Aquaculture 490 (2018) 19–24

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Influence of incubation temperature and time on the precision of MIC and disc diffusion antimicrobial susceptibility test data

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ARTICLE INFO

Keywords:
CLSI protocols
MIC
Disc diffusion
Incubation temperature
Precision
Aquatic animals

ABSTRACT

Normalised resistance interpretation was applied to measure the effects of incubation temperature and times on the precision of the data generated by MIC and disc diffusion antimicrobial susceptibility tests performed at 35 °C for 16 h, 28 °C for 24 h, at 22 °C for 24 h, at 22 °C for 48 h and < 19 °C for > 96 h. Analysis of 151 MIC data sets and 141 disc diffusion data sets demonstrated the precision of MIC data sets was not affected by the incubation conditions but that the precision of the disc data decreased significantly as the incubation temperature decreased and the time increased. For MIC data sets the standard deviation of the normalised distribution of the $\log_2$ transformed wild-type observations, which were independent of the numerical values of those sets, were applied as measures of the relative data set precision. The mean values of these standard deviations for 59 data sets generated at 35 °C, 27 data sets generated at 28 °C, 28 data sets generated at 22 °C and 37 data sets generated at 18–19 °C were $0.76 \pm 0.22 \log_2 \mu g\,ml^{-1}$, $0.76 \pm 0.26 \log_2 \mu g\,ml^{-1}$, $0.77 \pm 0.20 \log_2 \mu g\,ml^{-1}$ and $0.76 \pm 0.19 \log_2 \mu g\,ml^{-1}$ respectively. For the disc diffusion data sets the relative precision was quantified by calculating the standard deviation of the normalised distribution of the wild-type observations. The mean values of the standard deviation for 40 data sets generated at 35 °C, 50 data sets generated at 28 °C, 25 data sets generated at 22 °C and 26 data sets generated at ≤18 °C were 2.15 ± 0.61 mm, 2.65 ± 0.74 mm, 4.56 ± 1.09 mm and 6.70 ± 1.70 mm respectively. Because the incubation times increased as the temperatures decreased it was not possible in this work to determine whether changes in either or both were responsible for the reduction in precision observed in the disc zone data.

The low precision of disc diffusion data obtained after prolonged incubation at temperatures below 22 °C suggests, that for bacteria that require these conditions, preference should be given to MIC susceptibility testing methods.

1. Introduction

The incubation conditions recommended in the standard methods for antibiotic susceptibility testing of bacteria isolated from humans and farmed animals published by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucasr.org) and Clinical and Laboratory Standards Institute (CLSI, 2017) is 35 °C ± 2 °C for 16–20 h. However, many bacteria isolated from aquatic animals, and capable of causing infections in those animals, grow poorly or not at all under these conditions. As a consequence, the standard methods for antibiotic testing of bacteria from aquatic sources (CLSI, 2006a; CLSI, 2014) recommend incubation at 28 °C ± 2 °C for 24-28 h, 22 ± 2 °C °C for 24-28 h or 44–48 h and 18 °C ± 2 °C for 92–96 h depending on the species being examined.

Smith and Kronvall (2014a) investigated the performance characteristics of disc diffusion assays at these lower temperatures and longer incubation times. They demonstrated that the standard deviations of the normalised distribution of wild-type (WT) inhibition zones, calculated by normalised resistance interpretation (NRI) (Kronvall and Smith, 2016), provided a reasonable proxy for the relative precision of observational data sets. Analysing data sets composed from observations made on type or reference strains by various laboratories, they demonstrated that the precision of these data sets decreased as the temperature was reduced and as the incubation times were extended. They also demonstrated that these lower temperatures and longer times increased both intra- and inter-laboratory variation.

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https://doi.org/10.1016/j.aquaculture.2018.02.020
Received 20 September 2017; Received in revised form 10 February 2018; Accepted 15 February 2018
Available online 17 February 2018
0044-8486/

Two developments now allow an expansion of the initial work of Smith and Kronvall (2014a). The first is an increase in the data sets available that have been generated in single laboratory studies of susceptibilities at low incubation temperatures. The second is the development and publication of Excel spreadsheets that allow the rapid and automatic application of standardised NRI analysis to both disc and MIC data (http://www.bioscand.se/nri/).

In this work standardised NRI analyses were applied to measuring the precision of both disc diffusion and minimum inhibitory concentration (MIC) data generated in studies of the susceptibility of species or species groups performed at a variety of different incubation conditions by individual laboratories.

2. Materials and methods

2.1. Criteria for selection of susceptibility data sets

The susceptibility test data sets analysed in this work were obtained from the published literature. The data sets included were limited to those that were comprised of measurements made in a single laboratory of the susceptibility of members of a species, or on occasions a group of species, to a specific antimicrobial agent. In order to avoid the low precision that might be associated with data generated in studies made on a small number of strains (Smith and Kronvall, 2015) only those which reported the susceptibility of at least 30 strains were included for analysis. On rare occasions data sets were encountered where the distribution of susceptibility measure were widely dispersed and showed no evidence of a modal group. These data sets were not included in the analyses reported here.

2.2. MIC data sets

In compiling the MIC data sets those for which an excessive percentage of the WT observations were below the lowest limit of quantitation of the methods used were excluded. Equally those that generated in studies that did not employ a two-fold dilution series were also excluded. A total of 151 antibiotic/host specific MIC data sets, each obtained from single laboratories, were analysed.

Fifty-nine MIC data sets generated at 35 °C ± 2 °C with incubation for 16 h–18 h were accessed from the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/). These data were generated using the ISO standard test method (www.iso.org/standard/41630.html). These 59 data sets were referred to as the 35 °C/16 h sets.

