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Inoculum effect of β-lactam antibiotics

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The phenomenon of attenuated antibacterial activity at inocula above those utilized for susceptibility testing is referred to as the inoculum effect. Although the inoculum effect has been reported for several decades, it is currently debatable whether the inoculum effect is clinically significant. The aim of the present review was to consolidate currently available evidence to summarize which β-lactam drug classes demonstrate an inoculum effect against specific bacterial pathogens. Review of the literature showed that the majority of studies that evaluated the inoculum effect of β-lactams were *in vitro* investigations of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*. Across all five pathogens, cephalosporins consistently displayed observable inoculum effects *in vitro*, whereas carbapenems were less susceptible to an inoculum effect. A handful of animal studies were available that validated that the *in vitro* inoculum effect translates into attenuated pharmacodynamics of β-lactams *in vivo*. Only a few clinical investigations were available and suggested that an *in vitro* inoculum effect of cefazolin against MSSA may correspond to an increased likeliness of adverse clinical outcomes in patients receiving cefazolin for bacteraemia. The presence of β-lactamase enzymes was the primary mechanism responsible for an inoculum effect, but the observation of an inoculum effect in multiple pathogens lacking β-lactamase enzymes indicates that there are likely multiple mechanisms that may result in an inoculum effect. Further clinical studies are needed to better define whether interventions made in the clinic in response to organisms displaying an *in vitro* inoculum effect will optimize clinical outcomes.

Introduction

With the introduction of natural penicillins for civilian use in the 1940s, physicians very quickly accepted the use of β-lactams, which offered rapid bacterial killing and a large therapeutic window.[1] As time progressed, the β-lactam class expanded to include an array of agents that vary in their breadth of antimicrobial coverage, and the drug class arguably remains the most significant bacterial countermeasure in the early 21st century. In an analogous timeline, it was first noted in the 1940s that the size of the bacterial inoculum may affect *in vitro* susceptibility testing of penicillin.[2] The impact of bacterial density on the activity of β-lactams was further expanded throughout the 20th century as clinicians questioned whether the selection of a β-lactam should be modified based on the anticipated quantity of the bacteria at the site of infection.[3,4] Although numerous studies have addressed the potential impact of such an inoculum effect, the clinical implications remain ambiguous today.

Numerous mechanisms have been proposed to explain why the pharmacodynamics of antibacterials may be attenuated against high densities of bacteria. As the amount of bacteria within a single site increases, the concentration of antimicrobials that interact with individual bacterial cells decreases.[5] The ability of anti-infectives to interact with bacterial cells may be further hampered by biofilms that are constructed during high-burden infections and coordinated by quorum sensing pathways.[6] In some cases, quorum sensing at a high bacterial inoculum can directly mediate expression of proteins that decrease antibiotic susceptibility, such as resistance enzymes or efflux pumps.[7,8] Another potential explanation for the inoculum effect is the decreased expression of specific penicillin-binding proteins during stationary-phase growth.[9] High-inoculum infections more rapidly reach stationary phase, thus diminishing the effect of antibiotics targeting penicillin-binding proteins, such as the β-lactams. Higher concentrations of bacteria can increase the subpopulation of pre-existing resistant bacteria while also enhancing the chances of a population spontaneously acquiring a beneficial mutation capable of decreasing antibiotic susceptibility (i.e. bacterial density exceeds mutation frequency). Lastly, enzymatic degradation of the drug to a sub-lethal concentration may only occur with a high concentration of bacteria. With a large number of bacteria present at the site of infection, a subpopulation of bacteria may die initially and release defensive proteins and enzymes into the local environment that protect the remaining cells through a mechanism known as antibiotic-mediated altruistic death. The process of drug-hydrolysing enzymes remaining active *in vitro* after cell lysis has led some scientists to view the inoculum effect as a purely

in vitro artefact with minimal clinical significance, whereas other studies posit that the inoculum effect may have a substantial impact on clinical outcomes.[10,11]

Despite the lack of clarity surrounding the inoculum effect of β-lactams, it seems likely that a combination of mechanisms contributes to the phenomenon *in vitro* and *in vivo*, with β-lactamase production being the most prevalent. The inoculum effect has been observed in several high-burden animal infection models of endocarditis and pneumonia,[12–16] and it has been studied in human patients suffering from endocarditis as well.[3] Commonly studied β-lactamase producers include MSSA strains that utilize type A or C β-lactamases,[15,17] as well as the Gram-negative organisms *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Given the diversity of β-lactamase production with Gram-negative pathogens, the type of β-lactamase evaluated has varied from relatively narrow-spectrum enzymes such as TEM[13] and SHV[14] to extended-spectrum β-lactamases[18–21] or carbapenemases.[22]

Review methodology

This review seeks to unify the available studies that investigated the role of the inoculum effect for β-lactams against clinically relevant organisms. To evaluate the available literature, a PubMed search of English-language articles was conducted using the term 'inoculum effect'. Additionally, the references from relevant studies were manually reviewed to identify additional eligible studies. All articles published up to November 2018 were considered for inclusion. *In vitro* and *in vivo* articles were included in the review if the studies evaluated the pharmacodynamics of at least one β-lactam at multiple inocula. Alternatively, *in vivo* studies were also included if bacterial isolates that displayed an *in vitro* inoculum were further evaluated in an animal model. Retrospective and prospective clinical studies were included if *in vitro* inoculum effects detected during susceptibility testing were related to clinical outcomes. After the literature search was completed, the greatest number of studies were available for *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *H. influenzae* and *Staphylococcus aureus*, which prompted these five pathogens to be the focus of the review.

To summarize the findings of all studies for each pathogen, articles were divided into three categories based on the proportion of bacterial isolates within the study that displayed an inoculum effect for a given β-lactam (majority, minority or negligible). The determination of whether a bacterial isolate displayed an inoculum effect was based on the author's definition provided in each respective paper. The *in vitro* inoculum effect was most commonly defined as a ≥4- or 8-fold MIC increase at the higher bacterial inoculum. Definitions for the inoculum effect *in vivo* varied between studies.

Inoculum effects of β-lactams against specific pathogens

E. coli

E. coli is the leading cause of healthcare-associated infections and is capable of invading the blood, urinary tract, gastrointestinal tract, intra-abdominal cavity and lungs.[23] Infections caused by *E. coli* are associated with mortality rates that can exceed 25% and are becoming more difficult to treat owing to the increasing clinical prevalence of β-lactamase enzymes and other resistance mechanisms.[24] The inoculum of an *E. coli* infection varies; median concentrations of *E. coli* in urinary tract infections are typically $\sim10^6$–10^7 cfu/mL[25,26] while infections in the intra-abdominal cavity or lungs can have a significantly denser bacterial inoculum up to $\sim10^8$–10^9 cfu/mL.[27–29] β-Lactams remain an important therapeutic option for the treatment of *E. coli* infections, which highlights the importance of understanding the contribution of the bacterial inoculum to their efficacy.

Our literature search revealed 25 studies that examined the inoculum effect of β-lactams against *E. coli* (Table 1).[4,14,18,19,21,30–49] Of the 25 studies, 16 examined the inoculum effect using MICs, while 6 employed higher-level *in vitro* analyses (time–kill or hollow-fibre infection models) and the remaining 3 looked at the effect of bacterial inoculum in animal models. Of the inoculum effect studies we reviewed, a majority (80%; 20/25 studies) reported on the presence or absence of β-lactamases. ESBL-producing *E. coli* isolates were studied in 16 of the papers. Although a majority of the studies in *E. coli* to date have been conducted *in vitro*, their findings are primarily consistent with each other and align with the animal data available.

Carbapenems and cephamycins were the least likely β-lactam subclasses to display an inoculum effect for *E. coli* isolates. Only 25% (3/12)[4,18,19,21,31–33,35,39,41–43] and 20% (2/10)[18,32–35,41,43,45–47] of studies found that the carbapenem and cephamycin subclasses had at least a minority of isolates displaying an inoculum effect, respectively. The low-inoculum effect frequency was primarily driven by the high number of studies with ESBL-producing isolates since carbapenems and cephamycins are weakly hydrolysed by this β-lactamase enzyme.

In general, the inoculum effect was more common for β-lactam/β-lactamase inhibitor combinations against *E. coli* than it was for carbapenems or cephamycins. β-Lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are capable of protecting the β-lactams by binding to class A ESBL enzymes (e.g. TEM, SHV and CTX-M). Despite this predicted activity of β-lactam/β-lactamase inhibitor combinations against ESBL-producing *E. coli*, a majority of studies found diminished efficacy at higher *E. coli* inocula for at least some of the tested isolates (68.8%; 11/16 studies).[14,18,19,21,30–40] There are at least two important factors that may have contributed to the observation that the inoculum effect was more common for β-lactam/β-lactamase inhibitor combinations than it was for carbapenems and cephamycins: (i) AmpC, which is not robustly inhibited by the tested β-lactamase inhibitors, was investigated in three of the papers that found an inoculum effect;[31,33] and (ii) β-lactamase expression may exceed β-lactamase inhibitor concentrations at higher inocula, making the β-lactam more vulnerable to hydrolysis.

