# Effect of Inoculum, pH, and Cations on the *In vitro* Activity of OPT-80 vs. *Clostridium difficile*

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## Abstract

**BACKGROUND** OPT-80 is a new macrocyclic compound in clinical development for the treatment of *Clostridium difficile*-associated diarrhea. This study was conducted to investigate the influence of the growth medium (different commercial lots, pH, and concentration of divalent cations $Mg^{2+}$ and $Ca^{2+}$) and inoculum density on *in vitro* susceptibility testing of OPT-80.

**METHODS** The effect of inoculum, pH, different lots of commercial media and cations on susceptibility of ATCC strains of *Clostridium difficile* (*C. difficile*) to OPT-80 was determined using the CLSI (NCCLS) agar dilution method. The minimum inhibitory concentration (MIC) of OPT-80 was tested over an inoculum density range of $10^5$ – $10^8$ cfu/ml ($10^2$ - $10^5$ cfu/spot); pH range of 6.2 – 7.9; calcium concentration of 2.1 - 5.7 mg/dL; and magnesium concentration of 3.3 - 7.5 mg/dL. The effect of pH on the *in vitro* activity of OPT-80 was also tested via the broth dilution method. For pH experiments, buffer was added to media to minimize pH shifts caused by anaerobic equilibration. The MIC assays were controlled either by using vancomycin with in-house established OPT-80 MIC range of 0.5 – 1 µg/mL or by using the CLSI quality control organism (*Eubacterium lentum* vs. clindamycin).

**RESULTS** The susceptibility of *C. difficile* strains to OPT-80 was not dependent on the inoculum density, commercial lots of supplemented Brucella agar, or divalent cation concentrations within the media. In contrast, the MIC of OPT-80 vs. *C. difficile* increased with increasing pH. In both broth and agar dilution testing, the MIC of OPT-80 increased 8-fold from pH 6 to pH 8. The MIC values for vancomycin also increased with pH, and by a similar magnitude.

**CONCLUSIONS** These results indicate that the activity of OPT-80 is not affected by the size of inoculum or cation concentrations. In contrast, pH is a significant factor affecting the susceptibility of *C. difficile* to OPT-80 and vancomycin.

## Introduction

OPT-80 is a new antimicrobial compound with minimal activity against gram-negative bacteria, moderate activity against most gram-positive bacteria, and excellent activity toward *Clostridium difficile* (1 – 3). It is currently under development for the treatment of *C. difficile*-associated diarrhea (CDAD).

The MIC values measured for many antibiotics are known to be affected by environmental variables such as pH, the concentration of divalent cations such as calcium and magnesium, and the bacterial density. The dependence of the antibacterial activity on these factors is an important consideration, particularly for an antibiotic that targets bacteria in the gut, where these parameters can vary greatly with the diet and disease state.

The sensitivity of the MIC to these environmental variables may also be an important factor to consider when designing methodology for future *in vitro* testing. The Clinical and Laboratory Standards Institute, CLSI (formerly NCCLS) recommends using Brucella agar supplemented with vitamin $K_1$ and hemin for Minimal Inhibitory Concentration (MIC) determination for anaerobes. The level of divalent cations in this medium, however, is not standardized. Moreover, the pH of the media used under anaerobic glove box may also vary under different gas mixtures. Anaerobes are typically incubated in a mixture of nitrogen, hydrogen, and carbon dioxide, and the presence of $CO_2$ will acidify the medium and can be a significant source of variability. The inoculum size may also be difficult to standardize given the variety of atmospheric conditions available for anaerobic susceptibility testing ($H_2/CO_2$ generator, evacuation/replacement method, or anaerobic chamber). The anaerobic conditions available to each lab will determine the duration of organism exposure to aerobic atmosphere during bench top manipulations and anaerobic equilibration, and thus affect culture viability and experimental result.

In this study, we examined the effect on MIC of the level of the divalent cations calcium and magnesium, pH (from 5 – 8), inoculum density (over 3 orders of magnitude), and also the variability from lot to lot of Brucella broth.

