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Research Article

# In vitro activity of ceftobiprole against key pathogens associated with pneumonia in hospitalized patients: results from the PEG surveillance study, 2010

## Abstract

Empirical treatment of hospital-acquired pneumonia (HAP) has increasingly been threatened by methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug resistant Gram-negative pathogens. In contrast, empirical treatment of community-acquired pneumonia (CAP) is primarily impeded by antimicrobial-resistant pneumococci. Ceftobiprole, recently approved for the treatment of HAP and CAP in Europe, is active against a broad-spectrum of Gram-positive and Gram-negative pathogens, including MRSA and *Pseudomonas aeruginosa*. The objective of this study was to evaluate the susceptibility of ceftobiprole among 1,246 *S. aureus*, *Streptococcus pneumoniae*, Enterobacteriaceae species and *P. aeruginosa* isolated from respiratory tract and blood.

Isolates were collected in 25 laboratories across Germany, Switzerland and Austria. Minimum inhibitory concentrations (MICs) were determined using the microdilution method according to the standard ISO 20776-1:2006 and interpreted by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. Two-thirds of the isolates were obtained from respiratory specimens and one third from blood. There were 544 intensive care unit (ICU) isolates and 702 non-ICU isolates. The share of MRSA in *S. aureus* was 16%. Among pneumococci, 18.5% showed reduced susceptibility to penicillin. An extended-spectrum β-lactamase (ESBL) phenotype was confirmed for 18.4% of the *Escherichia coli* and 16.7% of the *Klebsiella pneumoniae* isolates. Of the *P. aeruginosa* isolates, 20.7% were ceftazidime-resistant.

$MIC_{50/90}$ values of ceftobiprole for methicillin-susceptible *S. aureus* (MSSA) and MRSA were 0.5/0.5 mg/L and 2/2 mg/L, respectively. All pneumococci were inhibited at 1 mg/L ceftobiprole. The activity of ceftobiprole against *E. coli* and *K. pneumoniae* was similar to that of ceftriaxone, but ceftobiprole showed superior activity against Enterobacteriaceae species known to produce chromosomally encoded AmpC-β-lactamases. $MIC_{50/90}$ values of ceftobiprole for ceftazidime-susceptible (4/16 mg/L) and ceftazidime-resistant *P. aeruginosa* (16/>32 mg/L) were comparable to those of cefepime (4/8 mg/L and 32/>32 mg/L, respectively). These findings suggest that ceftobiprole may represent a suitable option for the empirical treatment of HAP and CAP.

**Keywords:** respiratory tract infections, community-acquired pneumonia, CAP, hospital-acquired pneumonia, HAP

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# Introduction

Community-acquired pneumonia (CAP) represents a frequent infectious condition, with an incidence of 3–5 cases per 1,000 persons per year [1]. Mortality of CAP is around 13–14% in hospitalized patients, as opposed to 1% in ambulatory patients with mild CAP [2]. The most common microbial aetiology among hospitalized patients with CAP is *Streptococcus pneumoniae* followed by atypical pathogens, mixed aetiology and viruses [3]. Hospital-acquired pneumonia (HAP) has been reported to be the second most frequent healthcare-associated infection in European acute-care hospitals [4]. Mortality associated with HAP is limited in patients with reasonably good underlying status when an appropriate therapy is started immediately, but can be very high if initial antibiotic therapy is inappropriate [5], [6]. Most common pathogens in HAP are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and Enterobacteriaceae (predominantly *Klebsiella* species, *Escherichia coli*, and *Enterobacter* species), but *S. pneumoniae* may also be a causative agent [4], [7].

Empirical treatment of HAP has increasingly been threatened by the emergence and dissemination of methicillin-resistant *S. aureus* (MRSA) and multidrug resistant Gram-negative pathogens, while empirical treatment of CAP should take the prevalence of antimicrobial resistance in pneumococci into account [7].

Ceftobiprole medocaril (Zevtera®), a new generation β-lactam classified as group-5-cephalosporin by the Paul Ehrlich Society for Chemotherapy [8], has recently been approved in adults for the treatment of HAP, excluding ventilator-associated pneumonia (VAP), and CAP in various European countries, including Germany, Switzerland and Austria. Ceftobiprole, which is the active moiety of the pro-drug ceftobiprole medocaril, has been shown to have a broad-spectrum of *in vitro* activity against Gram-positive and Gram-negative pathogens, including MRSA, *P. aeruginosa* and Enterobacteriaceae (except *Proteus vulgaris*) [9], [10]. The antibacterial spectrum of ceftobiprole is based on its high affinity for essential penicillin-binding proteins, including PBP2' of MRSA, and its stability towards many β-lactamases [10]. Isolates of *Acinetobacter* spp., *Stenotrophomonas maltophilia* and extended-spectrum β-lactamase-producing (ESBL) Enterobacteriaceae, however, are poor targets for therapy with the drug, like other marketed broad-spectrum cephalosporins [9].

The objective of this study was to evaluate the comparative *in vitro* activity of ceftobiprole against key pathogens associated with HAP and CAP, i.e. *S. aureus*, *S. pneumoniae*, Enterobacteriaceae species and *P. aeruginosa*, collected during a resistance surveillance study conducted by the Paul Ehrlich Society in 2010.

# Material and methods

## Bacterial strains

Data of clinical isolates prospectively collected in 25 laboratories across Germany (n=21), Switzerland (n=3) and Austria (n=1) was analysed. Only first isolates from hospitalized patients recovered from respiratory tract and blood were included.

Demographic data requested from each participating laboratory included patients' age and gender, the location of the patient (i.e. medical department), type of ward (intensive care unit [ICU] or non-ICU), specimen type, and the collection date. Identification of the bacterial isolates at the study site was performed using routine laboratory procedures. Strains were shipped to a reference laboratory (Antiinfectives Intelligence, Rheinbach, Germany) for organism identification confirmation and susceptibility testing.

## Identification and susceptibility testing

Identification of isolates sent to the reference laboratory was confirmed to the species level by standard laboratory methods and by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper, Microflex, Bruker Daltonik GmbH, Bremen, Germany).