The inoculum effect against *E. coli* was most common for cephalosporins, monobactams and penicillins. Each of the studies that evaluated β-lactamase-producing *E. coli* found an inoculum effect for at least a minority of isolates exposed to cephalosporins (100%; 18/18 studies),[4,14,18,19,31–35,38,39,41,43–47,49] monobactams (100%; 8/8 studies)[33,34,38,39,43,45,46,48] and penicillins (80%; 4/5 studies).[34,36,37,39,46] The high inoculum effect rate for cephalosporins, monobactams and penicillins was likely due to the high prevalence of ESBL enzymes in the challenged isolates.

Table 1. Summary of studies evaluating the inoculum effect of β-lactams against *E. coli*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
β-Lactam/β-lactamase inhibitor combinations			
	MIC; piperacillin/tazobactam; 22 isolates with TEM, SHV or CTX-M BL (% unknown)[32]	MIC; piperacillin/tazobactam; 35 isolates with SHV, TEM, Toho or AmpC (% unknown)[33]	MIC; amoxicillin/clavulanate; TEM-1 (0/11)[36]
	time–kill; piperacillin/tazobactam; no BL (1/1)[30]	MIC; ampicillin/sulbactam, ticarcillin/clavulanate, piperacillin/tazobactam; 20 isolates with BL not reported (% unknown)[34a]	MIC; piperacillin/tazobactam; CTX-M (4/80)[18]
	time–kill; piperacillin/tazobactam; no BL (2/2), TEM (1/1)[31]	MIC; aztreonam/clavulanate, cefotaxime/clavulanate, ceftazidime/clavulanate, cefpodoxime/clavulanate, cefepime/clavulanate; 13 isolates with TEM, SHV, CTX-M, AmpC, ACT or no BL (% unknown)[38b]	MIC; mecillinam/clavulanate; CTX-M, OXA-1, SHV or VEB (11/48)[37]
	time–kill; ceftolozane/tazobactam; 1 no BL (1/1), AmpC (1/1), CMY (1/1), CTX-M (1/1)[121]	time–kill, MIC; ceftriaxone, sulbactam; SHV (1/2)[14c]	MIC; amoxicillin/clavulanate; SHV (0/1), TEM (0/4), other BL (0/4)[39]
	MIC; piperacillin/tazobactam; 99 isolates with CTX-M (100% of evaluable isolates)[19]	time–kill, MIC; amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanate; no BL (7/16), TEM (4/15)[35] murine sepsis model; piperacillin/tazobactam, amoxicillin/clavulanate; no BL (1/2), CTX-M (1/2)[21d]	hollow-fibre model; ampicillin/sulbactam; TEM (0/5)[40]
Carbapenems			
	MIC; imipenem; no BL (2/2)[41]	MIC; ertapenem, meropenem, imipenem; SHV (3/3), TEM (3/12), other BL (2/12)[39]	MIC; imipenem, meropenem; 6 isolates with BL not reported (0%)[42] MIC; imipenem; BL not reported (0/1)[43] MIC; imipenem; BL not reported (0/5)[4] MIC; meropenem; CTX-M (0/80)[18] MIC; ertapenem, imipenem, meropenem; 99 isolates with CTX-M (% unknown)[19]
	murine sepsis model; imipenem; no BL (1/1), CTX-M (1/1)[21d]		MIC; ertapenem, imipenem, meropenem; 22 isolates with TEM, SHV or CTX-M BL (% unknown)[32] MIC; meropenem; 35 isolates with SHV, TEM, Toho or AmpC (% unknown)[33] time–kill, MIC; imipenem; none (0/4), TEM (0/4)[35] time–kill; ertapenem; no BL (0/2), TEM (0/1), AmpC (0/1)[31]
Cephalosporins			
	MIC; ceftizoxime, cefotaxime; no BL (4/4)[41] MIC; cefamandole, cefalexin, cefalotin; 75 isolates with BL not reported (% unknown)[47]	MIC; ceftizoxime, ceftriaxone; 20 isolates with BL not reported (% unknown)[34]	–

Continued

Table 1. *Continued*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
	MIC; ceftazidime, cefoperazone, ceftriaxone, cefotaxime; BL not reported (4/4)[43]		
	MIC; cefoperazone, ceftazidime, ceftriaxone; BL not reported (9/14)[4]		
	MIC; cefotaxime; TEM (2/2)[44]	MIC; ceftazidime, cefepime, cefotaxime; CTX-M (35%–100%)[18e]	
	MIC; ceftibuten, cefotaxime, ceftazidime, cefixime, cefpodoxime; 4 isolates with TEM (9/12), SHV (3/4)[45]		
	MIC; ceftriaxone, ceftazidime, cefotaxime, cefepime; SHV (4/4), TEM (12/14), other BL (7/13)[39]	MIC; cefotaxime, ceftazidime, cefpodoxime, cefepime; 13 isolates with no BL, TEM, SHV, CTX-M, AmpC or ACT (% unknown)[38b]	
	MIC; cefotaxime, ceftazidime, cefepime; 99 isolates with CTX-M (100% of evaluable isolates)[19]		
	MIC; cefotaxime, ceftazidime, cefepime; 22 isolates with TEM, SHV or CTX-M BL (% unknown)[32]	time–kill, MIC; cefalotin, cefotaxime, cefpirome; no BL (2/12), TEM (5/12)[35]	
	time–kill; ceftriaxone; no BL (2/2), TEM (1/1)[31]		
	time–kill, MIC; ceftriaxone; SHV (2/2)[14c]	murine thigh infection model, MIC; cefepime; no BL, TEM, other ESBL (% unknown)[49g]	
	rat peritoneal infection model, MIC; cefuroxime, cefotaxime; no BL (1/2), BL (1/2)[46f]		
Cephamycins			
		MIC; cefoxitin; 22 isolates with TEM, SHV or CTX-M BL (% unknown)[32]	MIC; cefminox, cefoxitin; no BL (0/4)[41]
			MIC; cefoxitin; 75 isolates with BL not reported (% unknown)[47]
			MIC; cefoxitin; BL not reported (0/1)[43]
			MIC; cefoxitin; TEM (0/3), SHV (0/1)[45]
		MIC; cefoxitin, cefotetan; 20 isolates with BL not reported (% unknown)[34]	MIC; cefotetan; 35 isolates with SHV, TEM, Toho or AmpC (% unknown)[33]
			MIC; cefminox; CTX-M (0/80)[18]
			time–kill, MIC; cefoxitin; no BL (0/4), TEM (0/4)[35]
			rat peritoneal infection model, MIC; cefoxitin; no BL (0/1), BL (0/1)[46]
Monobactams			
	MIC; aztreonam; 20 isolates with BL not reported (% unknown)[34]	MIC; aztreonam; 13 isolates with no BL, TEM, SHV, CTX-M, AmpC or ACT (% unknown)[38b]	–
	MIC; aztreonam; SHV (1/1), TEM (2/3), other BL (2/3)[39]		
	MIC; aztreonam; BL not reported (1/1)[43]	hollow fibre model; aztreonam; NDM, OXA (1/1)[48h]	
	MIC; aztreonam; TEM(3/3)[45]		

Continued

Table 1. *Continued*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
	MIC; aztreonam; 35 isolates with SHV, TEM, Toho or AmpC (% unknown)[33]		
	rat peritoneal infection model, MIC; aztreonam; no BL (1/1), BL (1/1)[46]		
Penicillins			
	MIC; mecillinam; TEM-1 (10/11)[36]	MIC; ampicillin, ticarcillin, piperacillin; not reported (% unknown)[34a]	MIC; amoxicillin; other BL (0/4)[39]
	MIC; mecillinam; CTX-M, OXA-1, SHV or VEB (41/48)[37]	rat peritoneal infection model, MIC; ampicillin, piperacillin; no BL (1/2), BL (1/2)[46i]	

