David Sevillano
Lorenzo Aguilar
Luis Alou
María-José Giménez
Fabio Cafini
Natalia González
José Prieto

Protection of *Enterococcus faecalis* in mixed cultures with carbapenemase-producing *Escherichia coli* and *Bacteroides fragilis*: effect of the bacterial load

Microbiology Dpt., School of Medicine, Univ. Complutense, Avda. Complutense s/n, 28040 Madrid, Spain

ABSTRACT

Introduction. This study explores effects of pH and inoculum size on imipenem versus tigecycline activity against *E. coli*, *B. fragilis* and *E. faecalis*, both in individual and mixed cultures.

Methods. MIC/MBCs (mg/L) of tigecycline and imipenem were 0.12/≥16 and 4/4 for *E. coli*, 0.12/0.5 and ≥16/≥16 for *B. fragilis*, and 0.12/≥16 and 2/≥16 for *E. faecalis*, respectively. Killing curves in supplemented Brucella broth were performed at pH 7 or 5.8, with two final inocula (≈10^5 or ≈10^7 cfu/ml) of each isolate (individual cultures) and with 1:1:1 mixed inocula. Tubes were 48h incubated at 37ºC in anaerobiosis. Final concentrations (estimated concentrations in colon) were 1.50 mg/L for tigecycline and 26.40 mg/L for imipenem, with antibiotic-free curves as controls. Experiments were performed in triplicate.

Results. Imipenem showed inoculum effect against *E.coli* and *B. fragilis*, with reductions in initial inocula in experiments with standard inocula contrasting with increases in experiments with high inocula (both individual and mixed cultures). Against *E. faecalis* no inoculum effect for imipenem was observed in individual cultures, with marked reductions in initial inocula regardless inoculum size. However in mixed experiments the indirect protection of *E. faecalis* by the two gramnegatives resulted in bacterial regrowth. This protection was inoculum-dependant since it occurred with high but not with standard inocula. Tigecycline reduced initial inocula of the three isolates regardless culture type (individual/mixed) or experimental conditions (pH/inocula size), with lower reductions for the tolerant *E. faecalis*.

Conclusion. Carbapenemase activity was inoculum-dependant for self-protection and indirect protection of *E. faecalis*.

Key words: *B. fragilis*; *E. coli*; *E. faecalis*; Imipenem; Indirect pathogenicity; Inoculum effect; Mixed inocula; Tigecycline

Correspondence:
Lorenzo Aguilar
Microbiology Dept., School of Medicine, Universidad Complutense, Avda. Complutense s/n, 28040 Madrid, Spain.
Phone No.: 34-91-3941505
Fax: 34-91-3941511
E-mail: laguilar@med.ucm.es

Protección de *Enterococcus faecalis* en cultivo mixto con *Escherichia coli* y *Bacteroides fragilis* productores de carbapenemasa: efecto del inóculo bacteriano

RESUMEN

Introducción. Este estudio explora los efectos del tamaño del inóculo y el pH en la actividad de imipenem versus tigeciclina frente a *E. coli*, *B. fragilis* y *E. faecalis*, en cultivo individual y mixto.

Métodos. Los valores de CMI/CMB (mg/L) de tigeciclina e imipenem fueron 0,12/≥16 y 4/4 para *E. coli*, 0,12/0,5 y ≥16/≥16 para *B. fragilis*, y 0,12/≥16 y 2/≥16 para *E. faecalis*, respectivamente. Se realizaron curvas de letalidad en caldo Brucella suplementado a pH 7 o 5,8 con dos inóculos finales (≈10^5 o ≈10^7 ufc/ml) de cada aislado (cultivos individuales) y de un inóculo mixto en proporción 1:1:1. Los tubos se incubaron durante 48h a 37ºC en anaerobiosis. Las concentraciones antibióticas finales (concentraciones estimadas en colon) fueron 1,50 mg/L de tigeciclina y 26,40 mg/L de imipenem. Se usaron como control curvas de crecimiento bacteriano en medio sin antibiótico y los experimentos se realizaron por triplicado.