## Materials and Methods

**BACTERIAL STRAINS** Laboratory strains of *Clostridium difficile* 9689, 700057, 43255, 17857 and *Eubacterium lentum* 43055 were obtained from American Type Culture Collection (ATCC). All strains were streaked onto brucella agar plates, supplemented with hemin, and vitamin K from frozen stocks maintained at −78°C in 10% glycerol prior to use.

**MIC TESTING** Current CLSI procedures (4) for anaerobic broth and agar dilution were used for MIC evaluation. Broth dilution is not a validated method for MIC testing of *Clostridium*; however, due to potential inaccuracy of measuring the pH of solid agar after equilibration inside the anaerobic chamber, both methods were used and compared for the assessment of pH effects. All MIC runs were performed at least in duplicate. When replicates were different the mode was presented; if replicates were evenly split between two values, the higher one was given.

**INOCULUM DENSITY EFFECT ON MIC VALUES** The effects of inoculum density on susceptibility of *C. difficile* to OPT-80 and vancomycin were determined using the agar dilution method (4). The inocula were prepared by first making a suspension of ~$10^8$ cfu/mL and then serially diluting the suspension by 10-fold factors to obtain a culture density range between $10^5$ – $10^8$ cfu/mL, to give spot densities of $10^2$ – $10^5$ cfu/spot.

**PH EFFECT ON MIC VALUES** The susceptibility of *C. difficile* to OPT-80 was evaluated over a pH range of 6 – 8 using both agar dilution and microbroth dilution methods.

Using the agar dilution method, the MIC of OPT-80 was determined over a pH range of 6.2 – 8.0 against *C. difficile* strains in two separate experiments. In order to achieve the desired anaerobic pH for susceptibility testing, buffer (100 mM of $NaH_2PO_4$ or TAPS [N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic Acid]) was added to media at pH 7 and 8, respectively. Even with strong buffering, the pH shifted slightly following equilibration in the anaerobic gas, and thus in some cases media was titered in ambient air to above the desired anaerobic pH. The actual pH was always confirmed following equilibration inside the anaerobic chamber. Vancomycin, used as a control, was tested only at pH 7.

Using the broth microdilution method, the MIC values of OPT-80 and vancomycin were determined over a pH range of 6 – 8 against *C. difficile* strains in three separate series. In the first series, unbuffered Brucella broth was titrated in ambient air to obtain a pH range from 5 – 9. However, anaerobic equilibration of media in the glove box environment (10% $H_2$ / 5% $CO_2$ / 85% $N_2$) lowered the pH of the media, resulting in an anaerobic pH range from 5 – 7.5 (as tested using a portable pH meter with a flat-bottomed pH probe calibrated with buffer standards outside the glove box, then transferred inside). For subsequent experiments, buffer was added to media to resist pH shifts caused by anaerobic equilibration. In the second series, 10 mM buffer [$NaH_2PO_4 \cdot H_2O$ pH 7.0, MOPS pH 8.0, or TAPS pH 9.0, pH values in ambient air) was added to media with pH values greater than 6 to obtain a pH range from 6 – 7.6 after anaerobic equilibration. In the third series, the buffer concentration was increased to 100 mM for pH treatments above 6 to obtain an anaerobic pH range from 6 – 8.1.

**DIVALENT CATION CONCENTRATION EFFECT ON MIC VALUES** The agar dilution method was used to determine the effect of calcium and magnesium ion concentrations on susceptibility of *C. difficile* strains to OPT-80. The levels of divalent cations in the Brucella broth as acquired from the manufacturer were determined by the Laboratory Specialists, Inc. Additional amounts of divalent cations were added (in the form of calcium or magnesium chloride in order to give calcium ion concentrations of 2.1, 3.0 and 5.7 mg/dL and magnesium ion concentrations of 3.3, 4.5, and 7.5 mg/dL.

**REPRODUCIBILITY OF OPT-80 MIC VALUES WITH DIFFERENT COMMERCIAL LOTS OF MEDIA** Using the CLSI agar dilution method, susceptibility of *C. difficile* to OPT-80 was also examined with three different commercial lots of Brucella agar, from BBL (lot #30768960, 211086, and 3167036), supplemented with different lots of vitamin K (Sigma lot # V-3501 and 0214010) and hemin (Sigma lot # 072K1221 and 034K7656).