BL, β-lactamase.
Data are shown as: experimental system(s); antibiotic(s); β-lactamase(s) and number/% of isolates showing inoculum effect.
Articles were sorted by the β-lactam(s) investigated and then divided into three categories based on the proportion of isolates within the study that displayed an inoculum effect (majority, minority and negligible). Each line within a column describes the experimental system and β-lactamases investigated by a single study. Fractions indicate the number of isolates displaying an inoculum effect in the numerator and the total number of isolates in the denominator, whereas '% unknown' indicates that the exact percentage of isolates displaying the inoculum effect was not clear in the study.
[a]Inoculum effect was apparent for piperacillin and piperacillin/tazobactam but not ampicillin, ampicillin/sulbactam, ticarcillin or ticarcillin/clavulanate.
[b]No inoculum effect observed when comparing MICs conducted at 5×10^5 and 5×10^6 cfu/mL for non-β-lactamase-producing isolate. Minority of isolates displayed an inoculum effect when comparing MICs conducted at 5×10^5 and 5×10^6 cfu/mL for ESBL-producing isolates. Inoculum effect observed for all isolates when comparing MICs conducted at 5×10^5 and 5×10^7 cfu/mL.
[c]Ceftriaxone and ceftriaxone/sulbactam MICs at an inoculum of 5×10^7 cfu/mL were more predictive of outcomes in a rabbit endocarditis model than at 5×10^5 cfu/mL. Increased concentrations of sulbactam minimized the inoculum effect.
[d]Piperacillin/tazobactam was more affected than amoxicillin/clavulanate by bacterial inoculum in terms of bacterial killing and mortality for both ATCC 25922 and a CTX-M-producing *E. coli*. Imipenem showed reduced bacterial killing at high inoculum for both ATCC 25922 and a CTX-M-producing *E. coli* but mortality rates were minimally affected.
[e]35%, 85%, and 100% of isolates displayed an inoculum effect for ceftazidime, cefepime and cefotaxime, respectively.
[f]Inoculum effect was apparent in rats for cefotaxime but not cefuroxime.
[g]All isolates displayed an inoculum effect based on MIC tests. The $T_{>MIC}$ required to achieve similar effectiveness was not significantly different between the inocula. Higher doses were still required to achieve similar effectiveness at 10^7 cfu/mL for some of the isolates.
[h]Aztreonam exposure caused *E. coli* at both a low and high inoculum to have no viable bacterial counts; however, the investigators noted long filamentous, non-replicating cells in the high-inocula experiments but not the low-inocula experiments.
[i]Inoculum effect was apparent in rats for piperacillin but not ampicillin.

There were three *in vivo* studies that examined the effect of a high *E. coli* inoculum on the efficacy of various β-lactams. Docobo-Pérez *et al.*[21] used an *in vivo* murine sepsis model to analyse the effect of bacterial inoculum on carbapenem and β-lactam/β-lactamase inhibitor efficacy against WT and ESBL-producing *E. coli*. In contrast to the majority of *in vitro* studies, which showed no inoculum effect for carbapenems, Docobo-Pérez *et al.* found that imipenem activity was significantly reduced at a higher inoculum based on bacterial concentrations in the spleen following treatment. Despite reduced bacterial killing, the inoculum's effect on mortality was relatively small (mortality did not change for CTX-M-producing *E. coli* and increased from 0% to 6.7% for WT *E. coli* at the high bacterial inoculum). The diminished imipenem activity in the murine sepsis model was overcome by increasing the imipenem dose according to the *in vitro* MIC obtained at a higher inoculum. Docobo-Pérez *et al.* also found that piperacillin/tazobactam activity was significantly impacted by a high inoculum of WT *E. coli* while amoxicillin/clavulanate maintained its efficacy against this isolate. However, both β-lactam/β-lactamase inhibitor combinations displayed reduced activity at the high inoculum for the CTX-M-producing *E. coli*. Soriano *et al.*[46] employed a rat peritoneal infection model to define the minimum dosage of β-lactam antibiotics that reduced mortality from infection by either WT or β-lactamase-producing *E. coli*. *In vitro* susceptibility testing revealed that piperacillin, cefotaxime and aztreonam displayed a significant inoculum effect whereas cefuroxime, ampicillin and cefoxitin MICs were minimally affected by inoculum. The *in vitro* inoculum effect correlated well with *in vivo* outcomes in the rat peritoneal model as substantially higher doses of piperacillin, cefotaxime and aztreonam were required to reduce mortality in rats. Lastly, Maglio *et al.*[49] used a neutropenic thigh infection model to study the effectiveness of cefepime against two different inocula of WT ($n=2$) and ESBL-producing ($n=4$) *E. coli*. Cefepime doses administered to mice were selected based on the

MIC of the organisms at an inoculum of either 10^5 or 10^7 cfu/mL to match the infection inoculum. Therefore, the $T_{>MIC}$ required for an 80% effective dose (ED_{80}) could be compared between the inocula. All four ESBL-producing and one of the WT *E. coli* isolates required a lower $T_{>MIC}$ to achieve the target ED_{80} at the higher inoculum, which led the authors to conclude that cefepime may not be vulnerable to an inoculum effect against ESBL-producing *E. coli in vivo*. However, it appears that higher cefepime doses were required to achieve similar effectiveness at the higher inoculum for some *E. coli* isolates, though the specific doses were not reported.

While most data suggest that the β-lactamase enzyme is the probable cause of the *E. coli* inoculum effect, 66.7% (6/9 isolates) of *E. coli* without β-lactamases also showed an inoculum effect for at least one β-lactam.[30,31,35,38,41,46] The small number of *E. coli* isolates without β-lactamases prevents us from determining whether the inoculum effect in WT *E. coli* is specific to a β-lactam subclass. However, these limited data do support the notion that there are multiple mechanisms responsible for the inoculum effect and it is not only the result of higher concentrations of β-lactamase enzyme.

Overall, the inoculum effect was most commonly observed in β-lactamase-producing *E. coli* isolates when the tested β-lactam was susceptible to the β-lactamase present. Cephalosporins, monobactams and penicillins were most prone to the inoculum effect for ESBL-producing *E. coli*. β-Lactam/β-lactamase inhibitor combinations may also be less active at higher bacterial inocula while carbapenems and cephamycins were generally found to be immune to the effects of increased *E. coli* inocula. Since not all β-lactamase-producing *E. coli* isolates appear as phenotypically resistant at a standard susceptibility testing inoculum,[50] expanded use of rapid diagnostics or other β-lactamase screening approaches may prevent suboptimal drug selection for infections with a high bacterial inoculum. Considering that the available data discussed herein are either *in vitro* or in animal models, future studies are warranted that further examine the relevance of the β-lactam inoculum effect against *E. coli* in patients.

P. aeruginosa

P. aeruginosa is an opportunistic pathogen that has a tendency to cause infections in immunocompromised hosts associated with high rates of mortality that exceed 30%.[51] *P. aeruginosa* is a significant cause of pneumonia, urinary tract infections, intra-abdominal infections and surgical site infections. Infections by *P. aeruginosa* in the lung have been demonstrated to have a bacterial burden as high as 10^8 cfu/mL[52] while urinary tract infections typically have an inoculum closer to 10^6 cfu/mL.[26] The impact of the differences in burden of infection for *P. aeruginosa* on β-lactam activity has been frequently examined *in vitro*. We identified 17 studies (Table 2) that examined the inoculum effect for β-lactams against *P. aeruginosa*; 10 of these studies included only MIC data, 5 included time–kill experiments and 2 used animal models. Unfortunately, only 47.1% (8/17 studies) of the studies tested the included *P. aeruginosa* isolates for the presence of β-lactamases, which complicates interpretation of these data.

Although the inoculum effect data against *P. aeruginosa* are primarily from *in vitro* studies, the findings within each β-lactam subclass were generally consistent. Both studies that investigated the inoculum effect for β-lactam/β-lactamase inhibitor combinations against *P. aeruginosa* found an inoculum effect for piperacillin/tazobactam.[16,53] None of the studies (0/5 studies) that investigated carbapenems against *P. aeruginosa* found an inoculum effect in a majority of the tested isolates.[4,16,42,54–56] The two studies that reported some degree of an inoculum effect for carbapenems had weak supporting evidence. In the first study, Chow *et al.*[54] determined agar-dilution MICs on 270 *P. aeruginosa* isolates and found only a median 2-fold increase of imipenem MICs at the higher inoculum (traditional inoculum effect definition of ≥4- or 8-fold MIC change at higher inoculum). The second study, by Mimoz *et al.*,[16] found an *in vitro* inoculum effect for imipenem during susceptibility testing (4-fold MIC increase), but imipenem was still capable of significantly reducing bacterial burdens *in vivo* against a high-inoculum pneumonia in a rat infection model. Thus, the available *in vitro* and *in vivo* evidence does not suggest that carbapenems display a significant inoculum effect against *P. aeruginosa*.

The cephalosporin, monobactam and penicillin subclasses were particularly susceptible to the inoculum effect in *P. aeruginosa*. Each of the 11 *in vitro* studies and 1 animal model study that investigated cephalosporins against *P. aeruginosa* observed inoculum effects for at least a minority of the tested isolates.[4,16,53–62] Interestingly, five of the studies found an inoculum effect for cephalosporins against *P. aeruginosa* that did not report having a β-lactamase.[53,56–59] However, it is probable that these isolates had at least low-level expression of AmpC that was just not reported. The study by Mimoz *et al.*[16] also found that there was an *in vivo* inoculum effect for cefepime by MIC testing (>32-fold increase at higher inoculum) that corresponded to treatment failure in the rat pneumonia model. For the monobactam class, all four studies found an *in vitro* inoculum effect against *P. aeruginosa* by susceptibility testing. However, none of the studies reported using a β-lactamase-producing bacterial isolate or confirmed the inoculum effect *in vivo*. Penicillin inoculum effects were consistently observed against *P. aeruginosa*. All nine *in vitro* studies we reviewed (100%) found an inoculum effect for the penicillins against at least a minority of the tested bacterial isolates, but the results are hard to interpret owing to the limited antibiotic resistance information provided (i.e. β-lactamases not reported).[54–56,59,61,63–66] Furthermore, since penicillins without their respective β-lactamase inhibitors are not used clinically against *P. aeruginosa*, the relevance of this *in vitro* inoculum effect is likely minimal.