Resultados. Imipenem mostró efecto inóculo frente a *E.coli* y *B. fragilis*, con reducciones del inóculo inicial en los experimentos realizados con inóculo estándar en contraposición a los crecimientos del inóculo inicial observados en los experimentos realizados con inóculo alto, tanto en cultivos individuales como mixtos. Frente a *E. faecalis* imipenem no presentó efecto inóculo en cultivos individuales, con marcadas reducciones del inóculo inicial con independencia del tamaño del mismo. Sin embargo en cultivo mixto la protección indirecta de *E. faecalis* por los dos aislados gramnegativos produjo un recrecimiento bacteriano. Esta protección fue dependiente del tamaño del inóculo ya que ocurrió en los experimentos con inóculo alto pero no en los realizados con inóculo estándar. Tigeciclina redujo el inóculo inicial de los tres aislados con independencia del tipo de cultivo (individual/mixto) o las condiciones experimentales (pH/tamaño del inóculo), con menores reducciones en el caso de *E. faecalis* tolerante a este antibiótico.

Conclusión: La actividad carbapenemasa fue inóculo independiente para autoprotección y protección indirecta de *E. faecalis*.

Palabras clave: *B. fragilis*; *E. coli*; *E. faecalis*; Imipenem; Patógeno indirecto; Efecto inóculo; Inóculo mixto; Tigeciclina

INTRODUCTION

Acidic pH and low oxygen tension is the rule in mixed aerobic-anaerobic infections, and these conditions may affect antimicrobial susceptibility[1], as does the presence of high bacterial load. Classically, *Escherichia coli* and *Bacteroides fragilis* have been considered main responsible microorganisms of intra-abdominal infections. Enterococci is frequently co-isolated in secondary peritonitis and its isolation has been associated with comorbidities[2]. In secondary peritonitis that occurs several days after hospital admission, the potential presence of nosocomial bacteria exhibiting resistance traits (extended-spectrum β-lactamases -ESBL-, metallo- beta-lactamases, vancomycin-resistance...) should be suspected.

Carbapenemases have been known since the introduction of imipenem in 1980's[3]. Increased use of carbapenems following dissemination of multi-drug resistant ESBL- and AmpC- producing Enterobacteriaceae raised the fears of the diffusion of carbapenemase-producing strains among *B. fragilis*, non-fermenters and Enterobacteriaceae. Carbapenem high resistance in *B. fragilis* is emerging associated with *cfi*A-encoded class B metallo-beta-lactamase[4]. VIM- and IMP-carbapenemases are worldwide detectable, with an overall trend moving beyond *Pseudomonas aeruginosa* to Enterobacteriaceae[5]. Nowadays, there is a wide and rapid dissemination of multidrug resistant plasmids containing bla_{VIM-1} towards endemicity among different species[6].

This study explores the effect of different pH and inoculum sizes on the in vitro activity (in anaerobic conditions) of imipenem versus tigecycline against individual and mixed cultures of *E. coli*, *B. fragilis* and *Enterococcus faecalis*.

MATERIAL AND METHODS

Strains. Three clinical isolates were used: one VIM-1 producing *E. coli*, one imipenem-resistant *B. fragilis* and one *E. faecalis*.

Antibiotics. Powders of known potency of tigecycline (Pfizer Inc., Madrid, Spain) and of imipenem (Sigma-Aldrich Co., St. Louis, MO) were used.

In vitro susceptibility. MICs and MBCs were determined by microdilution following CLSI recommendations[7-9] with final inocula of 10^5 cfu/ml. Mueller-Hinton broth (Difco laboratories, Detroit, Mi, USA) supplemented with 25 mg/L of Ca^{2+} and 12.5 mg/L of Mg^{2+} and aerobic incubation was used for *E. coli* and *E. faecalis*, and Brucella broth (BD Diagnostics Co., Franklin Lake, NJ) supplemented with vitamin K1 (1 mg/L), haemin (5 mg/L) and 5% sheep blood (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA) and anaerobic incubation for *B. fragilis*. MICs and MBCs were determined in triplicate and modal values were considered. MICs were read at 24h for *E. coli* and *E. faecalis* and at 48h for *B. fragilis*. In addition MICs and MBCs for *E. coli* and *E. faecalis* were also aerobically determined in Brucella broth (BD Diagnostics) supplemented with vitamin K1 (1 mg/L), haemin (5 mg/L) and 5% sheep blood (Oxoid), and read at 24h.

