The biological reducing agent Oxyrase improves the resuscitation of dormant *Mycobacterium smegmatis* and *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the etiologic agent of Johne's disease, a chronic intestinal disease of cattle and other ruminants. Diagnostic culture for MAP is typically unrewarding until latter stages of the disease. This may be a consequence of oxidative damage to dormant organisms, which results from culture. The purpose of this investigation was to determine the effect of the commercial reducing agent Oxyrase® for Broth (OB) on the recovery of dormant *Mycobacterium smegmatis* (MS) and MAP. Dormant organisms were inoculated into medium only or that supplemented with media containing serial dilutions of OB. Growth was monitored by optical density for up to 21 days. Treatment of MAP and MS with OB led to significant increases in recovery and growth yield. However, the concentration of OB necessary to promote recovery was dependent on the number of viable organisms present in seed cultures. Mitigating oxidative damage by using OB can facilitate the recovery of dormant mycobacteria. Whether this is a direct or indirect effect has yet to be established.

Introduction

Johne's disease, which is caused by the acid-fast bacillus *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic intestinal disease that primarily affects domestic and wild ruminants worldwide. Johne's disease is particularly threatening to the economic viability of dairy farming in the US, wherein nearly 22% of the dairy herds are infected with MAP.[1] Cattle are usually infected shortly after birth and do not display signs of the disease until two or more years after the initial infection.[2] Because infection with MAP, which is shed in feces, is not reliably detected until hosts have entered the clinical stage of the disease,[3] one infected animal can infect many others in the herd before the first clinical case of Johne's disease is observed. It has been estimated that by the time the first animal develops clinical signs of Johne's disease, there may be as many as 20 other MAP-infected animals in the herd,[2] although this estimate is controversial.[4] The disease can be spread from herd to herd through the purchase of animals with undetectable infections.[5]

Following its establishment in the host, pathogenic mycobacteria are sequestered in granulomas in immunocompetent hosts.[6] Within these granulomas, it is believed that MAP, an aerobic organism, enters a dormant state as a result of nutrient and/or oxygen limitation.[7-9] When these dormant organisms are cultured directly in nutrient-rich media, they typically fail to grow. The exact reason for this is unclear. Work with other organisms has shown that dormant organisms are particularly susceptible to oxidative damage, and the sudden increase in aerobic metabolic rate may result in the formation of levels of oxygen free radicals that the organism at that instant is not capable of detoxifying.[10,11] Indeed, the inclusion of scavengers of reactive oxygen species, namely pyruvate and catalase, in culture media improved the recovery of viable but nonculturable (VNC) *Vibrio vulnificus*.[10]

Because progression into the clinical phase of Johne's disease results from MAP having resumed active growth within the host, the probability that MAP will be recovered from cattle exhibiting clinical signs is relatively high. In contrast, detecting infection in asymptomatic animals is problematic. It has been stated that many asymptomatically infected animals may be shedding MAP intermittently in feces;[2] however, the difficulty in culturing dormant organisms may indicate that the problem lies less with intermittent shedding than with intermittent detection. Indeed, evidence of a VNC state in MAP has recently been reported.[12] The failure to consistently detect infected animals compromises the success of strategies to combat MAP transmission.

Crow and colleagues[13] described the use of a sterile cytoplasmic membrane preparation obtained from *Escherichia coli* as a means to reduce oxygen in culture media. This preparation, which is available commercially as Oxyrase® for Broth (OB), has been used successfully to resuscitate VNC entero-hemorrhagic *E. coli* and *Salmonella*,[14] *Listeria monocytogenes*,[15] and *Campylobacter jejuni*.[16] Reducing oxygen tension in the culture environment may limit oxidative damage and ease the transition from dormancy into active aerobic metabolism during recovery in diagnostic culture procedures.[17] This could possibly decrease recovery time, allowing for earlier detection of MAP in fecal culture. Earlier detection of MAP infection could thus help to

reduce the spread of Johne's disease through and between herds.

Materials and Methods

Media and bacteria

Mycobacterium smegmatis mc²155 (MS) (kindly provided by Richard Groger, Washington University in St. Louis) was cultured in sealed 25×125 mm tubes containing 17 mL lysogeny broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.05% Tween 80 (Becton Dickinson) (LB/Tween) and 1.5 g/mL methylene blue (VWR International, Radnor, PA, USA), and incubated with gentle agitation at 37°C to

allow a slow transition to anaerobiosis.[7,18] Cultures were used no earlier than 4 days after methylene blue reduction was complete. For starvation dormant (SD) cultures, *Mycobacterium paratuberculosis* PAMSUM-8, isolated from a dairy cow with clinical paratuberculosis (Minnesota State University, Mankato culture collection) was cultured in sealed 75 cm^2 tissue culture flasks containing 30 mL Middlebrook 7H9 broth (Becton Dickinson) supplemented with 10% oleic acid, albumin, dextrose, and catalase (Becton Dickinson); 2 μg/mL mycobactin J (Allied Monitor, Fayette, MO, USA); and 0.05% Tween 80 (M7H9C) and incubated at 37°C for at least 9 months before use in experiments. Cultures were used at least 9 months after sealing flasks. To obtain anaerobically dormant (AD) cultures, MAP was cultured in rubber stoppered 30 mL serum vials containing 20 mL M7H9C and incubated at 37°C. In preliminary experiments incorporating 1.5 g/mL methylene blue in M7H9C as a redox indicator, it was observed that complete dye reduction required 8 weeks (Secott and Ghimire 2011, not published). Therefore, AD cultures were held for 3 months before use in experiments.