An *in vitro* inoculum effect against *P. aeruginosa* has been frequently reported for the cephalosporins, monobactams and penicillins. The *P. aeruginosa* chromosome encodes an inducible *ampC* gene that mitigates the potential importance of an inoculum effect of penicillins. Limited data suggest that carbapenems are not as susceptible to the inoculum effect in *P. aeruginosa* and more data are required for β-lactam/β-lactamase inhibitor combinations. Despite the fact that the current data are consistent for each β-lactam subclass, there remain few studies that attempt to investigate the role of the inoculum effect in animal models and no studies to our knowledge in humans. The currently available data support a future clinical study that investigates the predictive value of cephalosporin susceptibility tests conducted at a high inoculum (i.e. 5×10^7 cfu/mL). Future studies should also include carbapenemase-producing strains.

Table 2. Summary of studies evaluating the inoculum effect of β-lactams against *P. aeruginosa*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
β-Lactam/β-lactamase inhibitor combinations			
	rat pneumonia model, MIC; piperacillin/tazobactam; PER-1 (1/1)[16] time–kill; piperacillin/tazobactam; no BL (3/3)[53]	–	–
Carbapenems			
	–	MIC; imipenem; 270 isolates with BL not reported (% unknown)[54a] rat pneumonia model, MIC; imipenem; PER-1 (1/2)[16b]	MIC; imipenem; BL not reported (0/5)[4] MIC; imipenem, meropenem; BL not reported (0/6)[42] MIC; imipenem; 55 isolates with BL not reported (% unknown)[55] MIC, MBC, time–kill; imipenem; no BL (0/1)[56]
Cephalosporins			
	MIC; cefoperazone, moxalactam; BL not reported (2/2)[60] MIC, MBC; moxalactam; 10 isolates with BL not reported (% unknown)[61] MIC; cefepime, ceftazidime; 55 isolates with BL not reported (% unknown)[55] MIC; ceftazidime, cefoperazone, cefsulodin; 270 isolates with BL not reported (% unknown)[54] MIC; ceftazidime, cefoperazone, moxalactam, ceftizoxime; 92 isolates with BL not reported (% unknown)[62] rat pneumonia model, MIC; cefepime; PER-1 (1/1)[16] MIC, MBC, time–kill; cefoperazone, cefotaxime, ceftazidime, moxalactam; no BL (1/1)[56] time–kill; ceftazidime/cefepime; no BL (3/3)[53] time–kill, biofilm; ceftazidime; no BL (1/1), with BL (1/1)[57] time–kill; ceftazidime; no BL (1/1)[58] time–kill; cefepime; with BL (1/1)[55]	MIC; cefoperazone, ceftazidime, ceftriaxone; 5 isolates with BL not reported (2/5)[4]	–
Monobactams			
	MIC; aztreonam; BL not reported (2/2)[60] MIC, MBC; aztreonam; no BL (1/1)[56]	MIC; aztreonam; 55 isolates with BL not reported (% unknown)[55] MIC; aztreonam; 270 isolates with BL not reported (% unknown)[54]	–
Penicillins			
	MIC; carbenicillin; BL not reported (2/2)[64] MIC, MBC; ticarcillin; 10 isolates with BL not reported (% unknown)[61] MIC; carbenicillin, ticarcillin; 31 isolates with BL not reported (% unknown)[63]	MIC; azlocillin, carbenicillin, mezlocillin, ticarcillin; 255 isolates with BL not reported (% unknown)[66c] MIC; piperacillin, ticarcillin, carbenicillin; 270 isolates with BL not reported (% unknown)[54d] MIC; piperacillin; 55 isolates with BL not reported (% unknown)[55]	–

Continued

Table 2. *Continued*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
	MIC, time–kill; carbenicillin; with BL (4/4)[65]		
	MIC, MBC, time–kill; azlocillin, piperacillin, ticarcillin; no BL (1/1)[56]		
	MIC, time–kill; piperacillin; no BL (1/1)[59]		

BL, β-lactamase.
Data are shown as: experimental system(s); antibiotic(s); β-lactamase(s) and number/% of isolates showing inoculum effect.
Articles were sorted by the β-lactam(s) investigated and then divided into three categories based on the proportion of isolates within the study that displayed an inoculum effect (majority, minority and negligible). Each line within a column describes the experimental system and β-lactamases investigated by a single study. Fractions indicate the number of isolates displaying an inoculum effect in the numerator and the total number of isolates in the denominator, whereas '% unknown' indicates that the exact percentage of isolates displaying the inoculum effect was not clear in the study.
The *P. aeruginosa* chromosome encodes *ampC* and has not been individually listed for each study in this table.
[a]Median 2-fold increase in imipenem MICs for 270 isolates.
[b]The inoculum effect was observed in one of two experimental models. Imipenem MIC increased from 2 mg/L at an inoculum of 10^5 cfu/mL to 8 mg/L at an inoculum of 10^7 cfu/mL but was still able to significantly reduce bacterial burden in the rat pneumonia model following infection at a high inoculum.
[c]No inoculum effect observed against carbenicillin or ticarcillin; significant inoculum effect observed against azlocillin and mezlocillin.
[d]Minimal inoculum effect for ticarcillin or carbenicillin; significant inoculum effect observed for piperacillin.

K. pneumoniae

K. pneumoniae is part of the normal human flora and is associated with upwards of 8% of all healthcare-associated infections.[23,67] *K. pneumoniae* most commonly causes urinary tract infections, pneumonia and bacteraemia with mortality rates >30%.[68] Carbapenem-resistant isolates of *K. pneumoniae* have become increasingly prevalent and cause mortality rates as high as 50%.[69] The site of *K. pneumoniae* infection likely contributes to the size of the bacterial burden in a patient; for example, the inoculum can exceed 10^7 cfu/mL in the lungs but is typically orders of magnitude lower in the blood or urine.[70] Of the 19 studies we identified that examined the inoculum effect of β-lactams against *K. pneumoniae*, 17 were *in vitro* (15 MIC and 2 time–kill),[4,13,20,32–34,38,39,42,43,45,71–78] 2 used animal models[20,76] and none looked at the inoculum effect in patients (Table 3). Isolates were tested for the presence of β-lactamase enzymes in 83.3% (15/18 studies) of the studies. Of note, bla_{SHV-1} is almost universally found in *K. pneumoniae*; however, it is primarily a penicillinase and is unlikely to substantially affect the β-lactams that are reviewed herein.

There was not a clear trend between the few studies that examined the inoculum effect in *K. pneumoniae* for β-lactam/β-lactamase inhibitor combinations,[13,20,32–34,38,39] carbapenems[4,20,33,39,42,43,71–77] or cephamycins,[32–34,43,45] where at least a minority of isolates displayed an inoculum effect in 71.4% (5/7), 60% (9/15) or 60% (3/5) of studies, respectively. However, the cephalosporin subclass of β-lactams was consistently less active at a high *K. pneumoniae* inoculum. Nearly all of the studies that evaluated the effect of bacterial density on the activity of cephalosporins against *K. pneumoniae* (88.2%; 15/17 studies) found that a majority or minority of isolates displayed an inoculum effect for the β-lactamase-producing isolates.[4,13,20,33,38,39,43,45,73,74,76–78] There were three studies that also evaluated *K. pneumoniae* isolates without β-lactamases and two of these did not display an inoculum effect for cephalosporins.[13,77] There were a few studies that investigated the monobactams for an inoculum effect against predominantly ESBL-producing *K. pneumoniae* isolates and all of them (6/6 studies) found some degree of reduced antibacterial activity at the higher inoculum.[33,34,38,39,43,45]

There were only two studies that we found that investigated *in vivo* inoculum effects of β-lactams against *K. pneumoniae* in animal models. Harada *et al.*[20] discovered a pronounced inoculum effect for piperacillin/tazobactam, but not meropenem, against an ESBL-producing *K. pneumoniae* isolate in a mouse model of pneumonia. All of the piperacillin/tazobactam- and meropenem-treated mice lived following a low-inoculum infection ($n = 7$; 10^4 cfu/mouse), whereas only the meropenem-treated mice survived a higher *K. pneumoniae* inoculum ($n=7$; 10^6 cfu/mouse). The authors concluded that meropenem is more stable than piperacillin/tazobactam to the *in vivo* inoculum effect for ESBL-producing *K. pneumoniae*. Szabó *et al.*[76] evaluated the activity of imipenem and cefepime against a high-inoculum of SHV-5-producing *K. pneumoniae* in a murine sepsis model. Mice were infected with 10^7 cfu/g of *K. pneumoniae* and were treated with either cefepime or imipenem. After 24 h, 93.3% ($n=14/15$) of the untreated or cefepime-treated mice died whereas only 46.7% ($n=7/15$) of imipenem-treated mice died. The authors determined that MICs obtained at a higher inoculum of 10^7 cfu/mL (imipenem MIC=0.5 mg/L; cefepime MIC=0.125 mg/L) were better able to predict mouse survival than MICs at a standard inoculum of 10^5 cfu/mL (imipenem MIC=0.125 mg/L; cefepime MIC >256 mg/L). The currently available data *in vivo* are in agreement with the more extensive *in vitro* data; however, additional studies are warranted.