Killing activity. Killing curves[9] in Brucella broth (BD Diagnostics) supplemented with vitamin K1 (1 mg/L), haemin (5 mg/L) and 5% sheep blood (Oxoid) were performed with two different final inocula (≈10^5 and ≈10^7 cfu/ml). Individual inocula of each study strain and mixed inocula composed by the three isolates in a 1:1:1 proportion were used. The final concentration of tigecycline was 1.50 mg/L, the estimated concentration in colon using a penetration rate of 1.73 (obtained from the proportion AUC_{colon}/AUC_{serum})[10] for a C_{max} in serum of 0.87 mg/L[11]. For imipenem the estimated concentration in colon using a penetration rate of 0.4 (obtained from the proportion C_{serum}/C_{mucosa})[12] for a C_{max} in serum of 66 mg/L[13] was 26.40 mg/L, and this was the final concentration used in experiments with imipenem. Antibiotic-free growth curves were used as controls. All tubes containing individual or mixed cultures we-

Table 1 **Modal MICs and MBCs of tigecycline and imipenem for the study strains determined following CLSI recommendations (inoculum= 10^5 cfu/ml) using different media and pH.**

	MIC/MBC (mg/L)					
	Tigecycline			Imipenem		
Isolates	MHB* pH=7	BB† pH=7	BB† pH=5.8	MHB* pH=7	BB† pH=7	BB† pH=5.8
E. coli	0.12/≥16	0.25/0.25	0.5/0.5	4/4	4/4	0.5/1
B. fragilis	ND	0.12/0.5	0.5/0.5	ND	≥16/≥16	≥16/≥16
E. faecalis	0.12/≥16	0.25/≥16	0.5/≥16	2/>16	2/8	2/8

*MHB= Mueller-Hinton broth supplemented with 25 mg/L of Ca^{2+} and 12.5 mg/L of Mg^{2+};

†BB= Brucella broth supplemented with vitamin K1 (1 mg/L), haemin (5 mg/L) and 5% sheep blood;

ND= not determined

Table 2 **Increases (Δlog_{10}; mean ± SD) in initial inocula (log_{10}; mean ± SD) in antibiotic-free controls at 24h and 48h for the study strains in individual and mixed cultures using two pH values and two inocula sizes. Positive signs mean regrowth of initial inocula**

