

The *in Vitro* Effect of Imipenem and Ofloxacin on Enzymic Activity of *Klebsiella* Strains

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ABSTRACT. Fifty seven *Klebsiella* strains, viz. *K. pneumoniae* (28), *K. planticola* (19), *K. oxytoca* (6), *K. ornithinolytica* (3) and *K. terrigena* (1) possessed lipolytic and urealytic activity. The effect of imipenem and ofloxacin at subinhibitory concentrations (sub-MICs) on these enzymic activities of 4 strains was studied. At all the concentrations tested

(mainly at ¼ of the MICs) imipenem enhanced lipase activity manifested by cleavage of the substrate Tween 20. The effect of ofloxacin was strain- and concentration-dependent but in most cases lipolytic activity was also increased. The antibiotics practically did not affect the urease activity of the strains.

Klebsiellae are opportunistic pathogens associated with a variety of different severe diseases (septicæmia, pneumonia, urinary-tract infection and soft-tissue infection) mainly in hospitalized immunocompromised patients (Williams and Tomáš 1990; Stamm *et al.* 1991; Yinnon *et al.* 1996). The medically most significant *Klebsiella pneumoniae* and *K. oxytoca*, which are isolated to a smaller extent from human patients as compared with the former one, were regarded as the only pathogenic *Klebsiella* species. Recently further species (*K. planticola* and *K. terrigena*) were found in connection with human infectious diseases. Mainly *K. planticola* originally isolated from plant and aquatic environments has occurred in human clinical specimens at a relatively high frequency (Podschun and Ullmann 1994). The existence of multiply antibiotic-resistant *Klebsiella* strains mainly of the ESBL-producing ones is serious because it complicates therapy and is associated with high morbidity and mortality (Arlet *et al.* 1994; Smith and Chambers 1995).

A number of factors of *Klebsiella* species, mainly of *K. pneumoniae* that may contribute to the pathogenesis of diseases evoked by these bacteria are known (capsula, lipopolysaccharide, pili, extracellular toxic complex, siderophores, urease, hæmolysin, enterotoxin, serum resistance; Warren *et al.* 1982; Podschun and Ullmann 1998). Lipolytic activity found in *Klebsiella* strains may be another virulence factor, similar to those produced by other bacteria (Rollof *et al.* 1988; Straus *et al.* 1992).

Antibiotics at subinhibitory concentrations (sub-MICs) intermittently present during antibiotic therapy may interfere with many bacterial properties, including virulence parameters (Grimwood *et al.* 1989; Hošťacká and Majtán 1993; Tateda *et al.* 1994). In this study, the lipase and urease activity of *Klebsiella* species after treatment with imipenem and ofloxacin were studied.

MATERIAL AND METHODS

Bacterial strains. A total of 57 *Klebsiella* strains were studied. *K. pneumoniae* (28), *K. planticola* (19), *K. oxytoca* (6), *K. ornithinolytica* (3) and *K. terrigena* (1) were identified using a commercially available identification kit — Enterotest 24 (*Lachema*, Brno, Czechia). The microorganisms were isolated from the respiratory tract (17), urinary tract (28) and from other human body sites (12) of patients at the *Institute of Microbiology, Faculty of Medicine and Faculty Hospital* (Bratislava). Four strains were chosen to be assayed for changes in lipase and urease activity after treatment with imipenem and ofloxacin *in vitro*.

Antibiotics. Imipenem (*Merck & Co., Laboratoires Merck Sharp & DohmeChibret*, France) and ofloxacin (*Hoechst AG*, Germany) were used.

Antimicrobial susceptibility test. MICs were determined by the macrodilution broth method (Mueller–Hinton broth with 25 mg of Ca²⁺ and 12.5 mg Mg²⁺; MHB) using serial two-fold dilutions of the antibiotic. The lowest dilution of the antimicrobial drug that inhibited bacterial growth after incubation at 37 °C for 1 d was considered as MIC.

Cultivation of strains. Bacterial suspensions (0.2 mL, A₆₀₀ = 0.5) were incubated with 9.8 mL of MHB at 37 °C for 1 d. Supernatants (culture filtrates) obtained after centrifugation of bacterial suspensions were sterilized (0.22 µm, Millipore) and used for lipase determination in all strains.

