Agreement between the categorization of isolates of *Aeromonas salmonicida* and *Yersinia ruckeri* by disc diffusion and MIC tests performed at 22°C

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Abstract
Standard disc diffusion and MIC test procedure were used to investigate the susceptibility of two hundred and fifty-one isolates collected from infected fish in France to florfenicol, oxolinic acid and tetracycline. The tests were performed at 22 ± 2°C and for the 177 *Yersinia ruckeri* they were read after 24–28 hr incubation and for the 74 *Aeromonas salmonicida* isolates they were read after 44–48 hr. Applying epidemiological cut-off values to the susceptibility data generated in these tests, the isolates were categorized as wild-type or non-wild-type. The agent-specific categories into each isolate were placed on the basis of the data generated by the two methods were in agreement in 98% of the determinations made. It is argued that, with respect to categorising isolates, disc diffusion and MIC methods can be considered as equally valid at this temperature and after both periods of incubation.

KEYWORDS
*A. salmonicida*, categorical agreement, Clinical Laboratory Standard Institute, disc diffusion, Minimal inhibitory concentration, *Y. ruckeri*

1 | INTRODUCTION

With respect to non-fastidious species isolated from aquatic animals, the Clinical Laboratory Standard Institute (CLSI) have published standard test protocols for disc diffusion and Minimal Inhibitory Concentration (MIC) methods that specify incubation at 28 ± 2°C or 22 ± 2°C (CLSI, 2020a). Therefore, in designing studies of the antimicrobial susceptibility of bacteria that require these temperatures laboratories may choose to use either standard protocols for disc diffusion or those for MIC determinations. Logistical or economic factors may influence their decision, but they may also take into account the relative performance characteristics of the two methods (Smith, 2019).

One of the primary aims of susceptibility testing of isolates from aquatic animals is to categorize isolates as either wild-type (WT) or non-wild-type (NWT). Isolates that manifest a susceptibility within the range recorded for fully susceptible members of their species are
categorized as WT. Isolates that show a susceptibility outside this range which possess some mechanism that confers reduced susceptibility are categorized as NWT (Silley, 2012).

With respect to their relative performance characteristics, the original paper of Ericsson and Sherris (1971) argued that the precision of disc diffusion test data was slightly superior to that of MIC data. In a study of the influence of incubation temperature on the precision of susceptibility tests, Smith et al. (2018) also suggested that, when the data were obtained at 35°C, the precision of disc diffusion data was equal to or superior to that of MIC tests. However, they noted that the precision of disc diffusion data, but not those of MIC data, decreased when the temperature of incubation was decreased, and the incubation time was increased. They argued that putative epidemiological cut-off values calculated from data with low precision should not be applied or, at the very least, should be applied with extreme caution. They further argued that the extent of the loss of precision at lower incubation temperatures was such that for organisms requiring incubation at temperatures <22°C for >48 hr the disc diffusion method could not be recommended. This work was, therefore, undertaken to investigate the relative performance of disc diffusion and MIC methods when they were performed at 22 ± 2°C and incubated for either 24-28 or 44-48 hr.

In this work, the relative performance of disc diffusion and MIC methods was investigated by calculating the extent to which the categorization of isolates as WT or NWT, made from the data they generated, were in agreement with each other. These categorizations were generated by the application of epidemiological cut-off values to disc diffusion and MIC data for the susceptibility to florfenicol (FLO), oxolinic acid (OXO) and tetracycline (TET) obtained for isolates of Yersinia ruckeri and Aeromonas salmonicida collected from French laboratories. These susceptibilities were determined at 22 ± 2°C by the standard CLSI disc diffusion and MIC tests (CLSI, 2020a), and incubated for 24-28 and 44-48 hr. However, CLSI have not yet developed epidemiological cut-off values (ECVs) for any agents against Y. ruckeri or for TET against A. salmonicida. The guideline VET03Ed3 (CLSI, 2020b) gives ECVs for both inhibition zone and MIC data for FLO and OXO against A. salmonicida generated in tests at 22°C with incubation for 44-48 hr. However, the guideline M23Ed5 (CLSI, 2018) recommends that data from at least three laboratories are needed to set such ECVs and those for A. salmonicida MIC data were estimated using data from only a single laboratory and those for inhibition data from only two laboratories. Therefore, in this work the epidemiological cut-off values (COw) applied to the data sets for the French isolates studied in this work were calculated from those data themselves aggregated with additional published data sets that had been obtained using the same CLSI protocols.