		Low inocula				High inocula		
Strain	Culture	I. Inocula (log_{10})	Δlog_{10} at 24h	Δlog_{10} at 48h		I. Inocula (log_{10})	Δlog_{10} at 24h	Δlog_{10} at 48h
					pH = 7			
E. coli	Individual	5.42 ± 0.09	+3.35 ± 0.07	+3.23 ± 0.06		7.17 ± 0.14	+1.55 ± 0.07	+1.50 ± 0.26
	Mixed	5.43 ± 0.06	+3.27 ± 0.12	+3.27 ± 0.06		7.29 ± 0.05	+1.37 ± 0.15	+1.43 ± 0.25
B. fragilis	Individual	5.37 ± 0.05	+3.70 ± 0.14	+3.83 ± 0.15		7.33 ± 0.06	+2.10 ± 0.00	+1.90 ± 0.10
	Mixed	5.29 ± 0.04	+2.57 ± 0.45	+3.23 ± 0.15		7.15 ± 0.07	+1.53 ± 0.12*	+1.33 ± 0.21
E. faecalis	Individual	5.29 ± 0.09	+3.57 ± 0.06	+3.43 ± 0.12		7.24 ± 0.05	+1.70 ± 0.00	+1.63 ± 0.12
	Mixed	5.34 ± 0.10	+2.80 ± 0.30*	+2.43 ± 0.15*		7.29 ± 0.05	+1.10 ± 0.17*	+0.93 ±0.06*
					pH=5.8			
E. coli	Individual	5.46 ± 0.08	+3.15 ± 0.10	+3.03 ± 0.17		7.30 ± 0.12	+1.28 ± 0.10	+1.10 ± 0.26
	Mixed	5.42 ± 0.05	+3.20 ± 0.12	+3.10 ± 0.10		7.25 ± 0.11	+1.43 ± 0.13	+1.23 ± 0.22
B. fragilis	Individual	5.12 ± 0.08	+3.23 ± 0.45	+3.80 ± 0.00		7.11 ± 0.07	+2.07 ± 0.12	+2.08 ± 0.21
	Mixed	4.86 ± 0.18	+3.30 ± 0.14	+2.35 ± 0.50		6.87 ± 0.38	+1.23 ± 0.21*	+1.13 ± 0.68
E. faecalis	Individual	5.14 ± 0.08	+3.60 ± 0.14	+3.40 ± 0.08		7.15 ± 0.08	+1.30 ± 0.67	+1.48 ± 0.13
	Mixed	5.17 ± 0.05	+3.10 ± 0.18*	+2.60 ± 0.20*		7.14 ± 0.08	+1.45 ± 0.10	+1.03 ± 0.05*

*P<0.01 vs. reduction in individual culture

re incubated at 37ºC in anaerobic atmosphere (GasPack Ez, BD Diagnostics). Samples for colony counting were collected at 0, 24 and 48 h and plated onto McConkey agar (BD Diagnostics) incubated in environmental atmosphere as selective media for *E. coli*, onto colistin-nalidixic acid (CNA) agar (BD Diagnostics) incubated under 5% CO_2 as selective media for *E. faecalis*, and onto *Bacteroides* Bilis Esculin (BBE) agar supplemented with kanamycin (BD Diagnostics) incubated in anaerobiosis as selective media for *B. fragilis*. All experiments were performed in triplicate.

Data analysis. Log_{10} reductions (log_{10} colony counts at time 0 – log_{10} colony counts at each sampling time) were calculated. Comparisons between log_{10} reductions were performed by ANOVA with the Tukey's test for multiple comparisons. Due to multiple comparisons a P<0.01 was considered statistically significant.

RESULTS

In vitro susceptibility. Table 1 shows modal MIC and MBC values determined in the two media (at two pH for determinations in Brucella broth). When tested in the supplemented Mueller-Hinton broth, *E. coli* and *E. faecalis* (MIC= 0.12 mg/L) were tolerant to tigecycline (MBC/MIC ≥133), but when tested in the supplemented Brucella broth (pH=7) tolerance was only found for *E. faecalis* (tigecycline MBC/MIC ≥32). In the case of imipenem, *E. coli* was susceptible (susceptibility breakpoint: ≤4 mg/L), with higher MIC values in determinations at pH=7 (MIC= 4 mg/L) than at pH=5.8 (MIC= 0.5 mg/L). *B. fragilis* was fully resistant, both at pH 5.8 and 7, and *E. faecalis* was tolerant when tested in the supplemented Mueller-Hinton broth.

Antibiotic-free experiments. Table 2 shows mean initial inocula of the three study strains used in individual and mixed cultures in all experiments (with or without antibiotics).

All study isolates showed increases in initial inocula at 24h and 48h both in individual and mixed cultures regardless the initial inocula size or the pH.

By comparing growth rates in individual vs. mixed cultures, different patterns were found for the three isolates. The growth rate of *E. faecalis* in mixed cultures was significantly (P<0.01) lower than in individual cultures, at both pH values, and with both initial inocula. For *B. fragilis* this occurred only in high inocula experiments, while for *E. coli*, growth curves of individual cultures were similar than those of mixed cultures.