## Results

**INOCULUM DENSITY EFFECT ON MIC VALUES**

Tables 1 and 2 demonstrate the effect of inoculum density on the MIC of OPT-80 and vancomycin against *C. difficile* ATCC 9689 and ATCC 700057. Susceptibility of both *C. difficile* strains to OPT-80 was unaffected by inoculum concentration from $10^5$ – $10^8$ cfu/ml ($10^2$ – $10^5$ CFU/spot), as shown by identical MIC values obtained for all inoculum concentrations tested. The MIC of vancomycin, however, increased progressively with increasing inoculum concentration, with the highest inoculum density showing a fourfold increase in MIC over the lowest inoculum density. These results demonstrate that inoculum density is not a significant factor affecting the outcome of OPT-80 susceptibility testing of *C. difficile*.

**Table 1.** *In vitro* activity of OPT-80 (µg/mL) at different inoculum densities of *C. difficile* ATCC 9689 ($10^2$-$10^5$ CFU/spot)

| Inoculum Density | | ATCC 9689 | |
|---|---|---|---|
| (CFU/ml) | CFU/spot | OPT-80 | Vancomycin |
| 1.92 x $10^8$ | 1.92 x $10^5$ | 0.063 | 2 |
| 1.92 x $10^7$ | 1.92 x $10^4$ | 0.063 | 1 |
| 1.92 x $10^6$ | 1.92 x $10^3$ | 0.063 | 1 |
| 1.92 x $10^5$ | 1.92 x $10^2$ | 0.063 | 0.5 |

**Table 2.** *In vitro* activity of OPT-80 (µg/mL) at different inoculum densities of *C. difficile* ATCC 700057 ($10^2$-$10^5$ CFU/spot)

| Inoculum Density | | ATCC 700057 | |
|---|---|---|---|
| (CFU/ml) | CFU/spot | OPT-80 | Vancomycin |
| 1.48 x $10^8$ | 1.48 x $10^5$ | 0.125 | 1.2 |
| 1.48 x $10^7$ | 1.48 x $10^4$ | 0.125 | 1 |
| 1.48 x $10^6$ | 1.48 x $10^3$ | 0.125 | 1 |
| 1.48 x $10^5$ | 1.48 x $10^2$ | 0.125 | 0.5 |

**PH EFFECT ON MIC VALUES**

Table 3 depicts the effect of various pH values on susceptibility of *C. difficile* to OPT-80 as measured by agar dilution method in two separate runs. During the first run, the highest pH treatment (pH 7.9) showed an 8-fold increase in MIC values over the lower pH treatments (pH 6.2 & pH 7.2) for both strains of *C. difficile*. When a confirmatory run was repeated at the highest pH (pH 8.0), the MIC value remained high for both strains. No increase in OPT-80 MIC was observed between pH 6.2 and pH 7 for either strain.

The increase in MIC values with pH did not consistently correlate with increased growth, thus the effect of pH on MIC did not appear to be merely due to the enhanced viability of the organism at higher pH. The pH 7 treatment had less dense organism spot growth relative to the pH 6.2 and pH 7.9 treatments.

**Table 3.** pH effects on agar dilution MIC values using buffered medium

| | | ANAEROBIC PH | | | |
|---|---|---|---|---|---|
| | | 6.2 | 7 | 7.9 | 8.0 |
| ORGANISM | DRUG | *Unbuffered* | *100mM $NaH_2PO_4$ pH 7.2 (Air)* | *100mM TAPS pH 9.2 (Air)* | *100mM TAPS pH 9.2 (Air)* |
| ATCC 9689 | OPT-80 | 0.063 | 0.063 | 0.5 | 1 |
| | Vancomycin | 1 | 4 | – | – |
| ATCC 700057 | OPT-80 | 0.125 | 0.125 | 1 | 2 |
| | Vancomycin | 2 | 4 | – | – |

Table 4, 5 and 6 summarize MIC data from the broth microdilution susceptibility method performed in three separate runs with pH ranges from 5 to 8.1. In the first series, in which the medium was unbuffered, the MIC of OPT-80 at pH 7.5 was ≥8x greater than the MIC at pH 5.9 for both *C. difficile* strains (Table 4).

