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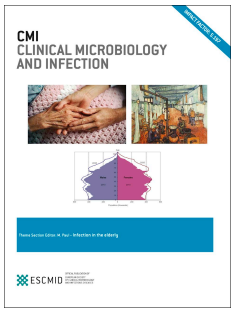

Burkholderia pseudomallei multi-centre study to establish EUCAST MIC and zone diameter distributions and epidemiological cut-off (ECOFF) values

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Title Page

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ABSTRACT

Objectives: Melioidosis, caused by *Burkholderia pseudomallei,* requires intensive antimicrobial treatment. However, standardised antimicrobial susceptibility testing (AST) methodology based on modern principles for determining breakpoints and ascertaining performance of methods are lacking for *B. pseudomallei*. This study aimed to establish MIC and zone diameter distributions on which to set epidemiological cut-off (ECOFF) values for *B. pseudomallei* using standard EUCAST methodology for non-fastidious organisms.

Methods: Non-consecutive, non-duplicate clinical *B. pseudomallei* isolates (9-70 per centre) were tested at eight study centres against eight antimicrobials by broth microdilution (BMD) and the EUCAST disc diffusion method. Isolates without and with suspected resistance mechanisms were deliberately selected. The EUCAST Development Laboratory ensured the quality of study materials, provided guidance on performance of the tests and interpretation of results. Aggregated results were analysed according to EUCAST recommendations to determine ECOFFs.

Results: MIC and zone diameter distributions were generated using BMD and disc diffusion results obtained for 361 *B. pseudomallei* isolates. MIC and zone diameter ECOFFs (mg/L–mm) were determined for amoxicillin-clavulanic acid (8–22), ceftazidime (8–22), imipenem (2–29), meropenem (2–26), doxycycline (2–none), tetracycline (8–23), chloramphenicol (8–22) and trimethoprim-sulfamethoxazole (4–28).

Conclusions: We have validated the use of standard BMD and disc diffusion methodology for AST of *B. pseudomallei*. The MIC and zone diameter distributions generated in this study allowed us to establish MIC and zone diameter ECOFFs, respectively, for the antimicrobials studied. These

ECOFFs served as background data for EUCAST to set clinical MIC and zone diameter breakpoints for *B. pseudomallei*.

Introduction

Melioidosis is a bacterial infection caused by the soil saprophyte *Burkholderia pseudomallei* [1]. The disease is estimated to affect approximately 165,000 people each year worldwide, causing nearly 90,000 deaths [2]. In some parts of the tropics, *B. pseudomallei* is one of the commonest isolates from clinical samples, particularly during the rainy season [3]. A series of randomised controlled trials have shown that the mortality from melioidosis can be substantially reduced by appropriate antibiotic treatment [4], and the overall mortality in northern Australia is now only approximately 10% [5]. However, if appropriate antibiotic treatment is delayed, the mortality rates may exceed 50% [6].

Due to numerous intrinsic resistance mechanisms harboured by the organism, treatment options are limited and these are sometimes further challenged by acquired resistance [7]. Treatment failure due to primary resistance to therapeutic agents is a well-documented problem in *B. pseudomallei* infections [8] which requires laboratories to establish antimicrobial susceptibility testing (AST) methods in order to inform treatment. Since the 1940s there have been numerous studies of the *in vitro* action of antimicrobial agents against *B. pseudomallei* using either broth or agar dilution or gradient diffusion to determine minimum inhibitory concentrations (MICs) [9–15]. Laboratories in endemic areas, however, usually use disc diffusion methods for routine AST of clinical isolates. To date, there have been no internationally accepted criteria published to assist with the interpretation of such tests. The Clinical and Laboratory Standards Institute (CLSI) recommends only the broth microdilution (BMD) method for testing *B. pseudomallei* [16] and EUCAST have not published any recommendations for this species prior to this study. Laboratories have therefore either used interpretative criteria for other species, such as Enterobacterales, *Pseudomonas aeruginosa* or *Burkholderia cepacia*, or developed their own in-house criteria [9–15].

In order to address the need for standardised AST methodology for *B. pseudomallei*, we have undertaken a multi-centre study. Following consultation with clinical colleagues and careful review of the current treatment guidelines, we identified eight clinically relevant antimicrobial agents against *B. pseudomallei*. In this study, we aimed to establish MIC and zone diameter distributions for eight antimicrobials tested against an international collection of *B. pseudomallei* isolates on which to set

epidemiological cut-off (ECOFF) values and interpretative criteria for AST of *B. pseudomallei* using EUCAST methodology for non-fastidious organisms.