**Table 1: Minimum inhibitory concentration breakpoints for ceftobiprole [12]**

| Organism group | EUCAST breakpoint (mg/L) | |
|---|---|---|
| | S≤ | R> |
| PK/PD breakpoint* | 4 | 4 |
| Enterobacteriaceae | 0.25 | 0.25 |
| *Pseudomonas aeruginosa* | IE | IE |
| *Staphylococcus aureus* | 2 | 2 |
| *Streptococcus pneumoniae* | 0.5 | 0.5 |
| All other organisms | – | – |

* Pharmacokinetic/pharmacodynamic breakpoint

S, susceptible; R, resistant; IE, insufficient evidence that the organism is a good target for therapy for the agent (hence no breakpoint was defined); EUCAST, The European Committee on Antimicrobial Susceptibility Testing; –, breakpoint not defined

Minimum inhibitory concentrations (MICs) of ceftobiprole and comparators were determined by the microdilution method, using commercially manufactured panels (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany) with geometric two-fold serial dilutions of each antimicrobial agent and cation-adjusted Mueller-Hinton broth as nutrient medium, according to the standard ISO 20776-1:2006 [11]. MICs were interpreted by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) species-related clinical breakpoints, if applicable [12]. Breakpoints of ceftobiprole are displayed in Table 1.

*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 29213 were used as quality control (QC) strains. The QC ranges of the MICs were those listed in the document ISO 20776-1:2006 [11]. Inoculum density was verified by manual counting of colony forming units (CFUs) for all QC strains and 10% of the clinical isolates. Gram-positive isolates which showed ceftobiprole MICs above the respective EUCAST resistance breakpoint (2 mg/L for *S. aureus* and 0.5 mg/L for *S. pneumoniae*) were also tested for ceftobiprole susceptibility by an agar gradient diffusion test (Etest®) according to the manufacturer's instructions (bioMérieux, Marcy-L'Etoile, France).

## Phenotypic and molecular detection of extended-spectrum β-lactamases

Isolates of *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus mirabilis* with resistance to cefotaxime and/or ceftazidime (MIC >1 mg/L) were screened for ESBL production. The Clinical and Laboratory Standards Institute (CLSI) ESBL phenotypic confirmatory test with cefotaxime ± clavulanic acid and ceftazidime ± clavulanic acid was performed on all isolates of these four species meeting the screening criterion [13]. Isolates with a confirmed ESBL phenotype were further characterized by PCR amplification and sequencing of β-lactamase genes ($bla_{\text{TEM-like}}$, $bla_{\text{SHV-like}}$ and $bla_{\text{CTX-M-1-2-8-9-25 group}}$) as described previously [14].

## Molecular detection of carbapenemases

Isolates showing MICs of meropenem >8 mg/L were tested for the presence of carbapenemases. For carbapenemase detection in Enterobacteriaceae, a modified Hodge test [13] and combined disk tests with EDTA and boronic acid were performed in addition to PCRs for $bla_{\text{KPC}}$, $bla_{\text{OXA-48}}$, $bla_{\text{VIM}}$, $bla_{\text{IMP}}$ and $bla_{\text{NDM}}$ [15]. In *P. aeruginosa*, a modified Hodge test on MacConkey agar, a combined disk test with EDTA and PCRs for $bla_{\text{KPC}}$, $bla_{\text{VIM}}$, $bla_{\text{IMP}}$ and $bla_{\text{NDM}}$ as well as for $bla_{\text{GES}}$ [16] were performed. All amplicons were sequenced. If the phenotypic tests suggested the presence of a carbapenemase, further PCRs for rarely occurring carbapenemase genes such as GIM were performed [17]. If negative, a microbiological bioassay was performed similarly as described previously [18].

## Typing of MRSA strains

MRSA isolates were characterized by amplifying and sequencing of the polymorphic X-region of the protein A gene (*spa*) as described previously [19]. The resulting *spa* types were assigned by using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany).

## Data processing and statistical evaluation

Data were processed using Microsoft Excel. Statistical significance of differences in resistance rates was judged by comparing 95% confidence intervals (95% CI). Intervals were constructed using the Newcombe-Wilson method without continuity correction. If a calculated resistance rate did not fall within the CI of the comparator, a significance of $p<0.05$ was assumed. No further statistical analysis was performed, considering the descriptive nature of the study.

# Results

A total of 1,246 clinical isolates were analysed. Of these, 813 (65.2%) were recovered from respiratory specimens and 433 (34.8%) from blood. 544 (43.7%) and 702 (56.3%) isolates were obtained from intensive care patients and patients on general wards, respectively. There were 254 *S. pneumoniae*, 241 *P. aeruginosa*, 188 *S. aureus*, 179 *E. coli*, 108 *K. pneumoniae*, 71 *Serratia marcescens*, 65 *Enterobacter cloacae*, 44 *K. oxytoca*, 29 *P. mirabilis*, 19 *Enterobacter aerogenes*, 13 *Citrobacter freundii*, 11 *Citrobacter koseri* and 24 isolates of other Enterobacteriaceae species. 588 (47.2%) isolates were recovered from patients with hospital-acquired infections and 283 (22.7%) isolates from patients with community-acquired infections (unknown source for the remaining patients). Almost two thirds of the isolates were recovered from male patients. Isolates were predominantly obtained from patients aged 60–79 years (n=616, 49.4%), followed by those aged 40–59 years (n=267, 21.4%) and then those aged ≥80 years (n=167, 13.4%). The median age was 66 years (range <1–100 years).
The MIC frequency distributions of ceftobiprole for the organism groups tested are presented in Table 2. Data on the antimicrobial activities of ceftobiprole in comparison to other antimicrobial agents, as well as the percentage of susceptible, intermediate and resistant strains, are displayed in Table 3.