In summary, the cephalosporins and monobactams were the only subclasses of β-lactams that consistently displayed an inoculum effect against *K. pneumoniae*. These studies suggest that for *K. pneumoniae* the expression of a β-lactamase active against cephalosporins or monobactams is predictive of at least an *in vitro* inoculum effect. There was not a clear inoculum effect pattern from the studies that evaluated β-lactam/β-lactamase inhibitor combinations, carbapenems and cephamycins. Interestingly, the inoculum effect for carbapenems was more commonly seen in *K. pneumoniae* than it was for any of the other Gram-negative

Table 3. Summary of studies evaluating the inoculum effect of β-lactams against *K. pneumoniae*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
β-Lactam/β-lactamase inhibitor combinations			
	MIC; piperacillin/tazobactam; 6 isolates with TEM BL (% unknown)[32]	MIC; piperacillin/tazobactam; 18 isolates with TEM, Toho or AmpC (% unknown)[33]	MIC; amoxicillin/clavulanate; TEM, other ESBL (1/6)[39]
	murine pneumonia model, time–kill, MIC; piperacillin/tazobactam; CTX-M (1/1)[20]	MIC; ampicillin/sulbactam, ticarcillin/clavulanate, piperacillin/tazobactam; 20 isolates with BL not reported (% unknown)[34a]	MIC; aztreonam/clavulanate, cefotaxime/clavulanate, ceftazidime/clavulanate, cefpodoxime/clavulanate, cefepime/clavulanate; 10 isolates with TEM, MIR, K, ACT or FOX (% unknown)[38]
		MIC, MBC; ceftriaxone, sulbactam; TEM (1/3)[13b]	
Carbapenems			
	MIC; ertapenem, imipenem, meropenem; ESBL (11/24)[72c]	MIC; imipenem; no BL (1/1), CMY-2 (0/1)[74]	MIC; imipenem, meropenem; SHV (2/16), TEM (1/8), AmpC (3/14)[77]
		MIC; imipenem, meropenem; CTX-M (3/9)[71]	MIC; ertapenem, imipenem, meropenem; 6 isolates with TEM BL (% unknown)[32]
	MIC; imipenem, meropenem; KPC, OXA, VIM (23/24)[71]	rabbit endophthalmitis model, MIC; imipenem; BL not reported (3/11)[4]	MIC; imipenem; BL not reported (0/1)[43]
	MIC; imipenem; 33 isolates with ESBL (% unknown), AmpC (24/28)[73]		time–kill; imipenem; CMY, DHA (0/1)[75]
	MIC; ertapenem, meropenem, imipenem; TEM, other ESBL (15/18)[39]	MIC; meropenem; 18 isolates with TEM, Toho or AmpC (6/18)[33]	murine model, MIC; imipenem (0/1)[76]
	MIC; imipenem, meropenem; 6 isolates with BL not reported (100%)[42]		murine pneumonia model, time–kill; meropenem; CTX-M (0/1)[20]
Cephalosporins			
	MIC; cefepime; no BL (1/1), CMY-2 (1/1)[74]	MIC; ceftazidime, cefotaxime, cefpodoxime, cefepime; 45 isolates with ESBL (% unknown)[20]	MIC; cefepime, cefpirome; no BL (1/4) [77]
	MIC; cefepime, cefotaxime, ceftazidime; ESBL (33/33), AmpC (28/28)[73]	MIC; ceftizoxime, ceftriaxone; 20 isolates with BL not reported (% unknown)[34]	
	MIC; cefotaxime, cefepime, ceftazidime, ceftriaxone; 18 isolates with TEM, Toho or AmpC (18/18)[33d]	MIC; cefotaxime, ceftazidime, cefixime, ceftibuten; TEM, other ESBL (92/142)[45e]	
	MIC; cefepime, cefpirome; SHV (6/8), TEM (8/8), AmpC (10/13)[77]	MIC; cefotaxime, ceftazidime, cefpodoxime, cefepime; 10 isolates with either TEM, MIR, K, ACT or FOX (% unknown)[38f]	MIC, MBC; ceftriaxone; no BL (0/1)[13]
	MIC; ceftriaxone, ceftazidime, cefotaxime, cefepime; TEM, other ESBL (15/17)[39]	time–kill; cefaclor, ceftibuten; none (1/2)[78g]	
	MIC, MBC; ceftriaxone; TEM (1/1)[13]	rabbit endophthalmitis model, MIC; cefoperazone; BL not reported (5/10)[4]	
	MIC; ceftazidime, cefoperazone, ceftriaxone, cefotaxime; BL not reported (4/4)[43]		
	MIC; cefotaxime, ceftazidime, cefepime; 6 isolates with TEM BL (% unknown)[32]		
	mouse model, MIC; cefepime (1/1)[76]		
Cephamycins			
	–	MIC; cefoxitin; 6 isolates with TEM BL (% unknown)[32]	MIC; cefoxitin; BL not reported (0/1)[43]
		MIC; cefoxitin, cefotetan; 20 isolates with BL not reported (% unknown)[34]	MIC; cefoxitin; TEM, other ESBL (0/33)[45]

Continued

Table 3. *Continued*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
		MIC; cefotetan; 18 isolates with TEM, Toho or AmpC (6/18)[33]	
Monobactams			
	MIC; aztreonam; 20 isolates with BL not reported (% unknown)[34]	MIC; aztreonam; 19 isolates with TEM, Toho or AmpC (% unknown)[33]	–
	MIC; aztreonam; TEM, other ESBL (3/3)[39]	MIC; aztreonam; TEM, other ESBL (15/33)[45]	
	MIC; aztreonam; BL not reported (1/1)[43]	MIC; aztreonam; 10 isolates with TEM, MIR, K, ACT or FOX (% unknown)[38f]	

BL, β-lactamase.
Data are shown as: experimental system(s); antibiotic(s); β-lactamase(s) and number/% of isolates showing inoculum effect.
Articles were sorted by the β-lactam(s) investigated and then divided into three categories based on the proportion of isolates within the study that displayed an inoculum effect (majority, minority and negligible). Each line within a column describes the experimental system and β-lactamases investigated by a single study. Fractions indicate the number of isolates displaying an inoculum effect in the numerator and the total number of isolates in the denominator, whereas '% unknown' indicates that the exact percentage of isolates displaying the inoculum effect was not clear in the study.
K. pneumoniae nearly always harbours bla_{SHV-1} and therefore this β-lactamase has not been separately listed for each study.
[a]Inoculum effect was apparent for piperacillin and piperacillin/tazobactam but not ampicillin, ampicillin/sulbactam, ticarcillin or ticarcillin/clavulanate.
[b]Higher concentrations of sulbactam prevented an inoculum effect.
[c]Inoculum effect was observed for ertapenem (70%) and meropenem (57%) but not for imipenem (0%).
[d]100% of isolates displayed an inoculum effect for cefotaxime, ceftriaxone and cefepime. Only 33.3% of isolates displayed an inoculum effect for ceftazidime.
[e]Majority of isolates displayed inoculum effect for cefotaxime, cefixime, cefpodoxime and ceftibuten, whereas <40% of these isolates displayed an inoculum effect for ceftazidime.
[f]Significant inoculum effect observed when comparing MICs conducted at 5×10^5 and 5×10^7 cfu/mL but not when comparing 5×10^5 and 5×10^6 cfu/mL.
[g]Significant inoculum effect for cefaclor but no inoculum effect for ceftibuten.

bacteria reviewed herein. Additional studies are required to determine whether the inoculum effect translates to infection sites with a high bacterial burden in patients, since a majority of the studies to date were *in vitro*.

H. influenzae

Historically, *H. influenzae* type b was one of most common pathogens responsible for bacterial meningitis in young children, and the organism was also capable of causing pneumonia, bacteraemia, septic arthritis and respiratory tract infections.[79,80] With the introduction of a vaccine against *H. influenzae* type b, non-typeable *H. influenzae* is now the predominant clinical pathogen in developed countries, but unvaccinated populations and immunocompromised patients are still at risk of infections caused by *H. influenzae* type b. Clinicians have previously speculated that the quantity of *H. influenzae* in infections such as otitis media may be around 10^5–10^6 cfu/ml, but the inoculum size likely varies based on the site of infection.[81] Considering that first-line antibacterials against *H. influenzae* are often β-lactams, previous investigations have evaluated whether the inoculum size of *H. influenzae* alters the pharmacodynamics of cephalosporins and other β-lactams.