2 | MATERIAL AND METHODS

2.1 | Bacterial isolates

The 74 Aeromonas salmonicida isolates and 177 Yersinia ruckeri isolates studied in this work were collected as part of the DIAMIC project, supported by the French EcoAntibio Plan. They were contributed from eleven veterinarians and veterinary laboratories working in France. The criterion for inclusion was that the isolates had been obtained during clinical investigations of diseased fish in France during the period 1985-2020.

The Mueller–Hinton agar (MHA) used in this work was obtained from Bio-Rad, the cation-adjusted Mueller–Hinton broth (CAMHB), and Brain Heart Infusion broth (BHI) were obtained from Thermo Fisher. Each isolate received in the laboratory was first grown on MHA at 22°C to check purity and stored in duplicate at −70°C in BHI broth supplemented with 20% of glycerol.

Confirmation of the identification of the presumptive A. salmonicida isolates was performed by Maldi-Tof and the PCR method of Byers et al. (2002). Only isolates, which were identified A. salmonicida by Maldi-Tof with score higher than 2.0 and gave positive result with PCR, were included in this study. Confirmation of the classification of the presumptive Y. ruckeri was also performed by Maldi-Tof. Only isolates, which were identified as Y. ruckeri with a score higher than 2.3, were included in this study.

2.2 | Susceptibility testing

Using both disc diffusion and MIC methods, the antimicrobial susceptibilities of the French isolates of both species were tested in a single laboratory (Anses MBA) against three agents FLO, OXO and TET. These agents are used in aquaculture in France and susceptibility to them are routinely investigated in French diagnostic laboratories. As recommended by Smith (2019), the abbreviations for these agents were generated by applying the EUCAST rules (www.eucast.org/ast_of_bacteria/guidedocuments/).

For the disc diffusion tests, FLO (30 µg) discs were obtained from Mast. The OXO (2 µg) and the TET (30 µg) discs were obtained from Bio-Rad. The microdilution MIC tests were performed using 96-well plates made in the ANSES laboratory using a Biomek automated liquid handler (Beckman Coulter). The concentration ranges in these plates were 0.003–64 mg/L for FLO, 0.004–16 mg/L for OXO and 0.015–128 mg/L for TET.

The susceptibility testing protocols used in this work were those specified for non-fastidious organisms in the CLSI guideline VET03Ed2 (CLSI, 2020a). The MIC tests were performed using CAMHB and disc diffusion tests were performed using MHA with the results being recorded after either 24-28 or 44-48 hr incubation at 22° ± 2°C. Reading of inhibition zone size was performed using an automatic reader of discs diffusion susceptibility tests “Sirscan micro” (I2A Diagnostic) including a system of image capture. The reading of each agar plate controlled by the same technician who performed all analysis. Following the standard practice of French diagnostic laboratories, the Y. ruckeri tests were read after incubation for 24-28 hr and the A. salmonicida tests after 44-48 hr.

For both disc diffusion and MIC tests, the control quality (QC) reference strains Escherichia coli ATCC25922 and Aeromonas salmonicida ATCC33658 were tested using the same test protocols as
were used for the tests of the *Y. ruckeri* and *A. salmonicida* isolates. The acceptable ranges applied were those given for FLO and OXO in the CLSI supplement VET04Ed3 (CLSI, 2020b). This supplement does not however provide acceptable ranges for TET for these reference strains using either the disc or MIC tests at 22°C. Because of this absence of the necessary quality control requirements, the susceptibility tests with this agent, although were performed using the testing protocols specified in VET03Ed2 (CLSI, 2020a), cannot be considered as having been performed according to the full CLSI method.

### 2.3 | Data sets for calculating epidemiological cut-off values

The epidemiological cut-off values (CO\textsubscript{WT}) applied to the data sets for the French isolates generated in this work were calculated from those data themselves aggregated, when they were available, with additional data sets that had also been obtained at 22°C. These data sets had been generated using the CLSI protocols provided in VET03-A (CLSI, 2006). However, with respect to the non-fastidious bacteria studied in this work the protocols provided VET03-A (CLSI, 2006) and VET03Ed3 (CLSI, 2020b) were identical.