Effect of inocula size. In experiments with tigecycline (table 3), the effect of the use of different inocula sizes was observed for *E. coli*, but not for *B. fragilis* or *E. faecalis*. Significantly lower reductions in the initial inocula of *E. coli* were found in experiments with high inocula (vs. low inocula) at 24h in individual cultures (pH= 7) and at 48h in mixed cultures (pH= 5.8).

In experiments with imipenem (table 4), for *E. coli* and *B. fragilis* there was a marked effect of the inocula sizes, with reductions in initial inocula in experiments using low inocula contrasting with increases in experiments using high inocula, at both pH, and both in individual and mixed cultures. In the case of *E. faecalis*, this occurred only in mixed cultures.

Table 3 **Reductions (Δlog_{10}; mean ± SD) in initial inocula by tigecycline at 24h and 48h for the study strains in individual and mixed cultures using two pH values and two inocula sizes.**

Strain	Culture	Low inocula		High inocula	
		Δlog_{10} at 24h	Δlog_{10} at 48h	Δlog_{10} at 24h	Δlog_{10} at 48h
		pH = 7			
E. coli	Individual	4.10 ± 0.14	3.67 ± 0.67	1.03 ± 0.15*	2.23 ± 0.32
	Mixed	2.80 ± 1.14	2.10 ± 0.50	1.10 ± 0.20	1.97 ± 0.21
B. fragilis	Individual	1.23 ± 0.50	1.30 ± 0.26	0.93 ± 0.32	2.27 ± 0.23
	Mixed	2.47 ± 0.46	2.80 ± 1.08	2.80 ± 0.85	3.80 ± 0.52
E. faecalis	Individual	1.07 ± 0.25	1.20 ± 0.10	1.05 ± 0.07	1.07 ± 0.21
	Mixed	1.20 ± 0.66	1.03 ± 0.21	0.60 ± 0.30	0.87 ±0.06
		pH=5.8			
E. coli	Individual	0.88 ± 0.99	3.03 ± 0.80	1.13 ± 0.17	1.27 ± 0.32
	Mixed	1.07 ± 0.65	3.20 ± 0.70	0.70 ± 0.22	1.30 ± 0.52*
B. fragilis	Individual	0.37 ± 0.15	0.57 ± 0.12	0.67 ± 0.38	1.50 ± 0.30
	Mixed	1.57 ± 0.81	1.75 ± 0.21	3.50 ± 0.36	3.65 ± 0.52
E. faecalis	Individual	0.85 ± 0.39	0.55 ± 0.37	0.93 ± 0.22	1.10 ± 0.16
	Mixed	1.03 ± 0.33	0.90 ± 0.08	0.83 ± 0.22	0.60 ± 0.59

*Significant (P<0.01) lower reduction with high inocula vs. low inocula

Individual vs. mixed cultures in the presence of antibiotics. In experiments with tigecycline (table 3), no marked differences were found between reductions in initial inocula by comparing individual and mixed cultures.

In experiments with imipenem (table 4), no differences were found for *E. coli* and *B. fragilis* between experiments using individual and mixed cultures regardless pH or initial inocula size. For *E. faecalis* marked differences were found between individual and mixed experiments using high inocula, with reductions in the initial inocula in individual cultures in contrast to increases in mixed cultures at both pH values.

DISCUSSION

Gut microbiota may represent one of the major reservoirs for antibiotic resistance genes, favored by extremely high population densities that make gut conditions very favorable for horizontal gene transfer[14,15]. The bacterial density, together with low oxygen tension and acidic pH, are the hallmark of intraabdominal mixed infections. *E. coli* and *B. fragilis* are the principal species isolated in this type of infections together with enterococci that is not frequent in community-acquired intraabdominal infections but commonly present in patients with nosocomial-acquired secondary peritonitis[2]. It has been postulated that by lowering the oxidation-reduction potential in the microenvironment, facultative organisms may promote more favorable conditions for anaerobic growth, and that *B. fragilis* produces detectable levels of β-lactamase that may protect normally susceptible components of mixed infections from the action of antimicrobials[16]. In this sense, the gram-negative isolates used in the present study were selected because of their production of carbapenemases. Imipenem was selected to explore the potential protection of the susceptible gram-positive by inactivating enzymes compared with tigecycline as potential drug not affected by carbapenemases. Although not affected by carbapenemases, tigecycline exhibits bacteriostatic activity against *E. coli* and *E. faecalis*, showing tolerance (MBC/MIC ≥32) under standard test conditions.