Methods

Study design, participants

Potential partners in melioidosis-endemic regions of Southeast Asia and northern Australia, together with reference laboratories in Europe experienced in testing this pathogen, were invited to take part in this multi-centre study. Since *B. pseudomallei* is a laboratory risk group 3 organism in most countries and a potential biothreat, all testing was planned to be performed on the sites where the organism was initially isolated or stored.

The flowchart displaying the stages of the study (carried out prospectively between March 2018 and January 2019) is detailed in the Supplementary material (Fig. S1). The EUCAST Development Laboratory (EDL) undertook the coordinating role in the study and ensured the quality and the representativeness of the data. Participating laboratories and numbers of isolates contributed per centre (*n*) were as follows: Cambodia Oxford Medical Research Unit, Cambodia (70), Mahidol-Oxford Tropical Medicine Research Unit, Thailand (65), Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Lao People's Democratic Republic (63), Royal Darwin Hospital, Australia (52), Townsville Hospital, Australia (49), Bundeswehr Institute of Microbiology, Germany (37), Robert Koch Institute, Germany (16), Public Health Agency of Sweden, Sweden (9).

Pre-study exercise to introduce EUCAST disc diffusion methodology in participating centres

A practical exercise was planned to introduce EUCAST disc diffusion methodology for non-fastidious organisms in the participating laboratories. For this purpose, the laboratories were asked to submit disc diffusion test results for *P. aeruginosa* ATCC 27853 with ceftazidime (10 or 30 µg), imipenem (10 µg) and meropenem (10 µg) discs for 10 consecutive days. The participating laboratories submitted their results together with pictures of disc diffusion plates taken on the first and last day of the testing.

Bacterial isolates

A total of 361 non-consecutive, non-duplicate *B. pseudomallei* clinical isolates (without and with suspected resistance to relevant agents) originating from human infections in different geographic

areas between 1986 and 2018 were selected (9-70 isolates per centre), see Supplementary material (Table S1).

Species identification

Participating centres had a long tradition of the isolation and identification of *B. pseudomallei*. A summary of methods used for identification at each centre is presented in the Supplementary material (Table S1).

Antimicrobial susceptibility testing

All isolates were tested with BMD in accordance with ISO 20776-1 standard [17] against amoxicillin-clavulanic acid (fixed clavulanic acid concentration at 2 mg/L), ceftazidime, imipenem, meropenem, doxycycline, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole. All isolates were tested in parallel with the EUCAST disc diffusion method for non-fastidious organisms [18,19]. Quality control (QC) of the BMD panels (Merlin Diagnostika, Bornheim-Hersel, Germany) and antimicrobial discs (Oxoid, Basingstoke, UK) was performed at the EDL before they were shipped to the participating centres where QC was repeated before testing of clinical isolates. Following a practice period, during which guidance on performance of the tests and interpretation of results was provided by EDL, each centre tested clinical isolates together with four QC strains (*Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213). Disc diffusion AST was performed using Mueller-Hinton agar plates that were routinely used at each participating laboratory, see Supplementary material (Table S2).

ECOFF determination

Each centre submitted their results to the EDL on a spreadsheet where aggregated results were analysed and ECOFFs determined according to EUCAST Standard Operating Procedure (SOP) 10.1 "MIC distributions and the setting of epidemiological cut-off (ECOFF) values" [20]. Consensus from visual estimation and the ECOFFinder program (version 2.1, available on the EUCAST website: https://www.eucast.org/mic_distributions_and_ecoffs/) was used to determine ECOFFs.

Results

The pre-study exercise with *P. aeruginosa* ATCC 27853 allowed the introduction of the EUCAST disc diffusion methodology in the participating centres. See Supplementary material (Table S2) for a summary of results achieved in the pre-study exercise.

MIC and disc diffusion results for eight antimicrobials were collected from the eight centres for 361 *B. pseudomallei* isolates. The pooled MIC and zone diameter distributions are displayed in **Table 1** and **Table 2**, respectively, whereas distributions for the individual centres are available in the Supplementary material (Table S3-S17).

Graphs of MIC-zone diameter correlation were prepared for each antimicrobial agent (see Fig. S2–S9 in the Supplementary material). As an example, the distribution of inhibition zone diameters vs. MICs for ceftazidime is presented in **Fig. 1.**

The MIC distribution histograms are displayed in the Supplementary material for each antimicrobial agent as (1) aggregated data from all laboratories (Fig. S10–S17) and (2) data from individual laboratories (Fig. S18–S25), respectively.