For *Y. ruckeri*, additional data sets accessed were those for the MIC values for 83 isolates with respect to FLO and TET (Huang et al., 2014).

For *A. salmonicida*, the additional data sets accessed were the MIC and disc diffusion data for 217 isolates with respect to FLO and OXO (Miller & Reimschuessel, 2006) and the data for disc diffusion 107 isolates with respect to FLO (Smith et al., 2007) and OXO (Ruane et al., 2007). All these data sets had been used to establish the ECV published in VET04Ed3 (CLSI, 2020b).

### 2.4 | Calculation of epidemiological cut-off values

Epidemiological cut-off values (CO\textsubscript{WT}) were calculated using the automatic Excel spreadsheets for normalized resistance interpretation analysis (NRI) (Kronvall, 2010; Kronvall & Smith, 2016) (www.biocand.se/nri/). Isolates were categorized as wild-type (WT) or non-wild-type (NWT) by application of these cut-off values to the disc zone and MIC values determined in this work. The acronyms used to refer to epidemiological cut-off values followed the recommendations of Smith (2019). The acronym ECV was reserved for cut-off values published by CLSI and for all other epidemiological cut-off values the acronym CO\textsubscript{WT} was used.

### 2.5 | Categorical agreement

For each agent/species combination, the categorization of each isolate as WT or NWT on the basis of disc diffusion data was compared with the categorization of the same isolate based on the MIC data. The percentage categorical agreement (CA) was calculated as the number of isolates which were placed in the same category by both disc and MIC methods as a percentage of the total number of isolates tested.

### 3 | RESULTS AND DISCUSSION

The zones of inhibition generated by FLO (30 µg) and OXO (2 µg) discs were determined for both QC reference strains on five occasions during this work. The MIC values for these agents against the reference strains were determined on four...
occasions. All these susceptibility measures were within the acceptable ranges set in VET04Ed3 (CLSI, 2020b). Full details of these results are available in the supplementary files (Table S1 and Table S2).

3.1 | NRI analysis

Table 1 presents a summary of the NRI analysis of the disc diffusion and MIC data obtained for 177 Y. ruckeri and 74 A. salmonicida isolates.

![FIGURE 2](image_url) Frequency distribution for MICs and diameters of the zone of inhibition for isolates of A. salmonicida (n = 74) when tested against florfenicol (30 µg; A), oxolinic acid (2 µg; B) and Tetracycline (30 µg, C) — Provisional Epidemiological cut-off values are indicated for MICs (horizontal dashed lines) and diameters of the zones of inhibition (vertical dashed lines) for each antimicrobial.

| Species           | Agent | Method | Isolates (labs) | CO_{WT}   | SD       | Source   |
|-------------------|-------|--------|-----------------|-----------|----------|----------|
| Y. ruckeri (read at 24−28 hr) | FLO   | Disc   | 177 (1)         | ≥27 mm    | 2.8 mm   | a        |
|                   | MIC   |        | 260 (2)         | ≤8 mg/L   | 0.37 log² mg/L | a, b     |
|                   | OXO   | Disc   | 177 (1)         | ≥28 mm    | 3.6 mm   | a        |
|                   | MIC   |        | 177 (1)         | ≤0.063 mg/L | 0.51 log² mg/L | a        |
|                   | TET   | Disc   | 177 (1)         | ≥26 mm    | 3.1 mm   | a        |
|                   | MIC   |        | 260 (2)         | ≤1 mg/L   | 0.53 log² mg/L | a, b     |
| A. salmonicida (read at 44−48 hr) | FLO   | Disc   | 398 (3)         | ≥29 mm    | 4.4 mm   | a, c, d  |
|                   | MIC   |        | 291 (2)         | ≤4 mg/L   | 1.0 log² mg/L | a, c     |
|                   | OXO   | Disc   | 398 (3)         | ≥31 mm    | 3.6 mm   | a, c, e  |
|                   | MIC   |        | 291 (2)         | ≤0.063 mg/L | 0.55 log² mg/L | a, c     |
|                   | TET   | Disc   | 74 (1)          | ≥29 mm    | 2.9 mm   | a        |
|                   | MIC   |        | 74 (1)          | ≤0.5 mg/L | 0.41 log² mg/L | a        |