## In vitro activity of ceftobiprole against Gram-positive isolates

Of the *S. aureus* isolates, 158 (84%) were methicillin-susceptible *S. aureus* (MSSA) and 30 (16%) were MRSA. *spa* typing revealed, that 16 of the 30 MRSA isolates belonged to either *spa* type t003 (EMRSA-3; New York clone; German designation, Rhine Hesse MRSA [n=9]) or t032 (EMRSA-15; German designation, Barnim MRSA [n=7]). Ceftobiprole demonstrated good activity against *S. aureus*, with $MIC_{50/90}$ values of 0.5/0.5 mg/L and 2/2 mg/L for MSSA and MRSA isolates, respectively. All MSSA isolates, and 27/30 (90.0%) of MRSA isolates, were considered ceftobiprole-susceptible. MICs of the three ceftobiprole-resistant MRSA were 4 mg/L, one dilu-

**Table 2: Distributions of ceftobiprole MICs for bacterial isolates collected from the respiratory tract or blood of hospitalized patients**

| Organism / phenotype (n) | Number (cumulative %) of isolates inhibited by ceftobiprole at MIC (mg/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ≤0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | >32 |
| ***Staphylococcus aureus* (188)** | 41 (21.8) | 115 (83.0) | 3 (84.6) | 26 (98.4) | 3[a] (100) | | | | |
| Methicillin-susceptible (158) | 41 (25.9) | 115 (98.7) | 1 (99.4) | 1 (100) | | | | | |
| Methicillin-resistant (30) | | | 2 (6.7) | 25 (90.0) | 3[a] (100) | | | | |
| ***Streptococcus pneumoniae* (254)** | 241 (94.9) | 10 (98.8) | 3[b] (100) | | | | | | |
| Penicillin-susceptible (207) | 207 (100) | | | | | | | | |
| Penicillin-non-susceptible (47) | 34 (72.3) | 10 (93.6) | 3[b] (100) | | | | | | |
| ***Escherichia coli* (179)** | 143 (79.9) | 3 (81.6) | 2 (82.7) | | | | 2 (83.8) | 2 (84.9) | 27 (100) |
| ESBL-negative (146) | 141 (96.6) | 3 (98.6) | 2 (100) | | | | | | |
| ESBL-positive (33) | 2 (6.1) | | | | | | 2 (12.1) | 2 (18.2) | 27 (100) |
| ***Klebsiella pneumoniae* (108)** | 86 (79.6) | 3 (82.4) | | 2 (84.3) | | | | 1 (85.2) | 16 (100) |
| ESBL-negative (90) | 86 (95.6) | 3 (98.9) | | 1 (100) | | | | | |
| ESBL-positive (18) | | | | 1 (5.6) | | | | 1 (11.1) | 16 (100) |
| ***Klebsiella oxytoca* (44)** | 19 (43.2) | 11 (68.2) | 4 (77.3) | | | | | | 10 (100) |
| ***Enterobacter* spp. (89)[c]** | 66 (74.2) | 3 (77.5) | 1 (78.7) | 3 (82.0) | 7 (89.9) | 3 (93.3) | 1 (94.4) | 1 (95.5) | 4 (100) |
| ***Serratia* spp. (74)[d]** | 63 (85.1) | 5 (91.9) | 3 (95.9) | 1 (97.3) | | 1 (98.6) | | 1 (100) | |
| ***Citrobacter* spp. (26)[e]** | 22 (84.6) | 2 (92.3) | | | | | | | 2 (100) |
| **Proteeae (43)[f]** | 33 (76.7) | | 1 (79.1) | | 1 (81.4) | 1 (83.7) | | 2 (88.4) | 5 (100) |
| ***Pseudomonas aeruginosa* (241)** | | 2 (0.8) | 10 (5.0) | 58 (29.0) | 69 (57.7) | 48 (77.6) | 23 (87.1) | 7 (90.0) | 24 (100) |
| Ceftazidime-susceptible (191) | | 2 (1.0) | 9 (5.8) | 58 (36.1) | 61 (68.1) | 39 (88.5) | 15 (96.3) | 3 (97.9) | 4 (100) |
| Ceftazidime-resistant (50) | | | 1 (2.0) | | 8 (18.0) | 9 (36.0) | 8 (52.0) | 4 (60.0) | 20 (100) |

[a] Etest® MICs were 3 mg/L (resistant) for one isolate and 1–1.5 mg/L (susceptible) for two isolates.
[b] Etest® MICs were 0.5 mg/L (susceptible) for the three isolates.
[c] Includes *E. cloacae* (n=65), *E. aerogenes* (n=19), and *E. asburiae* (n=5)
[d] Includes *S. marcescens* (n=71), *S. liquefaciens* (n=2), and *S. ureilytica* (n=1)
[e] Includes *C. freundii* (n=13), *C. koseri* (n=11), *C. braakii* (n=1), and *C. farmeri* (n=1)
[f] Includes *Proteus mirabilis* (n=29), *Morganella morganii* (n=9), and *Proteus vulgaris* (n=5)

**Table 3: In vitro activity of ceftobiprole and comparators against clinical isolates of most frequent bacterial species collected from the respiratory tract or blood of hospitalized patients**