ECOFFs were the consensus from visual estimation and the ECOFFinder program with one slight discrepancy of one dilution with imipenem between visual estimate (2 mg/L) and ECOFFinder program (1 mg/L). The determined ECOFF values and recently published EUCAST clinical breakpoints [21] for *B. pseudomallei* are listed in **Table 3.**

Discussion

In this multi-centre study, we validated the use of standard MIC broth microdilution and disc diffusion methodology for AST of *B. pseudomallei*. MIC and zone diameter ECOFFs for 361 *B. pseudomallei* clinical isolates were determined for eight antimicrobials. The ECOFFs and MIC distributions, served as background data for EUCAST when determining clinical MIC breakpoints and corresponding zone diameter breakpoints [21].

Current recommended treatment for all except mild localised infections is divided into two phases, the initial (intravenous intensive) phase lasts at least ten days (up to eight weeks), and the second (oral eradication) phase lasts at least 12 weeks (up to six months) [4,5]. Following a randomised controlled study published in 1989 [22], ceftazidime became the mainstay antimicrobial with carbapenems (imipenem and meropenem) as a backup option for more severe infections or treatment failures with ceftazidime [5]. Intravenous amoxicillin-clavulanic acid is an option as a second-line therapy during the initial phase where it is available [23], although it is associated with a

higher rate of treatment failures. Trimethoprim-sulfamethoxazole, with amoxicillin-clavulanic acid as an alternative, remains the first-line drug for the eradication phase therapy [24].

Even though primary resistance is uncommon for beta-lactam agents, emergence of resistance is a well-documented, albeit relatively rare, problem for all agents used in the treatment of melioidosis [7]. This increases the importance of performing AST of the organism before the initiation of treatment and monitoring the susceptibility of the isolate if treatment failure is suspected. However, the only recommended method for AST of *B. pseudomallei* is broth microdilution [16] which is cumbersome, especially when considering the high number of cases in endemic areas. The CLSI provides clinical MIC breakpoints for amoxicillin-clavulanic acid (2:1 ratio), ceftazidime, imipenem, doxycycline, tetracycline and trimethoprim-sulfamethoxazole, but not for meropenem which is the drug of choice in severe melioidosis in some centres [5], and chloramphenicol which is sometimes used in eradication therapy.

Due to the lack of a practical standardised method for AST, many laboratories in endemic areas have opted to develop their own in-house criteria for disc diffusion AST of *B. pseudomallei* by adapting clinical breakpoints available in CLSI guidelines for Enterobacterales, *P. aeruginosa* and *B. cepacia* complex [25]. Gradient strip tests are also widely used for determination of MICs of antimicrobials listed in the CLSI guideline. However, in a recent three-centre study, poor correlation with the reference BMD method was found for tetracycline and trimethoprim-sulfamethoxazole Etest strips (bioMérieux, France) for AST of *B. pseudomallei* [26].

Reader subjectivity and, as a consequence, difficulty in determining MIC endpoints for trimethoprim-sulfamethoxazole with *B. pseudomallei* was described previously [12]. In our study, investigators were advised to read the BMD MIC of trimethoprim-sulfamethoxazole at the lowest concentration that inhibited ≥80% of growth as compared to the growth control which corresponds to EUCAST and CLSI recommendations for this agent. The aggregated data from eight centres yielded an MIC distribution in which 91.4% (330/361) of isolates had an MIC between 0.25 and 2 mg/L (see Fig. S17 in the Supplementary material), showing that by standardisation of test procedures and reading practices among investigators, reader subjectivity can be minimised.

The lack of standardised methodology and interpretative criteria for disc diffusion testing of trimethoprim-sulfamethoxazole with *B. pseudomallei*, has resulted in misleading figures for trimethoprim-sulfamethoxazole resistance in *B. pseudomallei* in the literature [27,28]. For example, the national antimicrobial resistance surveillance program in Thailand reported the percentage of trimethoprim-sulfamethoxazole susceptible *B. pseudomallei* isolates between 39.8% and 52.8% for a

total of 4019 isolates collected between 2000 and 2004, which is probably misleadingly low [25]. Laboratories in the national network had submitted susceptibility data for trimethoprim-sulfamethoxazole obtained by disc diffusion methods which were interpreted according to CLSI criteria published for organisms other than *B. pseudomallei*. The failure to follow standardised methodology resulted in erroneous data and the authors described the results as unreliable.