Note: a; this work, b; Huang et al. (2014), c; Miller & Reimschuessel, 2006, d; Smith et al. (2007), e; Ruane et al. (2007)

Abbreviations: CO_{WT}, epidemiological cut-off values calculated by NRI analysis; Disc, Disc diffusion inhibition zones; FLO, florfenicol; MIC, minimal Inhibitory concentrations; OXO, oxolinic acid; SD, standard deviation of the normalized distributions for putative WT isolates; TET, tetracycline.
For all twelve data sets analysed, the standard deviations (SD) of the distributions of normalized WT observations, calculated by NRI analysis, were within the limits suggested by Smith et al. (2018).

3.2 | NRI analysis of the Y. ruckeri isolates

Scatter plots of the paired disc zones and MIC values for the Y. ruckeri isolates are shown for each of the three agents in Figure 1. The frequency of isolates categorized as NWT phenotype and the agreement between the categorization based on disc zone data and MIC values are shown in Table 2.

Analysis of the FLO disc data obtained in this work generated a CO\(_{WT}\) of ≥27 mm and analysis of the MIC data obtained in this work aggregated with those of Huang et al. (2014) generated a CO\(_{WT}\) of ≤8 mg/L. Application of these cut-off values to the data obtained for the 177 French isolates studied in this work categorized the same two isolates as NWT and the CA between the disc and MIC methods was 100%.

Analysis of the OXO disc data obtained in this work generated a CO\(_{WT}\) of ≥28 mm and applying this cut-off categorized 154 of the French isolates as NWT. Analysis of the MIC data generated a CO\(_{WT}\) of ≤0.063 mg/L which categorized 156 of the French isolates as NWT. With respect to OXO, the CA between the disc and MIC methods was 98%.

Analysis of the TET disc data obtained in this work generated a CO\(_{WT}\) of ≥26 mm and application of this cut-off value categorized one French isolate as NWT. The MIC data obtained in this work aggregated with those of Huang et al. (2014) generated a CO\(_{WT}\) of ≤1 mg/L which categorized all the French isolates as WT. With respect to TET, the CA between the disc and MIC methods was 99%.

Combining the Y. ruckeri data for all three agents, the total number of paired comparisons of the categorizations of isolates generated by the disc and MIC methods was 531. The categorizations were in agreement 528 (99%) of these pairs.

### TABLE 2 Summary of the categorization 177 Yersinia ruckeri and 74 Aeromonas salmonicida isolates as WT or NWT by applying the CO\(_{WT}\) generated in this work (Table 1) and the agreement between the categorizations made on the basis of MIC and disc diffusion data

| Species (number tested) | Agent | Method | NWT Isolates (%) | Categorical agreement |
|-------------------------|-------|--------|------------------|----------------------|
| Y. ruckeri (n = 177)    | FLO   | Disc   | 2 (1)            | 177 (100)            |
|                         |       | MIC    | 2 (1)            |                      |
|                         | OXO   | Disc   | 154 (87)         | 175 (98)             |
|                         |       | MIC    | 156 (88)         |                      |
|                         | TET   | Disc   | 1 (1)            | 176 (99)             |
|                         |       | MIC    | 0 (0)            |                      |
| A. salmonicida (n = 74) | FLO   | Disc   | 18 (24)          | 72 (97)              |
|                         |       | MIC    | 20 (27)          |                      |
|                         | OXO   | Disc   | 70 (95)          | 73 (99)              |
|                         |       | MIC    | 71 (96)          |                      |
|                         | TET   | Disc   | 44 (59)          | 69 (93)              |
|                         |       | MIC    | 49 (66)          |                      |

Abbreviations: Disc, Disc diffusion inhibition zones; FLO, florfenicol; MIC, minimal inhibitory concentrations; NWT, non-wild-type; OXO, oxolinic acid; TET, tetracycline.

3.3 | NRI analysis of the A. salmonicida isolates

Scatter plots of the paired disc zones and MIC values for the A. salmonicida isolates are shown for each of the three agents in Figure 2. The frequency of isolates categorized as NWT phenotype and the agreement between the categorization based on disc zone data and MIC values are shown in Table 2.