| Organism / phenotype (n) | Antibacterial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) | MIC Range (mg/L) | Proportion of isolates that are S/I/R (%)° | | |
|---|---|---|---|---|---|---|---|
| | | | | | S | I | R |
| ***Staphylococcus aureus* (188)** | Ceftobiprole | 0.5 | 2 | ≤0.25–4 | 98.4 | – | 1.6[#] |
| | Daptomycin | 0.5 | 0.5 | 0.125–1 | 100 | – | 0 |
| | Levofloxacin | ≤0.25 | >8 | ≤0.25–>8 | 77.7 | 0.0 | 22.3 |
| | Erythromycin | 0.5 | >32 | ≤0.25–>32 | 75.5 | 0.5 | 23.9 |
| | Linezolid | 1 | 1 | ≤0.25–2 | 100 | – | 0 |
| | Vancomycin | 0.5 | 1 | ≤0.25–2 | 100 | – | 0 |
| *Staphylococcus aureus* Methicillin-susceptible (158) | Ceftobiprole | 0.5 | 0.5 | ≤0.25–2 | 100 | – | 0 |
| | Daptomycin | 0.5 | 0.5 | 0.125–1 | 100 | – | 0 |
| | Levofloxacin | ≤0.25 | 0.5 | ≤0.25–>8 | 92.4 | 0.0 | 7.6 |
| | Erythromycin | 0.5 | 32 | ≤0.25–>32 | 86.7 | 0.0 | 13.3 |
| | Linezolid | 1 | 1 | ≤0.25–2 | 100 | – | 0 |
| | Vancomycin | 0.5 | 1 | ≤0.25–2 | 100 | – | 0 |
| *Staphylococcus aureus* Methicillin-resistant (30) | Ceftobiprole | 2 | 2 | 1–4 | 90.0 | – | 10.0[#] |
| | Daptomycin | 0.5 | 1 | 0.25–1 | 100 | – | 0 |
| | Levofloxacin | >8 | >8 | 4–>8 | 0 | 0 | 100 |
| | Erythromycin | >32 | >32 | 0.5–>32 | 16.7 | 3.3 | 80.0 |
| | Linezolid | 1 | 1 | ≤0.25–2 | 100 | – | 0 |
| | Vancomycin | 0.5 | 1 | 0.5–1 | 100 | – | 0 |
| ***Streptococcus pneumoniae* (254)** | Ceftobiprole | ≤0.25 | ≤0.25 | ≤0.25–1 | 98.8 | – | 1.2[§] |
| | Ceftriaxone | ≤0.125 | ≤0.125 | ≤0.125–1 | 97.2 | 2.8 | 0.0 |
| | Levofloxacin | 1 | 1 | ≤0.25–>8 | 98.0 | – | 2.0 |
| | Erythromycin | ≤0.25 | 32 | ≤0.25–>32 | 79.9 | 0.0 | 20.1 |
| | Penicillin | ≤0.063 | 0.25 | ≤0.063–2 | 81.5 | 18.5 | 0.0 |
| *Streptococcus pneumoniae* Penicillin-susceptible (207) | Ceftobiprole | ≤0.25 | ≤0.25 | ≤0.25 | 100 | – | 0 |
| | Ceftriaxone | ≤0.125 | ≤0.125 | ≤0.125–0.5 | 100 | 0 | 0 |
| | Levofloxacin | 1 | 1 | ≤0.25–2 | 100 | – | 0 |
| | Erythromycin | ≤0.25 | 4 | ≤0.25–>32 | 88.9 | 0.0 | 11.1 |
| *Streptococcus pneumoniae* Penicillin-non-susceptible (47) | Ceftobiprole | ≤0.25 | 0.5 | ≤0.25–1 | 93.6 | – | 6.4[§] |
| | Ceftriaxone | 0.25 | 1 | ≤0.125–1 | 85.1 | 14.9 | 0.0 |
| | Levofloxacin | 1 | 8 | ≤0.25–>8 | 89.4 | – | 10.6 |
| | Erythromycin | 8 | >32 | ≤0.25–>32 | 40.4 | 0.0 | 59.6 |
| ***Escherichia coli* (179)** | Ceftobiprole | ≤0.25 | >32 | ≤0.25–>32 | 79.9 | – | 20.1 |
| | Ceftriaxone | ≤0.125 | >16 | ≤0.125–>16 | 81.0 | 1.7 | 17.3 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | 64 | ≤1–>64 | 85.5 | 3.4 | 11.2 |
| | Ciprofloxacin | 0.125 | >8 | ≤0.063–>8 | 57.0 | 2.2 | 40.8 |
| | Gentamicin | 1 | >16 | ≤0.25–>16 | 87.7 | 0.0 | 12.3 |
| *Escherichia coli* ESBL-negative (146) | Ceftobiprole | ≤0.25 | ≤0.25 | ≤0.25–1 | 96.6 | – | 3.4 |
| | Ceftriaxone | ≤0.125 | ≤0.125 | ≤0.125–2 | 97.9 | 2.1 | 0.0 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | 16 | ≤1–>64 | 89.7 | 2.7 | 7.5 |
| | Ciprofloxacin | ≤0.063 | >8 | ≤0.063–>8 | 65.8 | 2.7 | 31.5 |
| | Gentamicin | 1 | 2 | ≤0.25–>16 | 93.2 | 0.0 | 6.8 |

(Continued)

**Table 3: In vitro activity of ceftobiprole and comparators against clinical isolates of most frequent bacterial species collected from the respiratory tract or blood of hospitalized patients**