Twenty-seven MIC data sets generated at 28 °C ± 2 °C with incubation for 24 h–28 h were obtained from studies of Uhland (2010) and Declercq et al. (2013). Uhland (2010), who investigated the susceptibilities of *Aeromonas* spp. and *Vibrio* spp., used the micro dilution protocol recommended in the CLSI guideline M49-A (CLSI, 2006b) and Declercq et al. (2013), who investigated *Flavobacterium columnare*, used the protocol suggested for this species in the same guideline. These 27 data sets were referred to as the 28 °C/24 h sets.

Fourteen MIC data sets generated at 22 °C ± 2 °C with incubation for 24 h–28 h were obtained from studies of *Aeromonas* spp. by Baron et al. (2017) who used the protocol recommended in the CLSI guideline VET04-A2 (CLSI, 2014). These 14 data sets were referred to as the 22 °C/24 h sets.

Fourteen MIC data sets generated at 22 °C ± 2 °C with incubation for 44 h–48 h were obtained from studies of *A. salmonicida* by Miller and Reimschuessel (2006) and Uhland and Higgins (2006) and the studies of various species by Michel et al. (2003). Miller and Reimschuessel (2006) used the micro dilution test protocols recommended in the CLSI guideline M49-A (CLSI, 2006b). Uhland and Higgins (2006) and Michel et al. (2003) used the agar dilution protocol of Alderman and Smith (2001). These 14 data sets were referred to as the 22 °C/48 h sets.

Thirty-seven MIC data sets generated at 18 °C ± 2 °C with an incubation time of 96–240 h were analysed. Twenty-seven were obtained from studies of *F. psychrophilum* by Michel et al. (2003), Miranda et al. (2016), Ngo et al. (2018), Smith et al. (2016) who reported data from 2 laboratories, and Van Vliet et al. (2017). The data of Ngo et al. (2018), Smith et al. (2016) and Van Vliet et al. (2017) were all generated using the appropriate micro-dilution protocols recommended in the CLSI guideline VET04-A2 (CLSI, 2014). The agar dilution protocol used by Miranda et al. (2016) was based on that suggested in M49-A (CLSI, 2006b) and that of Michel et al. (2003) was based on the suggestions in Alderman and Smith (2001). An additional six MIC data sets that had been generated in studies of *Piscirickettsia salmonis* were analysed (Henríquez et al., 2015; Contreras-Lynch et al., 2017). There is currently no standard test protocol for determining MIC values for *P. salmonis* and both authors used their own media. Four of the data sets were obtained from Henríquez et al. (2015) who used incubation at 19 °C for 5–7 days and two from Contreras-Lynch et al. (2017) who used incubation at 18 °C for 7–10 days. These 37 data sets were referred to as the 18 °C/96 + h sets.

2.3. Disc diffusion zone data sets

A total of 141 antibiotic/host specific disc diffusion data sets were accessed from the published literature. Each of these data sets was generated in a single laboratory.

Forty disc diffusion data sets generated at 35 °C ± 2 °C with incubation for 16-18 h were accessed from the EUCAST website (www.eucast.org/mic_distributions_and_ecoffs/). These 40 data sets were referred to as the 35 °C/16 h sets.

Fifty disc diffusion data sets generated at 28 °C ± 2 °C with incubation for 24-28 h were obtained from studies of *Aeromonas* spp. by Smith et al. (2012) studies of *Aeromonas* spp. and *Vibrio* spp. by Uhland (2010), from studies of *V. anguillarum* by Smith and Christofilogiannis (2007) and from studies of *Edwardsiella tarda* and *V. harveyi* by Lim et al. (2016). These studies used the test protocol recommended in the CLSI guideline M42-A (CLSI, 2006a). For some agents, however, Smith et al. (2012) used discs with contents other than those specified in this guideline. In addition data sets were obtained from Kwon et al. (2016) who studied *Photobacterium damselae* using the M42-A (CLSI, 2006a) protocol modified by the addition of 1%NaCl to the test agar. These 50 data sets were referred to as the 28 °C/24 h sets.

Twenty-five disc diffusion data sets generated at 22 °C ± 2 °C with incubation for 44 h–48 h were obtained from studies of *A. salmonicida* by Miller and Reimschuessel (2006) and Smith et al. (2007), who reported data from two laboratories. These studies used the test protocols recommended in the CLSI guideline M42-A (CLSI, 2006a). These 25 data sets were referred to as the 22 °C/48 h sets.

In addition twenty-six disc diffusion data sets generated at low temperatures (≤18 °C) with prolonged incubation times (≥96 h) were analysed. These were accessed from four sources. Ten were accessed from the studies of *F. psychrophilum* of Ngo et al. (2018) and Miranda et al. (2016) and 16 were abstracted from the susceptibility test records for *F. psychrophilum* and *Piscirickettsia salmonis* of ADL Diagnostic Chile Ltd.©, Puerto Montt, Chile. There are currently no standard test protocols for determining disc diffusion values for species that require prolonged incubation at temperatures ≤18 °C. Each of these four studies used slightly different testing protocols. These 26 data sets were referred to as the 18 °C/96 + h sets.

2.4. NRI analysis of data sets

The NRI method was used with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European patent No. 1,383,913, US Patent No. 7,465,559).

For the purposes of analysis, all MIC susceptibility measures were recorded as $\log_2 \mu g\, ml^{-1}$ values. All antibiotic/host specific data sets were analysed by NRI and the means and standard deviation of the