT) (Carattoli et al. 2005) was applied to type the resistance plasmids carrying carbapenemase and ESBL genes in *Enterobacteriaceae*. Plasmid extractions from transconjugant strains were subjected to PCR for detection of ESBLs and carbapenemases (MBL and OXA-48) in order to determine the location of *bla* genes. PBRT according to Bertini was applied to type the resistance plasmids carrying carbapenemase genes for A. *baumannii* (Bertini et al. 2010).

Molecular typing of *A. baumannii* isolates

Sequence groups (SGs 1-3) corresponding to international clonal lineages (ICL I-III) determination was performed according to the procedure described by Turton (Turton 2007).

Author's contribution:

Eva Pleško: laboratory analysis of the isolates

Branka Bedenić: laboratory analysis of the isolates, writing of the manuscript

Vesna Bratić: sample collection and study design

Slobodan Mihaljević, Marko Čaćić and Željko Verzak: manuscript design and critical review of the manuscript

Anita Lukić-chart preparation and statistical analysis

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