The MIC at pH 5 could not be determined, because the organism failed to grow at this pH. The buffered (10 mM) pH 7.6 treatment showed 8-fold and 16-fold increases in OPT-80 MIC over the pH 6 treatment for *C. difficile* ATCC 9689 & ATCC 700057, respectively (Table 5). In the third, strongly buffered (100 mM) series, similar results were seen with the highest pH treatment (pH 8.1) showing a 16-fold increase in MIC over the lowest pH treatment (pH 6) for both organisms (Table 6). Vancomycin showed a similar trend with the highest pH treatment producing MICs 4 – 8 fold greater than the lowest pH treatment in all three experiments.

**Table 4.** pH effects on MIC using unbuffered media

| | | ANAEROBIC PH (unbuffered) | | | | |
|---|---|---|---|---|---|---|
| ORGANISM | DRUG | 5 | 5.9 | 6.6 | 7.1 | 7.5 |
| ATCC 9689 | OPT-80 | *no growth* | ≤ 0.016 | 0.031 | 0.063 | 0.125 |
| | Vancomycin | | 1 | 1 | 2 | 4 |
| ATCC 700057 | OPT-80 | *no growth* | 0.031 | 0.063 | 0.125 | 0.25 |
| | Vancomycin | | 0.5 | 1 | 1 | 4 |

**Table 5.** pH effects on MIC values using weakly buffered medium (10mM)

| | | ANAEROBIC PH | | | |
|---|---|---|---|---|---|
| | | 6 | 6.7 | 7.2 | 7.6 |
| ORGANISM | DRUG | *pH 6.0 (Air)* | *10mM $NaH_2PO_4$ pH 7.0 (Air)* | *10mM MOPS pH 8.0 (Air)* | *10mM TAPS pH 9.0 (Air)* |
| ATCC 9689 | OPT-80 | ≤0.016 | 0.031 | 0.063 | 0.125 |
| | Vancomycin | 0.5 | 1 | 2 | 4 |
| ATCC 700057 | OPT-80 | 0.031 | 0.063 | 0.25 | 0.5 |
| | Vancomycin | 0.5 | 1 | 2 | 4 |

**Table 6.** pH effects on MIC values using strongly buffered medium (100mM)

| | | ANAEROBIC PH | | | | |
|---|---|---|---|---|---|---|
| | | 6 | 6.8 | 7.5 | 8 | 8.1 |
| ORGANISM | DRUG | *pH 6.0 (Air)* | *100mM $NaH_2PO_4$ pH 7.0 (Air)* | *100mM MOPS pH 8.0 (Air)* | *100mM TAPS pH 9.0 (Air)* | *100mM TAPS pH 9.5 (Air)* |
| ATCC 9689 | OPT-80 | ≤0.016 | 0.031 | 0.125 | 0.25 | 0.25 |
| | Vancomycin | 1 | 1 | 4 | >8 | >8 |
| ATCC 700057 | OPT-80 | 0.031 | 0.125 | 0.25 | 1 | 0.5 |
| | Vancomycin | 1 | 2 | 4 | 8 | 8 |

Assay plates at all pH treatments were also visually examined for overall growth. In the first series, which utilized unbuffered broth, overall culture turbidity increased with increasing pH. The same trend was observed in the second series, which utilized 10 mM buffered broth, except the culture turbidity was the same for pH 7.2 and pH 7.6. In the third series, culture turbidity was more equivalent across the pH treatments, with the exception of pH 7.5, which was the most turbid.

Overall, with both methods of susceptibility testing and across varying concentrations of buffer salts, the MIC values of OPT-80 and vancomycin increased with increasing pH for both strains of *C. difficile*.

**DIVALENT CATION CONCENTRATION EFFECT ON MIC VALUES** Measurement of the calcium and magnesium levels in commercial Brucella agar showed calcium and magnesium ion concentration of 21 and 33 mg/L, respectively. Various additional amounts of divalent cations were added, and OPT-80 MIC values for *C. difficile* strains were tested at three different concentrations of calcium ions (21, 30 and 57 mg/L) and three different concentrations of magnesium ions (33, 45 and 75 mg/L). The MIC values remained the same in all types of media. *C. difficile* 9689 had MIC value of 0.063 µg/ml and *C. difficile* 700057 with MIC value of 0.125 µg/ml in media with varying concentrations of cations. Vancomycin, which was tested as a control with supplemented Brucella agar without any extra calcium or magnesium as control during the experiments, demonstrated the expected MIC value of 1 µg/ml for all runs (Tables 7 and 8).