The inoculum effect of β-lactams against *H. influenzae* has been studied exclusively in the context of *in vitro* susceptibility testing or time–kill analyses. Substantial *in vitro* inoculum effects were noted for aminopenicillins[82–87] and cephalosporins,[81,84,86,88–91] whereas carbapenems[87,92] and cefoxitin[88] were only evaluated in a few studies and did not demonstrate a substantial inoculum effect *in vitro* (Table 4). Two studies detected an *in vitro* inoculum effect for aminopenicillins against β-lactamase-producing strains of *H. influenzae* but did not observe an inoculum effect in β-lactamase-deficient strains.[83,85] In contrast, cephalosporins frequently demonstrated an inoculum effect regardless of β-lactamase production, with ceftriaxone, ceftazidime and cefaclor displaying a substantial inoculum effect in multiple studies.[81,84,86,88,89,91] In addition, β-lactamase-deficient strains of *H. influenzae* that were resistant to ampicillin mediated an *in vitro* inoculum effect during susceptibility testing of carbapenems, cephalosporins and aminopenicillins.[90,92] The authors attributed the ampicillin resistance and inoculum effects to mutations in the PBP3 enzyme that decrease the target affinities of cephalosporins and other β-lactams. Regardless of the mechanisms behind the *in vitro* inoculum effects observed for *H. influenzae*, there were no animal models or human studies to indicate that the *in vitro* inoculum effects observed for cephalosporins and aminopenicillins are clinically significant. Adhering to standardized susceptibility testing methods proposed by organizations such as CLSI will likely minimize variance in how β-lactam susceptibilities are reported against *H. influenzae* strains displaying reduced susceptibilities at high inocula.

S. aureus

S. aureus is one of the most clinically relevant pathogens in both community and nosocomial settings. By utilizing an array of

Table 4. Summary of studies evaluating the inoculum effect of β-lactams against *H. influenzae*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
β-Lactam/β-lactamase inhibitor combinations			
	MIC; amoxicillin/clavulanate; 8 BL isolates (% unknown)[83a]	–	MIC, time–kill; amoxicillin/clavulanate; 4 isolates with no BL (% unknown)[83]
	MIC; amoxicillin/clavulanate; 15 BL isolates, 57 no BL isolates (% unknown)[89]		MIC; piperacillin/tazobactam; 10 isolates (% and BL status unknown)[122]
Carbapenems			
	MIC; meropenem; BLNAR (5/9)[92]	MIC; imipenem; BLNAR (3/9)[92]	MIC; meropenem (0/6)[87]
Cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefuroxime or cefaclor)[b]			
	MIC; ceftriaxone; 7 BLNAR isolates (% unknown)[90]	MIC; ceftazidime; BLNAR (4/9)[92]	MIC, time–kill; cefuroxime; 8 BL isolates, 4 no BL isolates (% unknown)[83]
	MIC; ceftriaxone; BLNAR (5/9)[92]		
	MIC; ceftriaxone; 19 BL isolates, 19 no BL isolates (% unknown)[86c]		MIC; ceftriaxone; BL status unknown (0/6)[87]
	MIC; ceftriaxone, ceftazidime; BL (10/10), no BL (10/10)[91]		
	MIC; ceftriaxone, ceftazidime, cefotaxime; 18 BL isolates, 15 no BL isolates (% unknown)[84]		
	MIC; cefuroxime; 15 BL isolates, 57 no BL isolates (% unknown)[89]	MIC; cefaclor; 31 BL isolates, 33 no BL isolates (% unknown)[85]	MIC; ceftriaxone, cefotaxime, cefuroxime; 4 BL, 7 BLNAR, 5 no BL (% unknown)[90d]
	MIC; cefotaxime; BL (10/10), no BL (9/10)[91]		
	MIC; cefaclor; BL (15/15), no BL (15/15)[81]		time–kill; cefaclor; no BL (0/5)[81]
	time–kill; cefaclor; BL (5/5)[81]		
Ampicillin (or amoxicillin if stated)			
	MIC, time–kill; TEM, ROB (% unknown)[83]	–	MIC, time–kill; 8 BL isolates, 4 no BL isolates (% unknown)[83]
	MIC; BLNAR (5/9)[92]		
	MIC (5/6, BL status unknown)[87]		
	MIC; 18 BL isolates, 15 no BL isolates (% unknown)[84]		
	MIC; 19 BL isolates, 19 no BL isolates (% unknown)[86c]		MIC; 33 no BL isolates (% unknown)[85]
	MIC; 31 BL isolates (% unknown)[85]		
	time–kill; amoxicillin; 2 BL isolates, 2 no BL isolates (% unknown)[82]		
Cefoxitin			
	–	–	MIC; 37 BL isolates, 20 no BL isolates (% unknown)[88]

Data are shown as: experimental system(s); antibiotic(s); β-lactamase(s) and number/% of isolates showing inoculum effect.
Articles were sorted by the β-lactam(s) investigated and then divided into three categories based on the proportion of isolates within the study that displayed an inoculum effect (majority, minority and negligible). Each line within a column describes the experimental system, the drug being investigated if relevant and the number of β-lactamase-positive (BL), β-lactamase-negative (no BL) or β-lactamase-negative but ampicillin-resistant (BLNAR) isolates included in each study. Fractions indicate the number of isolates displaying an inoculum effect in the numerator and the total number of isolates in the denominator, whereas '% unknown' indicates that the exact percentage of isolates displaying the inoculum effect was not clear in the study.
[a]Addition of clavulanate in a 1:1 fashion with amoxicillin reversed the inoculum effect.
[b]Cephalosporins were chosen for inclusion in the table based on frequency of appearance in *H. influenzae* studies. Some of the studies included other β-lactams that were not included in the table for the sake of brevity.
[c]Ampicillin also displayed an inoculum effect in time–kill experiments whereas ceftriaxone did not display an inoculum effect.
[d]Cefotaxime, ceftriaxone and cefuroxime did not display an inoculum effect for β-lactamase-negative (ampicillin-susceptible) or β-lactamase-positive isolates, whereas ceftriaxone displayed an inoculum effect against the β-lactamase-negative but ampicillin-resistant isolates.

virulence factors, *S. aureus* has been implicated in infections that involve nearly every organ system in the body, including pneumonia, bacteraemia, gastrointestinal infections and skin and soft tissue infections to name a few.[93,94] Common sites of infection, such as the urinary tract, may be associated with an inoculum of $\sim10^6$ cfu/mL, whereas peritoneal fluid has been associated with bacterial burdens that sometimes exceed 10^8 cfu/ml.[95,96] *S. aureus* is also the most common cause of infective endocarditis in the USA; this is a high-burden infection associated with inocula of 10^8–10^{11} cfu/g.[97,98] Although rates of methicillin resistance have increased over time, β-lactams remain important treatment options for MSSA infections.

The majority of studies that investigated the inoculum effect of β-lactams against MSSA focused on the *in vitro* inoculum effect of cefazolin during susceptibility testing and sometimes included nafcillin as a comparator. Overall, 15 studies were identified that observed an *in vitro* inoculum effect during cefazolin susceptibility testing at high inocula (Table 5).[3,15,17,99–110] There were no investigations that reported the absence of an *in vitro* inoculum effect for cefazolin. In contrast, all of the *in vitro* studies that investigated nafcillin (6/6 studies) were unable to detect an inoculum effect for nafcillin during susceptibility testing at high inocula.[3,15,17,99,103,107] Based on the isolate collections that were studied, the prevalence of the cefazolin inoculum effect varied between 13% and 58% of the organisms evaluated during susceptibility testing.[100,107]

Many of the studies that evaluated the cefazolin inoculum effect also characterized the β-lactamase production of the MSSA isolates in the investigations. The prevalence of β-lactamase production in MSSA was reported to be as high as 92% in a South Korean study and <80% in investigations based in the USA.[17,107,110] Due to different efficiencies of hydrolysing cefazolin, many authors distinguished between four staphylococcal β-lactamases encoded by *blaZ* genes, known as type A, B, C or D. The distribution of β-lactamase types varied considerably between studies, with the most prevalent enzyme being a type A β-lactamase, [104] a type C β-lactamase[108] or a relatively even distribution between type A, B and C β-lactamases.[109] Whereas one study found that MSSA isolates possessing the type C β-lactamase displayed the most substantial inoculum effect for cefazolin,[109] five studies identified MSSA isolates expressing the type A β-lactamase as the most likely to display a cefazolin inoculum effect during susceptibility testing.[104–108] An analysis by Lee *et al.*[100] concluded that the quantity of the type A β-lactamase did not significantly impact the *in vitro* cefazolin inoculum effect; however, type A β-lactamases that displayed the proline 226-tyrosine 229 genotype were more likely to result in an inoculum effect during cefazolin susceptibility testing than those with the serine 226-cysteine 229 genotype. Taken together, the results of the *in vitro* investigations suggest that the likeliness of cefazolin displaying an inoculum effect during susceptibility testing is based on the production of specific β-lactamases that will vary in their ability to hydrolyse cefazolin due to SNPs.