Previous studies have shown that in vitro conditions in MIC determination affect susceptibility results[1,17]. For this reason, in the present study MICs and MBCs were determined not only following standard laboratory conditions but also under the same conditions (media and pH) of killing curves. By changing test conditions results for tigecycline differed since the tolerance observed in *E. coli* in determinations performed in Mueller-Hinton broth disappeared when the MIC and MBC were determined in Brucella broth (the media used in the killing curves). This did not occur for *E. faecalis* that was tolerant regardless the media used in the determination. In the case of imipenem and *E. faecalis* tolerance observed in Mueller-Hinton broth also disappeared when tested in Brucella broth.

In antibiotic-free experiments no great differences were observed between bacterial growth in individual and mixed cultures for *E. coli* and *B. fragilis*, but not for *E. faecalis* that showed significant lower growth rates in the presence of the other bacteria in experiments with low and high inocula, suggesting worse fitness for *E. faecalis* in polymicrobial niches.

Table 4 **Reductions (Δlog_{10}; mean ± SD) in initial inocula by imipenem at 24h and 48h for the study strains in individual and mixed cultures using two pH values and two inocula sizes. Regrowth of initial inocula is shown by positive signs.**

		Low inocula		High inocula	
Strain	Culture	Δlog_{10} at 24h	Δlog_{10} at 48h	Δlog_{10} at 24h	Δlog_{10} at 48h
		pH = 7			
E. coli	Individual	4.10 ± 0.10	4.10 ± 0.10	+1.80 ± 0.57*	+1.33 ± 0.12*
	Mixed	4.13 ± 0.06	3.60 ± 0.26	+1.03 ± 0.12*	+1.03 ± 0.06*
B. fragilis	Individual	3.23 ± 0.15	1.30 ± 1.37	+1.30 ± 0.36*	+1.23 ± 0.67
	Mixed	3.30 ± 0.75	0.63 ± 0.42	+1.00 ± 0.52*	+0.40 ± 0.56
E. faecalis	Individual	2.83 ± 0.29	3.03 ± 0.46	3.70 ± 0.10	4.43 ± 0.21
	Mixed	2.90 ± 0.28	2.83 ± 0.40	+0.75 ± 0.07*,†	+0.57 ± 0.15*,†
		pH=5.8			
E. coli	Individual	4.15 ± 0.10	4.17 ± 0.12	+1.20 ± 0.22*	+1.33 ± 0.28*
	Mixed	4.13 ± 0.05	3.63 ± 0.95	+0.85 ± 0.19*	+1.08 ± 0.29*
B. fragilis	Individual	2.00 ± 0.40	+0.20 ± 1.08	+1.47 ± 0.21*	+1.77 ± 0.12
	Mixed	2.73 ± 0.55	0.53 ± 0.50	+1.73 ± 0.23*	+1.75 ± 0.07*
E. faecalis	Individual	2.13 ± 0.13	2.63 ± 0.45	3.97 ± 0.12	4.53 ± 0.64
	Mixed	1.93 ± 0.28	2.40 ± 0.80	+0.65 ± 0.10*,†	+0.38 ± 0.17*,†