The difficulty of reading disc diffusion results for this combination against *B. pseudomallei* is well known [29]. Prior to the start of the study, we requested pictures from the participating centres showing inhibition zones for *B. pseudomallei* with trimethoprim-sulfamethoxazole. Since the pictures often showed inhibition zones with poorly defined edges (and often with hazy growth within the zone, similar to that often observed for *Stenotrophomonas maltophilia* [21]), we asked all participants to read and record two zone diameters for trimethoprim-sulfamethoxazole; (1) the outer zone edge if an outer zone could be seen, and (2) an inner zone taking all growth into account (see Supplementary material Fig. S26 and specific reading instructions for *B. pseudomallei* in EUCAST clinical breakpoint tables [21]). Despite the reader subjectivity in determining zone edges, a satisfactory inhibition zone diameter distribution was obtained by reading the outer zone edge which showed good correlation with the MICs read at 80% inhibition. Results obtained by this specific reading method were used for analyses.

In EUCAST methodology, the tetracycline disc is used to predict susceptibility to doxycycline. The good correlation between doxycycline MIC ECOFF (2 mg/L) and tetracycline zone diameter ECOFF (23 mm) shown in our study (see Supplementary material Fig. S27) enabled EUCAST to recommend disc diffusion using tetracycline 30 µg disc as a screening test to predict doxycycline susceptibility in *B. pseudomallei* [21].

An earlier study by Maloney et al. generated MIC distributions of *B. pseudomallei* for ceftazidime, meropenem, doxycycline and trimethoprim-sulfamethoxazole [30]. The researchers used the reference BMD method to test 234 consecutive, clinical *B. pseudomallei* isolates. They produced MIC histograms for each antimicrobial agent and proposed ECOFFs by visual inspection. The ECOFFs proposed agree with our ECOFFs for ceftazidime, meropenem and trimethoprim-sulfamethoxazole, but the proposed ECOFF for doxycycline is one dilution higher than our ECOFF.

For a given microbial species and antimicrobial agent, the ECOFF is the highest MIC (and corresponding zone diameter) for organisms devoid of phenotypically-detectable acquired resistance mechanisms. It defines the upper end of the wild-type MIC distribution. The ECOFF provides an opportunity to compare rates of acquired resistance in situations where clinical breakpoints differ (e.g. between organisations, between humans and animals), change over time or have not been set. Our

data meet the criteria in the EUCAST SOP for defining MIC wild-type distributions and determining ECOFFs [20]. Obtaining MIC distributions from eight centres ensured that inter-laboratory variation was factored into the definition of the reference MIC distribution. The aggregated MIC distributions for each antimicrobial contained >100 MIC values in the putative wild-type distribution and >15 MIC values were available for each antimicrobial from seven participating centres. Since the data generated in this study fulfilled the standardised criteria for setting ECOFFs, we managed to establish ECOFFs for all targeted antimicrobials listed in Table 3.

Similarly, the zone diameter distributions generated in this study allowed us to establish zone diameter ECOFFs for all antimicrobials included in the study. This also enabled us to demonstrate that EUCAST standard disc diffusion methodology for non-fastidious organisms is applicable for *B. pseudomallei*.

The treatment of infections with *B. pseudomallei* requires high doses of antimicrobial agents. This is reflected by the fact that most wild-type isolates would be placed in the second EUCAST susceptible category, “susceptible, increased exposure (I)”, and should therefore be reported “I”, the exceptions being imipenem and meropenem. Laboratories adopting this approach will need to devote time and resources to educating clinicians in how to interpret laboratory reports of susceptibility of the species.

Finally, it is important to note that the proportion of non-wild-type organisms in our collection appears spuriously high because a disproportionately high number of isolates with *in vitro* antimicrobial resistance were deliberately included in this study, thus the distributions in our study cannot be used to draw epidemiological conclusions.

Conclusions

The MIC and zone diameter ECOFFs determined in this study formed the basis for EUCAST MIC and zone diameter breakpoints for *B. pseudomallei* in the most recent version of EUCAST clinical breakpoint tables [21]. Determination of MICs is a costly procedure in many low and middle income countries, whereas disc diffusion serves as a cost-effective alternative. We conclude that by implementing the EUCAST standard disc diffusion methodology for *B. pseudomallei*, laboratories in endemic regions where disc diffusion is used routinely will be able to test and report susceptibility results for *B. pseudomallei*.