Only four studies were identified that evaluated a cefazolin inoculum effect using an *in vivo* animal model. Singh *et al.*[15] used a rat infective endocarditis model to demonstrate that nafcillin and ceftaroline achieved significantly greater killing against an MSSA strain expressing a type A β-lactamase in comparison with cefazolin; however, when the production of the β-lactamase was disrupted, cefazolin and ceftaroline achieved similar bacterial reductions. In a similar analysis utilizing a rat infective endocarditis model, Nannini *et al.*[111] found that nafcillin and daptomycin achieved superior reductions in MSSA vegetations in comparison with cefazolin when the MSSA produced a type A β-lactamase. Inactivation of the type A β-lactamase allowed cefazolin to kill a significantly higher amount of MSSA in the animal model. A separate investigation by Fields *et al.*[112] evaluated the ability of cefazolin to kill type A β-lactamase-producing MSSA in a rabbit abscess model. Although the type A β-lactamase production did decrease the concentration of cefazolin in the abscesses, the authors concluded that the change in concentration was not substantial enough to alter the efficacy of cefazolin against the MSSA. Lastly, Miller *et al.*[113] used a rat infective endocarditis model to observe that addition of clavulanic acid to cefazolin significantly increased the *in vivo* killing of a type A β-lactamase-producing MSSA strain that demonstrated an *in vitro* inoculum effect.

A paucity of clinical trials exist that evaluate the clinical significance of the *in vitro* cefazolin inoculum effect. Chong *et al.*[107] retrospectively investigated MSSA isolates taken from patients with bacteraemia and determined that the presence of an *in vitro* inoculum effect was not associated with poorer clinical outcomes. Lee *et al.*[102] conducted a retrospective cohort study of patients with MSSA bacteraemia and found that the *in vitro* cefazolin inoculum effect was associated with persistent bacteraemia (P=0.04), but it did not significantly influence the likeliness of treatment failure ($P = 0.13$). A small retrospective analysis of haemodialysis patients with MSSA bacteraemia by Nannini *et al.*[108] concluded that the *in vitro* cefazolin inoculum effect may be associated with treatment failure (P=0.09); however, the study was limited by the comparison of only six cefazolin treatment failures and six clinical cures. A prospective cohort study by Song *et al.*[106] evaluated >300 MSSA bacteraemia cases to identify risk factors and clinical characteristics of patients infected with MSSA strains displaying an *in vitro* inoculum effect against cefazolin. The investigation found that clindamycin resistance and erythromycin resistance may be used as markers of the *in vitro* cefazolin inoculum effect with high specificities (92.9% and 90.9%, respectively) and negative predictive values (82.3% and 84.6%, respectively). A separate prospective observational cohort study compared the use of nafcillin and cefazolin for the treatment of MSSA bacteremia.[114] Overall, treatment failure was more likely to occur in the nafcillin group (P=0.015), due to a higher likeliness of discontinuing the drug because of adverse events. Interestingly, a stratified analysis found that patients receiving cefazolin for treatment of MSSA strains displaying the *in vitro* inoculum effect were more likely to experience a treatment failure (P=0.049) and mortality at 1 month (P=0.047) in comparison with patients infected with MSSA strains that did not display a cefazolin *in vitro* inoculum effect. Another prospective investigation by Miller *et al.*[11] evaluated 77 patients that received cefazolin or cefalotin for MSSA bacteraemia and found that MSSA strains demonstrating an *in vitro* cefazolin inoculum effect were significantly associated with 30 day all-cause mortality in a multivariate analysis (P=0.03).

To summarize, a plethora of studies suggest that cefazolin will demonstrate an *in vitro* inoculum effect during the susceptibility testing of a subset of MSSA clinical isolates. The increased MIC of cefazolin at high inocula is associated with the production of a type A or type C β-lactamase enzyme, and the activity of nafcillin appears to be unaffected by high inocula of β-lactamase-

Table 5. Summary of studies evaluating the inoculum effect of β-lactams against *S. aureus*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
Cefazolin (or cefalexin if stated)			
	MIC; cefalexin (90/118), cefazolin (79/118)[99]	MIC; cefalexin (21/61); *blaZ* unknown[101]	rabbit model; A (0/1)[112]
		MIC; cefalexin (36/98); A/C >B[103]	
	MIC; A (10/17), [10/11 Pro-Tyr and 0/6 Ser-Cys][100a]	MIC (2/5); *blaZ* unknown[3]	
		MIC (105/308); A >C[104]	
	MIC; A(16/17), C (44/46) >B (4/24), D (0/1)[102]	MIC; A (16/61), B (2/25), C (25/108), D (1/2), no *blaZ* (5/73)[110]	
		MIC; A (9/25) >B (0/15), C (10/45), no *blaZ* (0/13)[108]	retrospective clinical (10 CIE versus 67 non-CIE)[107]
	MIC; A (17/17), no *blaZ* (0/2)[15]	MIC; A (22/48) >B (3/43), C (22/49), D (0/2), no *blaZ* (0/43)[17]	
	rat model; A (1/1), no *blaZ* (0/1)[15]	MIC; A (16/60) >B (0/32), C (0/30), D (0/3), no *blaZ* (0/21)[105]	
	rat model; A (1/1), no *blaZ* (0/1)[111]	MIC; A (23/38) >B (2/43), C (3/117), D (0/3), no *blaZ* (0/17)[107]	
		MIC; A (23/41), C (37/132) >B (1/81), D (0/1), no *blaZ* (0/48)[106]	
	rat model; A (1/1), no *blaZ* (0/1)[113]	MIC; C (10/12) >A (6/12), B (4/13), D (1/1), no *blaZ* (0/14)[109]	retrospective clinical (65 CIE patients versus 48 non-CIE)[102c]
		retrospective clinical (3 CIE patients versus 9 non-CIE patients)[108b]	
	prospective clinical (42 CIE patients versus 35 non-CIE)[11]	prospective clinical (13 CIE patients versus 45 non-CIE)[114]	
Nafcillin			
	–	–	MIC; *blaZ* unknown[103]
			MIC (0/5); *blaZ* unknown[3]
			MIC (8/118); *blaZ* unknown[99]
			MIC; A (0/17), no *blaZ* (0/2)[15]
			MIC (0/185) [data not shown in article][17]
			MIC; A (0/38), B (0/43) (0/117), D (0/3), no *blaZ* (0/17)[107]
			rat model; A (0/1)[111]
			rat model; A (0/1)[15]
			prospective clinical (11 CIE patients versus 41 non-CIE)[114]
2nd- to 5th-generation cephalosporins			
	time–kill; cefaclor (1/1); *blaZ* unknown[78]	MIC; cefaclor (27/61); *blaZ* unknown[101]	MIC; ceftaroline; A (0/17), no *blaZ* (0/2)[15]
			MIC; cefpodoxime (0/61); *blaZ* unknown[101]
			MIC; cefuroxime; *blaZ* unknown[107]
			MIC; cefuroxime; A (0/25), B (0/15), C (0/45), no *blaZ* (0/13)[103]
	murine model; cefaclor (2/2 with *blaZ*, 0/2 no *blaZ*)[101]	MIC; cefepime; A (2/12), B (2/13), C (1/12), D (0/1), no *blaZ* (2/14)[109]	MIC; cefuroxime/ceftriaxone; with *blaZ* (0/14), no *blaZ* (0/38)[109]
			MIC; ceftriaxone; A (0/25), B (0/15), C (1/45), no *blaZ* (1/13)[103]
			MIC; ceftriaxone (7/302), cefepime (1/302), *blaZ* unknown[123]
	MIC; ceftriaxone; A (0/25), B (0/15), C (1/45), no *blaZ* (1/13)[103]	murine model; ceftobiprole (1/6); *blaZ* unknown[124]	murine model; cefpodoxime (0/2 with *blaZ*, 0/2 no *blaZ*)[101]
			murine model; ceftaroline (0/9); *blaZ* unknown[125]

Continued

Table 5. *Continued*

β-Lactam	Majority of isolates displayed an inoculum effect	Minority of isolates displayed an inoculum effect	Negligible number of isolates displayed an inoculum effect
			rat model; ceftaroline; A (0/1), *blaZ* negative (0/1)[15]
Carbapenems			
	–	–	MIC; imipenem/meropenem; with *blaZ* (0/14), no *blaZ* (0/38)[109]
			MIC; meropenem (0/302)[123]
β-Lactam/β-lactamase inhibitor combinations			
	MIC; ampicillin/sulbactam (199/302); *blaZ* unknown[123]	MIC; ampicillin/sulbactam; A (4/12), B (3/13), C (9/12), D (0/1), no *blaZ* (0/14)[109]	MIC; piperacillin/tazobactam (0/17); *blaZ* unknown[122]
		MIC; piperacillin/tazobactam (130/302); *blaZ* unknown[123]	rat model; cefazolin, clavulanic acid; A (0/1)[113]