Treatment of four strains with antibiotics. Bacterial suspensions of the strains (0.2 mL, $A_{600} = 0.5$) were incubated with 9.7 mL of MHB and 0.1 mL of antibiotic at the sub-MICs ($\frac{1}{4}$, $\frac{1}{8}$ or $\frac{1}{16}$ of the MIC) at 37 °C for 1 d. Control suspensions were cultured without the antibiotic. Bacterial cells obtained after centrifugation were washed and adjusted to A_{600} of 1 for the determination of urease. Culture filtrates (supernatants) were sterilized (0.22 μ m, Millipore) and used for lipase assay.

Determination of lipolytic activity. The spectrophotometric method using Tween 20 as substrate was applied (Tirunarayanan and Lundbeck 1968; Lonon *et al.* 1988). The reaction mixture (0.1 mL of 10 % Tween 20 in 50 mmol/L Tris-HCl, pH 7.6, 0.1 mL of 1 mol/L CaCl₂, 0.5 mL of culture filtrate) was incubated with 2.3 mL of buffer at 37 °C in a water bath for 2 h. The substrate was replaced with buffer in the blank. Fatty acid liberated by cleavage of Tween 20 in the presence of calcium formed an insoluble salt which increased the absorbance A_{400} of the reaction mixture. One unit of lipase activity was defined as the amount of enzyme which under these conditions increased the absorbance by 0.01. The activity was evaluated as units per mL culture filtrate (Table I).

Table I. Lipase activity of *Klebsiella* species exposed to imipenem and ofloxacin†

Strain	Imipenem					Ofloxacin				
	MIC mg/L	Fraction of MIC	μ g/L	Lipase activity		MIC mg/L	Fraction of MIC	μ g/L	Lipase activity	
				U/mL	%				U/mL	%
<i>K. pneumoniae</i> 751	1.56	0	—	34.7 ± 1.5	100	0.78	0	—	26.6 ± 2.5	100
		$\frac{1}{4}$	390	65.6 ± 6.1	189		$\frac{1}{4}$	190	31.4 ± 2.0	118
		$\frac{1}{8}$	190	59.5 ± 4.4	171		$\frac{1}{8}$	90	39.6 ± 0.7	149
		$\frac{1}{16}$	90	35.2 ± 1.4	101		$\frac{1}{16}$	40	32.7 ± 0.6	123
<i>K. pneumoniae</i> 1296	3.12	0	—	40.4 ± 3.0	100	1.56	0	—	37.0 ± 1.7	100
		$\frac{1}{4}$	780	83.7 ± 1.4	207		$\frac{1}{4}$	390	40.0 ± 1.1	108
		$\frac{1}{8}$	390	61.7 ± 2.5	153		$\frac{1}{8}$	190	44.4 ± 4.1	120
		$\frac{1}{16}$	190	41.5 ± 2.1	103		$\frac{1}{16}$	90	38.6 ± 2.4	104
<i>K. planticola</i> 251	1.56	0	—	30.2 ± 1.0	100	0.39	0	—	37.3 ± 3.6	100
		$\frac{1}{4}$	390	46.9 ± 4.8	155		$\frac{1}{4}$	90	26.6 ± 0.2	71.3
		$\frac{1}{8}$	190	43.1 ± 3.3	143		$\frac{1}{8}$	40	37.0 ± 3.1	99.2
		$\frac{1}{16}$	90	37.1 ± 2.2	123		$\frac{1}{16}$	20	32.0 ± 2.6	85.8
<i>K. oxytoca</i> 852	1.56	0	—	54.3 ± 2.1	100	0.39	0	—	60.6 ± 2.7	100
		$\frac{1}{4}$	390	147 ± 1.2	271		$\frac{1}{4}$	90	42.8 ± 0.8	70.6
		$\frac{1}{8}$	190	94.9 ± 5.8	175		$\frac{1}{8}$	40	69.8 ± 2.5	115
		$\frac{1}{16}$	90	85.3 ± 1.1	157		$\frac{1}{16}$	20	62.5 ± 4.4	103

†Means of three measurements ± SD.

Urease assay. Urea agar (Christensen) (*Imuna*, Slovakia) was used to prepare urea agar slants and plates. Hydrolysis of urea by urease leads to a pH increase which is manifested in the presence of a pH indicator (phenol red) by a color change of urea agar. Urea agar slants were used to test all strains for production of urease (Cowan 1974). Urease activity of four selected strains after exposure to imipenem and ofloxacin was evaluated on urea agar plates. Briefly, 40 μ L of antibiotic treated as well as control samples ($A_{600} = 1$) was applied per well to urea agar plates and incubated for 8 h at 37 °C. Subsequent incubation at 4 °C (about 18 h) intensified the purple-red circular zone around the wells (local production of alkalinity) manifested against the yellow-pink background as a result of enzymic degradation of urea.