*Significant (P<0.01) lower reduction with high inocula vs. low inocula

†Significant (P<0.01) lower reduction in mixed vs. individual cultures

Classical studies have described the in vitro and in vivo inoculum effect shown by β-lactams[18-20]. In the present study, reductions in initial inocula of the two carbapenemase-producing gram-negatives observed in imipenem experiments with standard inocula contrast with increases in initial inocula in experiments using high inocula, both in individual and mixed cultures. This suggests that carbapenemases had not significant effect on imipenem activity in experiments with low inocula (>3 log_{10} reductions for *E. coli* in individual and mixed cultures), but marked effect when high inocula were used. Thus, it seems that, at least in vitro, carbapenemase activity is inoculum-dependant. In contrast, imipenem highly reduced the initial inoculum of *E. faecalis* in individual cultures, both using standard and high inocula (absence of inoculum effect). This was expected since the isolate used in the present study was not tolerant in the media used in killing curves. However in experiments using mixed inocula a regrowth was observed in experiments with high inocula, showing the indirect protection of *E. faecalis* from the action of imipenem attributable to carbapenemases produced by the gram-negative isolates. Indirect pathogenicity (protection of a susceptible microorganism by β-lactamases produced by other bacteria present in the niche) has been described in *in vitro* pharmacodynamic models (protection of *Streptococcus pyogenes* and/or *Streptococcus pneumoniae* by β-lactamase producing *Haemophilus influenzae*)[21], but with contradictory communications on its clinical relevance[22,23]. In the present study testing intraabdominal isolates, the indirect protection of *E. faecalis* by the two gram-negatives only occurred at high bacterial densities (not with standard inocula), indicating again that this indirect pathogenicity was inoculum-dependant.

In experiments with tigecycline, initial inocula of the three isolates were always reduced regardless the type of culture (individual/mixed) or experimental conditions (pH/inocula size). This occurred despite *E. faecalis* showed tolerance in the in vitro susceptibility determinations carried out using the conditions used in killing curves (Brucella broth and both pH). However reductions in initial inocula of *E. faecalis* were lower than those obtained for the two gram-negatives. Interestingly, in this study tigecycline highly reduced initial inocula of the *B. fragilis* isolate in mixed inocula experiments (>3.5 log_{10} when using high inocula with both pH). Although there is clinical evidence of the activity of tigecycline against anaerobic bacteria in mixed intra-abdominal infections, no correlation between MIC values, PK/PD data and clinical outcome has been found and therefore there are not defined breakpoints for susceptibility[24].

The results of this study show that, in vitro, carbapenemase activity was inoculum-dependant. This may be important in nosocomial intraabdominal infections where there is a high bacterial load and possible presence of multiresistance traits. The inoculum-dependant carbapenemase activity resulted not only in self-protection but also in indirect protection of other bacteria present in the niche (as *E. faecalis* in our study). This may have increasing importance since there is a worldwide trend of carbapenemases moving beyond *P. aeruginosa* to Enterobacteriaceae[5], and these enzymes are cause of concern in *B. fragilis*[25].

ACKNOWLEDGEMENTS

The authors thank L. Alcalá and E. Bouza (Microbiology Dpt., Hospital Universitario Gregorio Marañón, Madrid) for the supply of the *B. fragilis* isolate, and R. Cantón (Microbiology Dpt., Hospital Ramón y Cajal, Madrid) for the supply of the *E. coli* isolate used in this study.

This study was supported in part by an unrestricted educational grant from Pfizer S. L.U, Madrid, Spain.