Transparency declaration

The authors declare no conflict of interest.

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Author contributions

OK, EM, JÅ and GK had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis; and were responsible for drafting the manuscript together with DABD. GK and DABD were responsible for the study's conception or design. Acquisition, analysis or interpretation of data were by OK, DABD, EM, JÅ, PT, JH, PA, VW, TPC, RB, JH, RN, MA, SZ, LZ, TW, DJ, RG, and GK. Critical revision of the manuscript for important intellectual content was by OK, DABD, EM, JÅ and GK. OK and GK were responsible for supervising the study. All of the authors have contributed to writing the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at insert link here.

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Table 1. Minimum inhibitory concentration (MIC) distributions for *B. pseudomallei* isolates (*n* = 361; aggregated data from eight centres)

	MIC (mg/L)											
Antimicrobial agent	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**
Amoxicillin-clavulanic acid*				2	6	140	165	15	3	3	5	**34**
Ceftazidime				1	9	116	189	14	22	5	**17**	
Imipenem		9	58	209	70	18	6	1		**2**		
Meropenem				73	232	60	7	1				
Doxycycline		2	52	195	84	18	8	7	**7**			
Tetracycline				23	96	175	59	9	8	**3**		
Chloramphenicol					1	55	267	31	3	3	**13**	
Trimethoprim-sulfamethoxazole	2	8	32	127	136	47	6	6	8	1		

Underlined = the mode of respective distribution.

Bold underlined = truncation (higher than the highest concentration on the MIC panel).

* For susceptibility testing purposes, the concentration of clavulanic acid was fixed at 2 mg/L.

Table 2. Zone diameter distributions for *B. pseudomallei* isolates (*n* = 361; aggregated data from eight centres)

Antimicrobial agent	Disk content (µg)	Zone diameter (mm)																					
		6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Amoxicillin-clavulanic acid	20-10	8			3	5	1	6	2	4	3		3	4	6	8	5	3	5	19	31	59	64
Ceftazidime	10	23		2	2	4	5	3	4	7	3	3		2	3	7	7	12	17	31	60	65	68
Imipenem	10										2	2		4		4	2	3	6	2		2	
Meropenem	10							1				1	3	5	3	7	14	10	9	16	11	16	14
Tetracycline	30	1							3	2	2	3		3	2	4	6	5	13	26	60	65	62
Chloramphenicol	30	16		1				1			1	1		1	1	2	1	2	15	36	77	59	55
Trimethoprim-sulfamethoxazole	1.25-23.75	17				2	1	1	3	2	1		1	8	2	10	4	7	6	12	20	24	19

Antimicrobial agent	Zone diameter (mm)																						
	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Amoxicillin-clavulanic acid	78	31	13																				
Ceftazidime	27	6																					
Imipenem		2	6	2	12	7	37	55	61	49	50	20	21	5	6		1						
Meropenem	34	39	55	53	51	17	2																
Tetracycline	52	21	17	7	6		1																
Chloramphenicol	39	26	18	6	2				1														
Trimethoprim-sulfamethoxazole	31	15	31	6	26	33	32	27	13	4			2		1								

Underlined = the mode of respective distribution.

Table 3. Epidemiological cut-off (ECOFF) values for *B. pseudomallei* based on minimum inhibitory concentration (MIC) and disc diffusion data on 361 observations for each antimicrobial agent. For reference, MIC and zone diameter clinical breakpoints set by EUCAST are listed.

	MIC and zone diameter ECOFFs for *B. pseudomallei* determined in this study			**EUCAST MIC and zone diameter clinical breakpoints for *B. pseudomallei***			
Antimicrobial agent	**MIC ECOFF (mg/L)**	**Disc content (µg)**	**Zone diameter ECOFF (mm)**	**MIC breakpoints (mg/L)**		**Zone diameter breakpoints (mm)**	
				S ≤	**R >**	**S ≥**	**R <**
Amoxicillin-clavulanic acid	8	20-10	22	0.001	8	50	22
Ceftazidime	8	10	22	0.001	8	50	18
Imipenem	2	10	29	2	2	29	29
Meropenem	2	10	26	2	2	24	24
Doxycycline	2	-	Note*	0.001	2	Note*	Note*
Tetracycline	8	30	23	NA	NA	50	23
Chloramphenicol	8	30	22	0.001	8	50	22
Trimethoprim-sulfamethoxazole	4	1.25-23.75	28	0.001	4	50	17

NA: Not applicable.