RESULTS

Sterile culture filtrates prepared from all *Klebsiella* strains tested showed lipolytic activity against Tween 20. Lipase activity lay in the range of 25–61 U/mL (*K. pneumoniae*), of 19–61 U/mL (*K. planticola*), of 31–67 U/mL (*K. oxytoca*); 38.7 U/mL was found in *K. terrigena*. All strains pro-

duced urease after application to urea agar slants, too. The effect of imipenem and ofloxacin on lipase and urease activity of 4 strains (of 57 isolated) was evaluated. These strains were sensitive to imipenem and the MICs of ofloxacin lay in the range of 0.39–1.56 mg/L. The results showed that imipenem at all sub-MICs tested increased lipolytic activity (Table I), a concentration of $\frac{1}{4}$ of the MICs being the most effective. In this case the lipolytic activity lay between 155 and 271 %, after $\frac{1}{8}$ it was 143–175 % and after $\frac{1}{16}$ it was in the range of 101–157 % of the control values (without imipenem).

All sub-MICs of ofloxacin caused an increase of lipolytic activity in two strains of *K. pneumoniae*, and two concentrations ($\frac{1}{8}$ or $\frac{1}{16}$ of the MIC) in *K. oxytoca*. The values of lipolytic activity were 118 and 108 % after treatment with $\frac{1}{4}$ of the MICs, after $\frac{1}{8}$ they were in the range of 115–149 % and after $\frac{1}{16}$ between 103–123 % (as compared with control values). On the other hand, lipolytic activity was suppressed mainly after exposure to $\frac{1}{4}$ or $\frac{1}{16}$ of the MIC in *K. planticola* (71.3 % or 85.8 %) and after $\frac{1}{4}$ in *K. oxytoca* (70.6 %).

Urease activity after exposure to imipenem and ofloxacin was practically unaffected.

DISCUSSION

During the last decades, resistance to the newer β -lactams has been emerging among *Klebsiella* strains isolated in Europe (Legakis *et al.* 1993; Kolář *et al.* 1995; Hanberger *et al.* 1999). Very often mainly carbapenems (imipenem or meropenem) as well as quinolones are the drugs of choice in the treatment of severe infections caused by these multiresistant strains. The *in vitro* as well as the *in vivo* findings suggest the usage of sub-MICs of the antibiotics as a strategy for suppression of bacterial virulence (Grimwood *et al.* 1989; Sofer *et al.* 1999). To our knowledge, the published data do not show a production of lipolytic substances in *Klebsiella* strains, although lipase has been found to be one of the virulence factors of some other bacteria (Rollof *et al.* 1988; Straus *et al.* 1992). Our strains produced lipolytic activity that was enhanced after exposure of the strains to imipenem. In the majority of cases ofloxacin increased this activity, but to a lesser extent in comparison with imipenem. Only a few authors studied the effect of antimicrobials in connection with bacterial lipolytic activity. Molinari *et al.* (1993) showed that the extent of changes in lipase activity of *Pseudomonas aeruginosa* isolates was dependent on strain and type of macrolide antibiotic, but lipase in the majority of the strains was not substantially affected. No alterations or only weak changes in lipase were found in *Acinetobacter baumannii* after exposure to imipenem (Hošťacká 1999).

Urealytic activity of some bacteria, including *Klebsiella* strains, is implicated in kidney and bladder stone formation and contributes to the pathogenesis of several diseases (Takeuchi *et al.* 1980; Warren *et al.* 1982). Our *Klebsiella* strains treated with the antibiotics tested did not show changes in urealytic activity. Inhibition of urease activity observed in *Proteus vulgaris* was dependent on antibacterial agents (Hamilton-Miller and Gargan 1979). Several compounds, including β -lactam and aminoglycoside antibiotics caused less than 20 % inhibition of urease. Some others did bring about significant inhibition but it did not reach the activity of one of the widely used inhibitors of urease (acetohydroxamic acid) which caused 30 % reduction. Similar results were published by Coppi and Bonardi (1970).

Our results showed that imipenem and ofloxacin in the majority of cases stimulated lipolytic activity in *Klebsiella* strains and did not affect urease. Similarly to these results, several *in vitro* studies documented enhanced production of other bacterial virulence factors after treatment with sub-MICs of antimicrobials (Grif *et al.* 1998; Schmitz *et al.* 1998; Yoh *et al.* 1999). Conditions in an *in vivo* infection are more different than those of *in vitro* experiments. The potential effect of these findings for infected organism would be evaluated in further experiments.

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