CIE, *in vitro* cefazolin inoculum effect; with *blaZ*, sum of all isolates with a type A, B, C or D *blaZ* β-lactamase; no *blaZ*, isolates without a β-lactamase. Data are shown as: experimental system(s); antibiotic(s); β-lactamase(s) and number/% of isolates showing inoculum effect.
Articles were sorted by the β-lactam(s) investigated and then divided into three categories based on the proportion of isolates within the study that displayed an inoculum effect (majority, minority and negligible). Each line within a column describes the experimental system and the distribution of type A, B, C or D *blaZ* β-lactamases reported; the fraction of isolates displaying an inoculum is noted parenthetically. >indicates that the authors identified a higher incidence of the inoculum effect with either a type A or C (or both) β-lactamase relative to type B and D.
[a]Strains displaying the type A *blaZ* proline 226-tyrosine 229 genotypes demonstrated a greater inoculum effect than strains producing serine226-cysteine 229.
[b]Association between MSSA bacteraemia treatment failure and *in vitro* inoculum effect was not significant (P=0.09) but limited by a small sample size.
[c]Persistent bacteraemia was significantly higher in patients infected with a strain displaying an *in vitro* inoculum effect but other differences were not significant.

producing MSSA strains. A handful of *in vivo* studies and retrospective clinical studies give varying accounts of whether the inoculum effect of cefazolin observed during susceptibility testing has a meaningful impact on the performance of the drug *in vivo*. Importantly, two prospective studies determined that mortality was statistically worse when patients received cefazolin for MSSA strains that displayed an inoculum effect during cefazolin susceptibility testing.[11,114] Considering that one of the aforementioned studies also determined that clinical outcomes for the treatment of MSSA bacteraemia were superior when patients received cefazolin in comparison with nafcillin, identification of the cefazolin inoculum effect *in vitro* may provide insight into the comparative selection of cefazolin or nafcillin for high-burden MSSA infections. If clinical microbiology laboratories are not capable of performing susceptibility testing at high inocula, other indicators such as erythromycin or clindamycin resistance may be a pragmatic means of inferring the likeliness of an *in vitro* inoculum effect.[106] Rapid diagnostics that detect the production of specific β-lactamases may also be a useful method for the rational selection of cefazolin or nafcillin for the treatment of MSSA infections with high inocula. A randomized clinical trial is needed to definitively determine the clinical implications of the *in vitro* cefazolin inoculum effect.

Discussion

Based mainly on the *in vitro* studies included in the current review, cephalosporins were consistently found to demonstrate a substantial inoculum effect against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *H. influenzae* and *S. aureus* (Tables 1–5). In contrast, carbapenems displayed a less frequent inoculum effect during *in vitro* and *in vivo* animal testing. The magnitude of inoculum effects observed for other β-lactam subclasses varied based on the pathogen that was being investigated. Considering the diverse number of cephalosporins that were studied, there was variability in the frequency of the observed inoculum effects depending on the specific drug and pathogen pair. Despite being structurally similar to cephalosporins, the cephamycins consistently displayed less of an *in vitro* inoculum effect against Gram-negative pathogens, which likely is a result of ESBL enzymes and some other β-lactamases being unable to efficiently hydrolyse the cephamycins. However, because many of the *in vitro* investigations did not report whether an MIC increase at a high inoculum resulted in a different interpretation of whether an isolate was susceptible to a given antibacterial, the clinical significance of such MIC increases is ambiguous.

Studies that have evaluated piperacillin/tazobactam at various inocula have shown that it is prone to an inoculum effect, especially for ESBL-producing organisms. Tazobactam is capable of inhibiting class A ESBL enzymes, so diminished activity of piperacillin/tazobactam against high-bacterial burden infections is somewhat surprising. Piperacillin/tazobactam was recently compared with meropenem through a randomized controlled trial for definitive treatment of bloodstream infections caused primarily by ESBL-producing *E. coli* or *K. pneumoniae*.[115] The results of this trial, and other recent clinical studies,[116,117] favour the use of meropenem

over piperacillin/tazobactam for infections caused by ESBL-producing pathogens. Our review revealed that piperacillin/tazobactam was generally more prone to the inoculum effect than meropenem for ESBL-producing Enterobacteriaceae. Perhaps the inoculum effect provides at least a partial explanation of the worse clinical outcomes for the patients receiving piperacillin/tazobactam. Activity of the other traditional β-lactamase inhibitors, clavulanate and sulbactam, was found to be less affected by bacterial inocula in this review.

Although *in vitro* inoculum effects were more frequently observed against β-lactamase-producing organisms, there are likely alternative mechanisms of the *in vitro* inoculum effect aside from enzymatic degradation. An *in vitro* inoculum effect was observed against β-lactamase-deficient strains of *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *H. influenzae* (Tables 1–4). For example, aminopenicillins displayed an *in vitro* inoculum effect against β-lactamase-producing isolates of *H. influenzae* and also β-lactamase-deficient isolates of *H. influenzae* that were resistant to ampicillin.[92] The *in vitro* inoculum effect mediated by the β-lactamase-deficient isolates of *H. influenzae* was likely due to mutations in the protein targets of the aminopenicillins. Increasing the density of bacteria in an *in vitro* system will also likely decrease the average number of β-lactam molecules available to interact with a bacterial cell.[5] The *in vitro* inoculum effect of β-lactams is therefore likely mediated by several mechanisms, with the production of β-lactamase enzymes being the most prevalent.

Several of the studies included in the current article used animal models to investigate the presence of *in vivo* inoculum effects. The results of the animal models that investigated β-lactams against *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus* were largely consistent with the inoculum effects observed *in vitro* (Tables 1–3 and 5). Mimoz *et al.*[16] observed that isolates of *P. aeruginosa* that demonstrated an *in vitro* inoculum effect during cefepime susceptibility testing also resulted in cefepime treatment failures in a rat pneumonia model. Docobo-Pérez *et al.*[21] used an *in vivo* murine sepsis model to show that increasing imipenem doses were able to overcome an inoculum effect. The animal model work performed by various groups validates that the *in vitro* inoculum effects of several β-lactams translate into *in vivo* systems, but the significance of the *in vivo* inoculum effect appears to vary based on the drug and the pathogen. Furthermore, increasing antibiotic doses to combat isolates displaying an *in vitro* inoculum effect is a strategy that warrants additional *in vivo* study. Although the current review likely includes the majority of the *in vivo* studies relevant to the inoculum effect, the search methodology of the article may have excluded investigations that potentially offer some additional insight into the inoculum effect.

Despite the overwhelming amount of evidence for *in vitro* inoculum effects, there are only a few studies that evaluated the clinical significance of the *in vitro* inoculum effect. The clinical investigations to date have centred on the use of cefazolin against MSSA isolates that displayed an *in vitro* inoculum effect (Table 5). Given the lack of clinical studies evaluating the inoculum effect in Gram-negative pathogens, the role of modifying antibacterial selection or dose based on a suspected high-burden Gram-negative infection is still unclear. The currently available data suggest that it is generally prudent to directly confirm β-lactamase production and avoid using substrate drugs for infections that are suspected to have a high bacterial burden since the increased inoculum may potentiate the effects of β-lactamases, even in low-MIC pathogens. Furthermore, we recommend that for Gram-negative infections with a high bacterial burden where the organism is initially susceptible to a cephalosporin but the patient is not responding clinically, clinicians may consider the inoculum effect as a potential contributor to treatment failure and modify therapy. Considering that two prospective clinical studies observed poorer outcomes in patients that received cefazolin for bacteraemia caused by MSSA that displayed an *in vitro* inoculum effect, the medical community may consider encouraging microbiology laboratories to test for the cefazolin *in vitro* inoculum effect in patients with MSSA bacteremias.[11,114] If the resources needed to test for a cefazolin *in vitro* inoculum effect are not available, clinicians may consider using markers of the cefazolin *in vitro* inoculum effect (such as clindamycin and erythromycin resistance[106]) and maintain a high degree of suspicion for persistent MSSA bacteraemia in patients receiving cefazolin to determine when nafcillin may be a more suitable alternative.

Although the clinical role of the inoculum effect has yet to be fully elucidated, it is at least clear that precision of the bacterial inoculum during susceptibility testing is imperative to ensure accurate and reproducible testing of β-lactam MICs. EUCAST and CLSI both recommend targeting an inoculum of 5×10^5 cfu/mL for susceptibility tests.[118,119] However, EUCAST guidelines allow the inoculum to range between 3 and 7×10^5 cfu/mL and CLSI recommends testing between 2 and 8×10^5 cfu/mL. Our review suggests that deviations in β-lactam MIC as a result of slight changes in the bacterial inoculum are quite common. Inoculum variations, at least within the CLSI accepted range, can result in significant variation in MIC, particularly for resistant organisms.[120]

In closing, there is a plethora of studies that have investigated the inoculum effect of β-lactams. We recommend that microbiology laboratories strive to use precise inocula for susceptibility testing. Furthermore, it may be prudent to evaluate MSSA isolated from blood cultures for a cefazolin inoculum effect to inform β-lactam selection for the treatment of MSSA bacteraemias. For Gram-negative bacteria, we recommend direct identification of β-lactamases when possible to avoid using substrate β-lactams in suspected high-inoculum infections, even if the β-lactam MIC remains low. We conclude that the inoculum effect of β-lactams is likely meaningful clinically but further clinical investigations are needed before routine changes to clinical practice can be made with confidence.

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Disclaimer

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