REFERENCES

1. Falagas ME, McDermott L, Snydman DR. Effect of pH on in vitro antimicrobial susceptibility of the *Bacteroides fragilis* group. Antimicrob Agents Chemother 1997; 41:2047-49.
2. Cercenado E, Torroba L, Cantón R, Martínez-Martínez L, Chaves F, García-Rodríguez JA, et al. Multicenter study evaluating the role of enterococci in secondary bacterial peritonitis. J Clin Microbiol 2010; 48:456-9.
3. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm?. Clin Microbiol Rev 2005; 18:306-25.
4. Wybo I, De Bel A, Soetens O, Echahidi F, Vandoorslaer K, Van Cauwenbergh M, et al. Differentiation of cfiA-negative and cfiA-positive *Bacteroides fragilis* isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2011; 49:1961-4.
5. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev 2007; 20:440-58.
6. Tato M, Coque TM, Ruíz-Garbajosa P, Pintado V, Cobo J, Sader HS, et al. Complex clonal and plasmid epidemiology in the first outbreak of Enterobacteriaceae infection involving VIM-1 metallo-beta-lactamase in Spain: toward endemicity?. Clin Infect Dis 2007; 45:1171-8.
7. **Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Growth Aerobically - Seventh Edition: Approved Standard M7-A7. CLSI, Wayne, PA, USA, 2006.**
8. Clinical and Laboratory Standards Institute. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria- Seventh Edition: Approved Standard M11-A7. CLSI, Wayne, PA, USA, 2007.
9. National Committee for Clinical Laboratory Standards. Methods for Determining Bactericidal Activity of Antimicrobial Agents: Approved Guideline M26-A. NCCLS, Wayne, PA, USA, 1999.
10. Rubino CM, Ma L, Bhavnani SM, Korth-Bradley J, Speth J, Ellis-Grosse E, et al. Evaluation of tigecycline penetration into colon wall tissue and epithelial lining fluid using a population pharmacokinetic model and Monte Carlo simulation. Antimicrob Agents Chemother 2007; 51:4085-9.
11. US Food and Drug Administration. 2013. TYGACIL® prescribing information. http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021821s013s017s018lbl.pdf.
12. Kager L, Brismar B, Malmborg AS, Nord CE. Imipenem concentrations in colorectal surgery and impact on the colonic microflora. Antimicrob Agents Chemother 1989; 33:204-8.
13. US Food and Drug Administration. 2013. PRIMAXIN ® prescribing information. http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/050587s065,050630s028lbl.pdf.
14. Aminov RI. Horizontal gene exchange in environmental microbiota. Front Microbiol 2011; 2:158.
15. Salyers AA, Gupta A, Wang Y. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol 2004; 12:412-6.
16. Lorber B. *Bacteroides*, *Prevotella*, *Porphyromonas* and *Fusobacterium* species (and other medically important anaerobic gram-negative bacilli). En: Mandell GL, Bennett JE, Dolin R, editors. Mandell, Douglas, and Bennett Principles and Practice of Infectious Diseases, 6th ed. Philadelphia: Elsevier Churchill Livingstone, 2005; 2838-46.
17. Torrico M, González N, Giménez MJ, Alou L, Sevillano D, Navarro D, et al. Influence of media and testing methodology on susceptibility to tigecycline of Enterobacteriaceae with reported high tigecycline MIC. J Clin Microbiol 2010; 48:2243-6.
18. Soriano F, Ponte C. Comment on: Functional relationship between bacterial cell density and the efficacy of antibiotics. J Antimicrob Chemother 2009; 63:1301.
19. Soriano F, Edwards R, Greenwood D. Effect of inoculum size on bacteriolytic activity of cefminox and four other b-lactam antibiotics against *Escherichia coli*. Antimicrob Agents Chemother 1992; 36:223-6.
20. Soriano F, García-Corbeira P, Ponte C, Fernández-Roblas R, Gadea I. Correlation of pharmacodynamic parameters of five beta-lactam antibiotics with therapeutic efficacies in an animal model. Antimicrob Agents Chemother 1996;40:2686-90.
21. Sevillano D, Aguilar L, Alou L, Giménez MJ, González N, Torrico M, et al. Beta-lactam effects on mixed cultures of common respiratory isolates as an approach to treatment effects on nasopharyngeal bacterial population dynamics. PLoS One 2008; 3:e3846.
22. Pichichero ME, Casey JR, Mayes T, Francis AB, Marsocci SM, Murphy AM, et al. Penicillin failure in streptococcal tonsillopharyngitis: causes and remedies. Pediatr Infect Dis J 2000; 19:917-23.
23. Stjernquist-Desatnik A, Holst E. Tonsillar microbial flora: comparison of recurrent tonsillitis and normal tonsils. Acta Otolaryngol 1999; 119:102-6.
24. European Society of Clinical Microbiology and Infectious Diseases (ESCMID). 2013. Tetracyclines - EUCAST clinical MIC breakpoints. Available at: http://www.srga.org/eucastwt/MICTAB/MICtetracyclines.htm.
25. Livermore DM. Bacterial resistance to carbapenems. Adv Exp Med Biol 1995; 390:25-47.