| Organism / phenotype (n) | Antibacterial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) | MIC Range (mg/L) | Proportion of isolates that are S/I/R (%)° | | |
|---|---|---|---|---|---|---|---|
| | | | | | S | I | R |
| *Escherichia coli* ESBL-positive (33) | Ceftobiprole | >32 | >32 | ≤0.25–>32 | 6.1 | – | 93.9 |
| | Ceftriaxone | >16 | >16 | 0.5–>16 | 6.1 | 0.0 | 93.9 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | 64 | ≤1–>64 | 66.7 | 6.1 | 27.3 |
| | Ciprofloxacin | >8 | >8 | ≤0.063–>8 | 18.2 | 0.0 | 81.8 |
| | Gentamicin | 1 | >16 | 0.5–>16 | 63.6 | 0.0 | 36.4 |
| ***Klebsiella pneumoniae* (108)** | Ceftobiprole | ≤0.25 | >32 | ≤0.25–>32 | 79.6 | – | 20.4 |
| | Ceftriaxone | ≤0.125 | >16 | ≤0.125–>16 | 84.3 | 0.0 | 15.7 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5–1 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 4 | 32 | ≤1–>64 | 81.5 | 6.5 | 12.0 |
| | Ciprofloxacin | ≤0.063 | >8 | ≤0.063–>8 | 78.7 | 1.9 | 19.4 |
| | Gentamicin | 0.5 | 8 | ≤0.25–>16 | 88.9 | 0.0 | 11.1 |
| *Klebsiella pneumoniae* ESBL-negative (90) | Ceftobiprole | ≤0.25 | ≤0.25 | ≤0.25–2 | 95.6 | – | 4.4 |
| | Ceftriaxone | ≤0.125 | 0.25 | ≤0.125–0.5 | 100 | 0 | 0 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | 8 | ≤1–>64 | 91.1 | 3.3 | 5.6 |
| | Ciprofloxacin | ≤0.063 | 0.5 | ≤0.063–>8 | 91.1 | 2.2 | 6.7 |
| | Gentamicin | 0.5 | 0.5 | ≤0.25–16 | 96.7 | 0.0 | 3.3 |
| *Klebsiella pneumoniae* ESBL-positive (18) | Ceftobiprole | >32 | >32 | 2–>32 | 0 | – | 100 |
| | Ceftriaxone | >16 | >16 | 0.5–>16 | 5.6 | 0.0 | 94.4 |
| | Meropenem | ≤0.5 | 1 | ≤0.5–1 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 16 | >64 | 4–>64 | 33.3 | 22.2 | 44.4 |
| | Ciprofloxacin | >8 | >8 | ≤0.063–>8 | 16.7 | 0.0 | 83.3 |
| | Gentamicin | 1 | >16 | ≤0.25–>16 | 50.0 | 0.0 | 50.0 |
| ***Klebsiella oxytoca* (44)** | Ceftobiprole | 0.5 | >32 | ≤0.25–>32 | 43.2 | – | 56.8 |
| | Ceftriaxone | ≤0.125 | >16 | ≤0.125–>16 | 77.3 | 0.0 | 22.7 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | >64 | ≤1–>64 | 79.5 | 0.0 | 20.5 |
| | Ciprofloxacin | ≤0.063 | 4 | ≤0.063–>8 | 84.1 | 4.5 | 11.4 |
| | Gentamicin | 0.5 | 1 | ≤0.25–2 | 100 | 0 | 0 |
| ***Proteus mirabilis* (29)** | Ceftobiprole | ≤0.25 | 8 | ≤0.25–>32 | 82.8 | – | 17.2 |
| | Ceftriaxone | ≤0.125 | >16 | ≤0.125–>16 | 89.7 | 0.0 | 10.3 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | ≤1 | 2 | ≤1–64 | 96.6 | 0.0 | 3.4 |
| | Ciprofloxacin | ≤0.063 | 8 | ≤0.063–>8 | 79.3 | 3.4 | 17.2 |
| | Gentamicin | 1 | >16 | 0.5–>16 | 72.4 | 3.4 | 24.1 |
| ***Enterobacter cloacae* (65)** | Ceftobiprole | ≤0.25 | 8 | ≤0.25–>32 | 67.7 | – | 32.3 |
| | Cefepime | ≤0.25 | 4 | ≤0.25–>32 | 75.4 | 16.9 | 7.7 |
| | Ceftriaxone | 0.5 | >16 | ≤0.125–>16 | 64.6 | 4.6 | 30.8 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5–32 | 98.5 | 0.0 | 1.5 |
| | Piperacillin-tazobactam | 4 | >64 | ≤1–>64 | 73.8 | 3.1 | 23.1 |
| | Ciprofloxacin | ≤0.063 | 0.25 | ≤0.063–>8 | 92.3 | 1.5 | 6.2 |
| | Gentamicin | 0.5 | 0.5 | ≤0.25–>16 | 96.9 | 1.5 | 1.5 |

(Continued)

**Table 3: In vitro activity of ceftobiprole and comparators against clinical isolates of most frequent bacterial species collected from the respiratory tract or blood of hospitalized patients**

| Organism / phenotype (n) | Antibacterial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) | MIC Range (mg/L) | Proportion of isolates that are S/I/R (%)° | | |
|---|---|---|---|---|---|---|---|
| | | | | | S | I | R |
| ***Enterobacter aerogenes* (19)** | Ceftobiprole | ≤0.25 | 0.5 | ≤0.25–>32 | 89.5 | – | 10.5 |
| | Cefepime | ≤0.25 | 1 | ≤0.25–>32 | 94.7 | 0.0 | 5.3 |
| | Ceftriaxone | 0.5 | >16 | ≤0.125–>16 | 52.6 | 0.0 | 47.4 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 8 | >64 | 2–>64 | 52.6 | 15.8 | 31.6 |
| | Ciprofloxacin | ≤0.063 | 4 | ≤0.063–8 | 89.5 | 0.0 | 10.5 |
| | Gentamicin | 0.5 | 1 | ≤0.25–>16 | 94.7 | 0.0 | 5.3 |
| ***Serratia marcescens* (71)** | Ceftobiprole | ≤0.25 | 0.5 | ≤0.25–32 | 85.9 | – | 14.1 |
| | Cefepime | ≤0.25 | 0.5 | ≤0.25–8 | 95.8 | 2.8 | 1.4 |
| | Ceftriaxone | 0.25 | 2 | ≤0.125–>16 | 88.7 | 1.4 | 9.9 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5–16 | 98.6 | 0.0 | 1.4 |
| | Piperacillin-tazobactam | 2 | 4 | ≤1–>64 | 94.4 | 1.4 | 4.2 |
| | Ciprofloxacin | 0.125 | 0.125 | ≤0.063–>8 | 91.5 | 5.6 | 2.8 |
| | Gentamicin | 1 | 1 | 0.5–8 | 98.6 | 0.0 | 1.4 |
| ***Citrobacter freundii* (13)** | Ceftobiprole | ≤0.25 | 0.5 | ≤0.25–>32 | 76.9 | – | 23.1 |
| | Cefepime | ≤0.25 | ≤0.25 | ≤0.25–>32 | 92.3 | 0.0 | 7.7 |
| | Ceftriaxone | ≤0.125 | 8 | ≤0.125–>16 | 84.6 | 0.0 | 15.4 |
| | Meropenem | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0 | 0 |
| | Piperacillin-tazobactam | 2 | 8 | ≤1–16 | 92.3 | 7.7 | 0.0 |
| | Ciprofloxacin | ≤0.063 | 0.125 | ≤0.063–2 | 92.3 | 0.0 | 7.7 |
| | Gentamicin | 0.5 | 1 | 0.5–>16 | 92.3 | 0.0 | 7.7 |
| ***Pseudomonas aeruginosa* (241)** | Ceftobiprole | 4 | 32 | 0.5–>32 | –* | – | – |
| | Ceftazidime | 2 | 32 | 0.5–>32 | 79.3 | – | 20.7 |
| | Cefepime | 4 | 32 | 0.5–>32 | 74.7 | – | 25.3 |
| | Meropenem | 1 | 16 | ≤0.5–>32 | 71.0 | 14.9 | 14.1 |
| | Piperacillin-tazobactam | 8 | 64 | ≤1–>64 | 77.2 | – | 22.8 |
| | Ciprofloxacin | 0.25 | >8 | ≤0.063–>8 | 68.0 | 4.1 | 27.8 |
| | Gentamicin | 2 | 16 | ≤0.25–>16 | 86.7 | – | 13.3 |
| *Pseudomonas aeruginosa* Ceftazidime-susceptible (191) | Ceftobiprole | 4 | 16 | 0.5–>32 | –* | – | – |
| | Ceftazidime | 2 | 4 | 0.5–8 | 100 | – | 0 |
| | Cefepime | 4 | 8 | 0.5–32 | 92.1 | – | 7.9 |
| | Meropenem | ≤0.5 | 4 | ≤0.5–32 | 84.3 | 9.9 | 5.8 |
| | Piperacillin-tazobactam | 8 | 16 | ≤1–64 | 90.1 | – | 9.9 |
| | Ciprofloxacin | 0.25 | 4 | ≤0.063–>8 | 78.5 | 4.2 | 17.3 |
| | Gentamicin | 1 | 4 | ≤0.25–>16 | 94.8 | – | 5.2 |
| *Pseudomonas aeruginosa* Ceftazidime-resistant (50) | Ceftobiprole | 16 | >32 | 1–>32 | –* | – | – |
| | Ceftazidime | 32 | >32 | 16–>32 | 0 | – | 100 |
| | Cefepime | 32 | >32 | 2–>32 | 8.0 | – | 92.0 |
| | Meropenem | 8 | >32 | ≤0.5–>32 | 20.0 | 34.0 | 46.0 |
| | Piperacillin-tazobactam | 64 | >64 | 8–>64 | 28.0 | – | 72.0 |
| | Ciprofloxacin | 8 | >8 | 0.125–>8 | 28.0 | 4.0 | 68.0 |
| | Gentamicin | 4 | >16 | ≤0.25–>16 | 56.0 | – | 44.0 |