* In EUCAST methodology, tetracycline disc diffusion is used to infer doxycycline susceptibility.

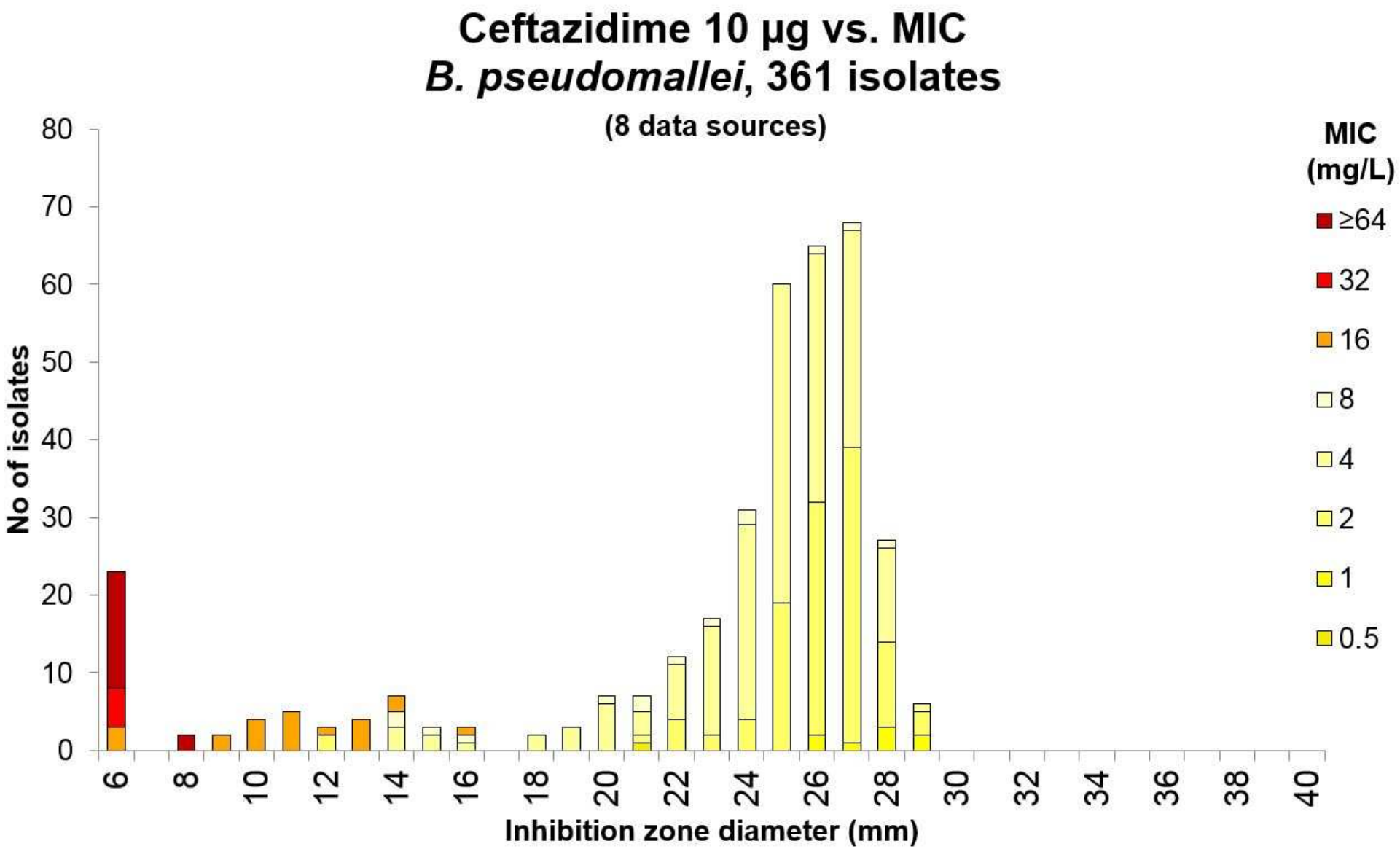

Fig. 1. Ceftazidime (10 µg disc) inhibition zone diameter distribution for *B. pseudomallei* isolates (*n* = 361; aggregated data from eight centres). Corresponding minimum inhibitory concentration (MIC) values are shown through the colouring of bars. The colours correspond to EUCAST ceftazidime MIC breakpoints for *B. pseudomallei* (S ≤ 0.001 mg/L, R > 8 mg/L): I = yellow and R = orange/red.