Analysis of the FLO disc data obtained in this work aggregated with those published by Miller and Reimschuessel (2006) and Smith et al. (2007) generated a CO\(_{WT}\) of ≥29 mm. Application of this cut-off value to the FLO data for the 74 French isolates categorized 18 of them as NWT. Analysis of the MIC data for FLO generated in this work aggregated with those published by Miller and Reimschuessel (2006) generated a CO\(_{WT}\) of ≤4 mg/L and application of this cut-off value to the FLO data for the French isolates categorized 20 of them as NWT. With respect to FLO, the CA between the disc and MIC methods for the French isolates was 97%.

Analysis of the OXO disc data obtained in this work aggregated with those published by Miller and Reimschuessel (2006) and Ruane et al. (2007) generated a CO\(_{WT}\) of ≥31 mm. Application of this cut-off value to the OXO data for the French isolates categorized 70 of them as NWT. Analysis of the MIC data for FLO generated in this work aggregated with those published by Miller and Reimschuessel (2006) generated a CO\(_{WT}\) of ≤0.063 mg/L and application of this cut-off value to the OXO data for the French isolates categorized 71 of them as NWT. With respect to OXO, the CA between the disc and MIC methods for the French isolates was 99%.

Analysis of the TET disc data obtained in this work generated a CO\(_{WT}\) of ≥29 mm. Application of this cut-off value to the TET data for the French isolates categorized 44 of them as NWT. Analysis of the MIC data for TET generated in this work generated a CO\(_{WT}\) of ≤0.5 mg/L and application of this cut-off value to the TET data for the French isolates categorized 49 of them as NWT. With respect to TET, the CA between the disc and MIC methods for the French isolates was 93%.

Combining the A. salmonicida data for all three agents, the total number of paired comparisons of the categorizations of isolates generated by the disc and MIC methods was 222. The categorizations were in agreement 214 (96%) of these pairs.

3.4 | Frequency of reduced susceptibility

In the isolates examined in this work, the frequencies of NWT phenotypes with respect to OXO were high in both species. Using the
MIC data, the frequencies of NWT phenotypes with respect to OXO were 96% for *A. salmonicida* and 88% for *Y. ruckeri*. With respect to TET and FLO, the frequencies of NWT were higher in the *A. salmonicida* isolates than in the *Y. ruckeri* isolates. Again using the MIC data, the frequencies with respect to TET were 59% for *A. salmonicida* and 0% for *Y. ruckeri* and with respect to FLO they were 27% for *A. salmonicida* and 1% for *Y. ruckeri*.

It should be noted, however, that the isolates studied in this work were collected from those held by eleven diagnostic laboratories operating in France. Smith et al., (2013) suggested that it is reasonable to assume that outbreaks which are not effectively controlled by chemotherapy with the first agent chosen would be investigated in more detail than those which were effectively controlled. Moreover, the diagnostic laboratories might have been more prone to keeping isolates with abnormal susceptibility profiles in their historical collection of strains. Thus, there is potential for isolates with reduced susceptibility among being over-represented in those collected by frontline diagnostic laboratories. As a result of the potential for this bias in the isolates studied in this work, the frequencies of NWT phenotypes reported here cannot be taken as providing an accurate picture of the frequency of reduced susceptibility in France as a whole.

**4 | CONCLUSIONS**

In the study of bacteria isolated from aquatic animals, the primary aim of either zone size or MIC data is to categorize isolates as either WT, fully susceptible members of their species, or as NWT isolates, that possess some mechanism that confers reduced susceptibility (Silley, 2012). In this work, the agreement between the categorization of isolates using disc diffusion data and that using MIC data both generated at 22°C according to the testing protocols specified by CLSI (2020a) was investigated for 735 sets of paired determinations. The overall categorical agreement between the two methods was 98%. The work reported here on susceptibility tests performed at 22°C suggests that disc diffusion or MIC methods were equally valid and found no reasons why one should be preferred over the other.

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**CONFLICT OF INTEREST**

There were no conflicts of interest.

**ETHICS APPROVAL, PATIENT CONSENT AND CLINICAL TRIAL REGISTRATION STATEMENT**

The work reported did not require ethical approval or patient consent and did not involve any clinical trials.

**PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES**

All material from other sources was available and accessed from published work that was in the public domain.

**DATA AVAILABILITY STATEMENT**

Data sharing not applicable to this article as no data sets were generated or analysed during the current study.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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