° Sensitivity categories defined by species-related breakpoints according to EUCAST
# Etest® MICs of the three ceftobiprole-resistant isolates were 3 mg/L (resistant) for one isolate and 1–1.5 mg/L (susceptible) for two isolates.
§ Etest® MICs of the three ceftobiprole-resistant isolates were 0.5 mg/L.
* No species-related breakpoint was defined by EUCAST [12].
MIC, minimum inhibitory concentration; $MIC_{50}$, minimum concentration required to inhibit 50% of the isolates; $MIC_{90}$, minimum concentration required to inhibit 90% of the isolates; S, susceptible; I, intermediate; R, resistant; EUCAST, The European Committee on Antimicrobial Susceptibility Testing

tion step above the breakpoint of 2 mg/L, two and one of which were associated with *spa* type t032 and t001 (ST228; Southern German MRSA), respectively. The three ceftobiprole-resistant isolates, as well as two randomly selected ceftobiprole-susceptible MRSA of t032 (MICs 2 mg/L), were re-tested by an agar gradient diffusion test (Etest®). Etest® MICs were 3 mg/L for the t001 isolate and 1–1.5 mg/L for the t032 isolates. Resistance rates to levofloxacin and erythromycin were 7.6% and 13.3%, respectively, for MSSA isolates and 100% and 80%, respectively, for MRSA isolates. All *S. aureus* isolates were susceptible to vancomycin, linezolid and daptomycin.

Of the 254 *S. pneumoniae* isolates, 47 (18.5%) exhibited reduced susceptibility to penicillin (PNSP, MIC >0,063 mg/L), however, none were found to be penicillin-resistant. Ceftobiprole inhibited all isolates at 1 mg/L, though three blood isolates (1.2%) exhibiting penicillin MICs of 1–2 mg/L were categorized as ceftobiprole-resistant. When the Etest® was applied, however, the ceftobiprole MIC for each of the three blood isolates was 0.5 mg/L (susceptible category). The highest MIC of ceftriaxone for PNSP was also 1 mg/L. Resistance to erythromycin and levofloxacin was observed in 51 (20.1%) and 5 (2%) isolates, respectively. All *S. pneumoniae* isolates were susceptible to vancomycin and linezolid (data not shown).

## In vitro activity of ceftobiprole against Gram-negative isolates

Fifty-nine (16.3%) of the tested Enterobacteriaceae isolates showed an ESBL phenotype, which included 33 *E. coli*, 18 *K. pneumoniae*, 6 *K. oxytoca* and 2 *P. mirabilis*. Proportions of isolates showing the ESBL phenotype were 18.4% (*E. coli*), 16.7% (*K. pneumoniae*), 13.6% (*K. oxytoca*) and 6.9% (*P. mirabilis*).