**Table 7.** *In vitro* activity of OPT-80 in supplemented Brucella agar with different divalent cation concentrations

| | | MIC VALUES (µg/mL) | |
|---|---|---|---|
| DRUG | Calcium Concentration (mg/L) | *C. difficile* (ATCC 700057) | *C. difficile* (ATCC 9689) |
| OPT-80 | 33 | 0.125 | 0.063 |
| | 45 | 0.125 | 0.063 |
| | 75 | 0.125 | 0.063 |
| Vancomycin | 33 | 1 | 1 |

**Table 8.** *In vitro* activity of OPT-80 in supplemented Brucella agar with different divalent cation concentrations

| | | MIC VALUES (µg/mL) | |
|---|---|---|---|
| DRUG | Magnesium Concentration (mg/L) | *C. difficile* (ATCC 700057) | *C. difficile* (ATCC 9689) |
| OPT-80 | 21 | 0.125 | 0.063 |
| | 30 | 0.125 | 0.063 |
| | 57 | 0.125 | 0.063 |
| Vancomycin | 21 | 1 | 1 |

**OPT-80 MIC VALUES WITH DIFFERENT COMMERCIAL LOTS OF MEDIA** Three different lots of supplemented Brucella agar media were used to compare the activity of OPT-80 against *C. difficile* strains. The MIC assays were controlled by testing the activity of the QC organism, *Eubacterium lentum* vs. clindamycin which was within the CLSI (NCCLS) acceptable ranges, i.e. 0.06 – 0.25 µg/mL. Another control step for the MIC assays was to include metronidazole and monitor its activity vs. *C . difficile* strains, which in our laboratory has been shown to have MIC values ranging between 0.25 – 0.5 µg/mL. As shown in Table 9, the activity of OPT-80 vs. *C. difficile* was not affected by different lots of supplemented Brucella agar. All controls demonstrated activities within established ranges.

**Table 9.** *In vitro* activity of OPT-80 tested with three different lots of media

| | | MIC VALUES (µg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Metronidazole | | | Clindamycin | | | OPT-80 | | |
| Bacteria | ATCC # | Lot 1 | Lot 2 | Lot 3 | Lot 1 | Lot 2 | Lot 3 | Lot 1 | Lot 2 | Lot 3 |
| *C. difficile* | 9689 | 0.5 | 0.5 | 0.5 | 4 | 4 | 4 | 0.25 | 0.25 | 0.125 |
| *C. difficile* | 43255 | 0.5 | 0.5 | 0.5 | 8 | 8 | 8 | 0.25 | 0.5 | 0.25 |
| *C. difficile* | 17857 | 0.25 | 0.5 | 0.5 | 4 | 2 | 4 | 0.25 | 0.25 | 0.125 |
| *Eubacterium lentum* | 43055 | 1 | 0.25 | 1 | 0.25 | 0.25 | 0.125 | 0.25 | 0.25 | 0.125 |

## Conclusions

- In contrast to vancomycin, the activity of OPT-80 vs. *C. difficile* was unaffected by inoculum concentrations, in the range of $10^2$ – $10^5$ cfu/spot.
- The susceptibility of *C. difficile* to OPT-80 was unaffected by cation concentrations (calcium ion in the range of 2.1 - 5.7 mg/dL and magnesium concentration of 3.3 – 7.5 mg/dL), and by various commercial lots of media.
- The MIC values for both OPT-80 and vancomycin increased with increasing pH over a pH range of 6 – 8. The high MIC values at basic pH may be due to deprotonated form of the phenolic hydroxyl groups of both compounds above their pKa, where they form a charged species that is expected to be less permeable to bacterial cells. In contrast, below the pKa (7.22 for OPT-80), the antibiotics will be mostly protonated, and thus should permeate the cell membrane more efficiently.
- Organism density generally increased with increasing pH and showed no growth at pH 5; the dependence of growth density, but not MIC, on pH was reduced in the presence of buffering agents.

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