Among the 563 Enterobacteriaceae isolates, 432 (76.7%) were considered ceftobiprole-susceptible. $MIC_{50/90}$ values of ceftobiprole for *E. coli* and *K. pneumoniae* isolates were comparable to those of ceftriaxone, but ceftobiprole was more active than ceftriaxone against isolates of Enterobacteriaceae species known to produce chromosomally encoded AmpC-β-lactamases. The higher *in vitro* activity of ceftobiprole against *C. freundii*, *E. cloacae* and *S. marcescens*, however, did not result in a greater proportion of ceftobiprole-susceptible isolates. $MIC_{50/90}$ values of ceftobiprole for 19 *E. cloacae* isolates exhibiting high-level resistance to ceftriaxone (MICs ≥16 mg/L) were 4/>32 mg/L, as compared to 4/16 mg/L for cefepime, but none of these isolates was classified as ceftobiprole-susceptible. Like other broad-spectrum cephalosporins, ceftobiprole showed poor activity against isolates with an ESBL phenotype. Two out of the 59 *E. coli* isolates with an ESBL phenotype, however, were ceftobiprole-susceptible. One isolate was inhibited by 2 mg/L cefotaxime, which was lowered to ≤0.25 mg/L when clavulanic acid was added; the other had a ceftazidime MIC of 16 mg/L, which was lowered to 0.5 mg/L in the presence of clavulanic acid. Neither isolate harboured an enzyme of the groups CTX-M-, TEM- or SHV-type. In contrast, five ESBL-negative *E. coli* and four ESBL-negative *K. pneumoniae* were considered ceftobiprole-resistant (MICs of 0.5–2 mg/L). Of interest, all five ESBL-negative *E. coli* and two out of the four ESBL-negative *K. pneumoniae* were resistant to amoxicillin-clavulanic acid (MICs >128 mg/L) and piperacillin-tazobactam (MICs 32–>64 mg/L).

For most Enterobacteriaceae species, susceptibility rates of ceftobiprole were slightly lower than those of ceftriaxone (differences not statistically significant), but the susceptibility rate of ceftobiprole for *E. aerogenes* was superior to ceftriaxone ($p<0.05$). On the contrary, *K. oxytoca* isolates were susceptible to ceftobiprole less often than ceftriaxone ($p<0.05$).

Resistance to ciprofloxacin was frequently seen in *E. coli* (40.8%) and varied between 2.8% (*S. marcescens*) and 19.4% (*K. pneumoniae*) in the other Enterobacteriaceae species. Meropenem inhibited all Enterobacteriaceae isolates at 1 mg/L except for two isolates, one isolate each of *E. cloacae* (MIC 32 mg/L) and *S. marcescens* (MIC 16 mg/L). The *E. cloacae* isolate produced the metallo-β-lactamase (MBL) VIM-1, while no carbapenemase was detected in the *S. marcescens* isolate. MICs of ceftobiprole for both isolates were >16 mg/L, as expected.

Of the 241 *P. aeruginosa* isolates, 191 (79.3%) were susceptible to ceftazidime. Susceptibility rates of ciprofloxacin (68.0%), meropenem (71.0%), cefepime (74.7%), and piperacillin-tazobactam (77.2%) were slightly lower while susceptibility to gentamicin (86.7%) was more widespread. A carbapenemase was detected in 14 (41.2%) of the 34 meropenem-resistant isolates (VIM-2 [n=7], VIM-1 [n=2], IMP-31 [n=2], GES-5 [n=1], GIM-1 [n=1], and IMP-7 [n=1]). $MIC_{50/90}$ values of ceftobiprole for ceftazidime-susceptible *P. aeruginosa* isolates (4/16 mg/L) were comparable to those of cefepime (4/8 mg/L), but both drugs showed poor activity against ceftazidime-resistant isolates, as expected.

# Discussion

*S. aureus*, *P. aeruginosa* and Enterobacteriaceae are considered the most common causative agents of HAP [4], [7], while *S. pneumoniae* is the leading aetiological agent among hospitalized patients with CAP [3]. Ceftobiprole is a broad-spectrum cephalosporin which demonstrated *in vitro* and *in vivo* activity against *S. aureus* (including MRSA), *S. pneumoniae*, Enterobacteriaceae (non-ESBL phenotype) and *P. aeruginosa* [9], [10], [20], [21], [22], [23]. The current study examined the *in vitro* activity of ceftobiprole against a collection of 1,246 respiratory and blood isolates of *S. aureus*, *S. pneumoniae*, *P. aeruginosa* and Enterobacteriaceae that were collected from hospitalized patients in Germany, Switzerland and Austria in 2010.

According to data of the Paul Ehrlich Society, the prevalence of MRSA rose from below 2% in 1990 to 17.9% in

2001. Subsequently, the rate of MRSA showed only marginal variations until 2010 (16.7%) [24]. ARS (Antibiotika-Resistenz-Surveillance) is a laboratory-based surveillance system that continuously collects resistance data from routine medical samples (currently 28 in-patient and out-patient care laboratories) on clinically relevant bacteria in Germany. The ARS data on MRSA prevalence indicates a downward trend after 2010 (2008: 20.6%; 2009: 20.1%; 2010: 22.4%; 2011: 17.4%; 2012: 17.0%; 2013: 13.9% for blood isolates) [25].

More than 98% of the *S. aureus* isolates (including 90% of MRSA isolates) in the current study were considered ceftobiprole-susceptible. However, based on $MIC_{50/90}$ values, MRSA isolates were four times less susceptible to ceftobiprole than MSSA (wild type) isolates, as previously observed by others [9], [10], [20].

The SENTRY Antibiotic Surveillance Program in Europe, comprising more than 60,000 clinical bacterial pathogens isolated in Europe, Turkey, and Israel from 2005 to 2010, observed susceptibility to ceftobiprole in >4,000 MRSA isolates (98.3%) [9], while 100% susceptibility to ceftobiprole was observed for a collection of 232 MRSA isolates from hospital-associated patients across Canada [20]. In both studies, $MIC_{50/90}$ values of ceftobiprole were 1/2 mg/L for MRSA, as compared to 2/2 mg/L in the present study. In contrast, Hebeisen et al. reported $MIC_{50/90}$ values of 2/4 mg/L for MRSA isolates [10]. The trend towards slightly elevated ceftobiprole MICs in the present study may have facilitated the finding of three ceftobiprole-resistant MRSA. All three MRSA isolates were inhibited at 4 mg/L ceftobiprole, two of which turned out to be susceptible when the Etest® was used, leaving one "true" ceftobiprole-resistant MRSA isolate remaining. The mechanism of ceftobiprole resistance was not investigated. The resistant isolate belonged to *spa* type t001 (ST228), harbouring the staphylococcal chromosomal cassette mec (SCCmec) type I [26]. Farrell et al. found SCCmec type I strains to be less susceptible to ceftobiprole ($MIC_{50/90}$, 2/4 mg/L) than SCCmec type II–IV strains ($MIC_{50/90}$, 1/1–2/2 mg/L) [27]. All MRSA isolates in the current study were considered ceftobiprole-susceptible at the pharmacokinetic/pharmacodynamic (PK/PD) breakpoint of 4 mg/L, which corresponds to a ceftobiprole dosage of 500 mg as a 2-hour intravenous infusion every 8 h.

Ceftobiprole also demonstrated high potency against *S. pneumoniae*, with 98.8% testing susceptible. Two penicillin-intermediate isolates (MICs 1 mg/L) and one penicillin-resistant isolate were considered ceftobiprole-resistant (MIC 1 mg/L), though Etest® results revealed MICs of 0.5 mg/L (susceptible category). Ceftobiprole also demonstrated high potency against *S. pneumoniae* isolates collected during the European SENTRY Antibiotic Surveillance Program, with 99.3% of 4,443 *S. pneumoniae* isolates testing susceptible [9].

Based on $MIC_{50/90}$ values, the activity of ceftobiprole against Enterobacteriaceae was comparable to those of ceftriaxone or cefepime and susceptibility rates ranged between 75% and 90% for most species. *K. oxytoca* isolates, however, were less often susceptible to ceftobiprole than ceftriaxone. We speculate that ceftobiprole is a stronger substrate than ceftriaxone for the chromosomally encoded class A OXY β-lactamases of *K. oxytoca* [28]. Furthermore, like other broad-spectrum cephalosporins, ceftobiprole showed poor activity against ESBL-positive Enterobacteriaceae isolates. Based on $MIC_{50/90}$ values, the activity of ceftobiprole against Enterobacteriaceae species known to produce chromosomally encoded AmpC-producing β-lactamases resembled that of cefepime, but because of the comparatively low breakpoint of resistance set for ceftobiprole (>0.25 mg/L as compared to >2 mg/L for cefepime) the resistance rates of ceftobiprole found for *E. cloacae*, *C. freundii*, and *S. marcescens* were considerably higher than those of cefepime.

The potency of ceftobiprole against *P. aeruginosa* was comparable to ceftazidime and cefepime, as shown in previous *in vitro* studies [9], [10], [20]; however, as EUCAST has not set a species-related clinical breakpoint for *P. aeruginosa*, we were not able to assess the rate of ceftobiprole-susceptible isolates. At the target concentration of 4 mg/L, susceptibility to ceftobiprole was achieved in 58% of the *P. aeruginosa* isolates tested, which was similar to the susceptibility rate (64.6%) found for 3,434 *P. aeruginosa* isolates in the European SENTRY Antibiotic Surveillance Program [9].

Based on the spectrum of pathogens recovered from the patients enrolled in the Phase 3 clinical trial of ceftobiprole medocaril versus ceftazidime plus linezolid for the treatment of HAP [29], and the susceptibility data found in this surveillance study, we predict that 80% of the microbiological aetiologies associated with HAP (excluding VAP) would be ceftobiprole-susceptible (Table 4). Identical coverage rates are predicted for piperacillin/tazobactam and cefepime, while imipenem and meropenem would cover almost 90% of the pathogen spectrum.

In conclusion, the results of this surveillance study demonstrate that ceftobiprole is active against key pathogens associated with HAP and CAP in hospitalized patients. Hence, ceftobiprole may represent a suitable option for the empirical treatment of HAP and CAP, especially for cases in which both MRSA and Gram-negative pathogens are suspected. One should be aware, however, of the lack of activity of ceftobiprole against ESBL-producing strains. Furthermore, the addition of a combination partner can be considered if the patient is at risk for a *P. aeruginosa* infection as susceptibilities of this species are difficult to predict [29].

**Table 4: Projection of the proportion of ceftobiprole-susceptible isolates among microbiological aetiologies associated with HAP (excluding VAP)**

| Organism | Isolates recovered n (%) [29] | Current study | | Projected proportion of ceftobiprole-susceptible isolates (%) |
|---|---|---|---|---|
| | | Breakpoint (mg/L) | Susceptible isolates (%) | |
| *Staphylococcus aureus* | 88 (33) | 2[a] | 99[b] | 33 |
| *Streptococcus pneumoniae* | 21 (8) | 0.5[a] | 100[c] | 8 |
| *Enterobacteriaceae* | 91 (34) | 0.25[a] | 77 | 26 |
| *Pseudomonas aeruginosa* | 36 (13) | 4[d] | 58 | 8 |
| *Acinetobacter baumannii* | 20 (7) | – | 0 | 0 |
| *Haemophilus influenzae* | 14 (5) | 0.5[e] | 100[f] | 5 |
| **Total** | **270 (100)** | | | **80** |

[a] Species-related clinical breakpoint set by EUCAST
[b] Two out of the three isolates with a MIC of 4 mg/L were considered ceftobiprole-susceptible (see text).
[c] The three isolates with a MIC of 0.5 mg/L were considered ceftobiprole-susceptible (see text).
[d] Pharmacokinetic/pharmacodynamic breakpoint set by EUCAST
[e] Presumed epidemiological cut-off value (ECOFF)
[f] *Haemophilus influenzae* was not tested in the current study, but clinical isolates are usually fully susceptible to ceftobiprole [9].

HAP, hospital-acquired pneumonia; VAP, ventilator-associated pneumonia; –, breakpoint not defined

# Notes

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## Competing interests

M. Kresken is a partner and CEO of Antiinfectives Intelligence GmbH, a research organization providing services to pharmaceutical companies; B. Körber-Irrgang is an employee of Antiinfectives Intelligence GmbH; M. Kaase has received speaker or consultancy fees or research grants from Amplex, AstraZeneca, Bayer Vital, Becton Dickinson, BioMérieux, Bio-Rad, Bruker Daltonics, Cepheid, Infectopharm, MSD, Pfizer, Roche Diagnostics and Siemens Healthcare. All other authors declare no competing interests.

## Ethical approval

Not required.

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## Erratum

The reference in Tab. 4 was corrected from [23] to [29].

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