Preparation of microplates

Two fold serial dilutions (1:2-1:2048) of OB (Oxyrase, Mansfield, OH, USA) were prepared in the appropriate medium (LB/Tween for MS; M7H9C for MAP). One hundred microliters of each dilution was added to eight wells of a sterile 96-well microplate (Corning, New York, NY, USA) (columns 1-11). The twelfth column contained medium only and therefore served as a negative control. After centrifugation of bacterial cultures at 1600 × *g* for 20 minutes, cell pellets were resuspended in 0.01 M phosphate-buffered saline (pH 7.4) with 0.05% Tween 20 (VWR, USA) (PBST). Bacterial suspensions were adjusted to a density equivalent to 2×10^8 colony forming units (CFU)/mL using a colorimeter (Vitek, bioMérieux, Marcy-l'Étoile, France). The cultures were further diluted to a final concentration of 2000 CFU/mL in the appropriate culture medium, and 100 L of bacterial suspension was added to microplate wells containing medium only or OB in medium to yield a final cell density of 1000 cfu/mL. The plate was then covered with a transparent seal (Whatman, Maidstone, UK) and incubated with gentle agitation in a 37°C incubator. Optical density (590 nm) was measured using a microplate reader (Thermo Fisher, Waltham, MA, USA) every 24-96 hours until growth ceased or contamination was observed.

Viability estimation

The BacLight Live/Dead bacterial viability kit (Life Technologies, Thermo Fisher, USA) was used to estimate MAP viability. Included in the kit are SYTO® 9 and propidium iodide (PI)

nucleic acid stains. Because intact cytoplasmic membranes are non-permeable to PI, only dead or dying cells will be stained with PI. In contrast, SYTO® 9 is membrane-permeable, and cells exhibiting high levels of SYTO® 9 fluorescence were regarded as alive. Attempts were made to prepare a standard curve to measure viability according to the manufacturer's guidelines; however, correlation between expected and observed results was not satisfactory. It was decided to use the ratio of SYTO® 9 fluorescence to that of PI to categorize cultures as high (≥0.4) or low (≤0.01) viability. MAP cultures were harvested by centrifugation as described above and resuspended in PBST to a density equivalent to 1×10^5 CFU/mL. A 0.5 mL aliquot of the suspension was stained for 10 minutes with an equal volume of a 1:1 mixture of SYTO® 9:PI prepared according to the manufacturer's instructions. Staining was analyzed using a Guava EasyCyte Mini flow cytometer (Millipore, Merck, Darmstadt, Germany), and cultures were then further diluted for use in OB resuscitation assays.

Data analysis

The optical densities of cultures were normalized by averaging the optical density of all wells after experimental setup (typically Day 0), and subtracting this value from readings at all subsequent time points. This allowed for the subtraction of the signal given by the medium and initial inoculum alone and therefore quantified only the growth of the organism. Data were analyzed using two-way repeated measures ANOVA and Bonferroni *post hoc* tests. The number of positive wells within a treatment was also determined, where wells yielding OD$_{590}$ values greater than 0.085 were defined as positive.

Results

General comments

For each of the experiments described below, two-way repeated measures ANOVA revealed significant interactions between time and OB concentration (P<0.0001), complicating accurate interpretation of P values corresponding to independent contributions of those variables. The effect of matching was significant (P<0.0001), supporting the use of the repeated measures ANOVA for analysis.

Oxyrase® for Broth improved the recovery of dormant *Mycobacterium smegmatis*

The inclusion of OB in LB/Tween improved the rate of recovery of AD MS. However, the concentration of OB that enhanced resuscita-

tion varied between experiments. In the first experiment with MS, significant (P<0.05 for Bonferroni *post hoc* tests) improvement in recovery was observed on days 2-4 in cultures treated with 1:2 OB, day 3 in those treated with 1:4 OB, and days 3 and 4 for those treated with 1:8 OB (Figure 1A). OB also increased the number of wells that were growth-positive and reduced the time needed for recovery for MS treated with 1:2, 1:4, and 1:8 OB (Figure 2A).

In the second experiment, low dilutions (high concentrations) had no effect on the recovery of dormant MS (data not shown). Instead, enhanced resuscitation was observed only when intermediate concentrations of OB were used (Figure 1B). There was a drop in the mean optical density for all cultures on day 6. Statistically significant enhancement of dormant MS recovery was seen on day 12 for cultures treated with 1:128 OB (P<0.05 for Bonferroni *post hoc* tests). Again, OB increased the number of culture-positive wells and reduced the time needed to reach positive status for MS cultures treated with 1:64 and 1:128 OB (Figure 2B).

Oxyrase® for Broth improved the recovery of dormant *Mycobacterium avium* subsp. *paratuberculosis*

M7H9C supplemented with OB improved the rate of recovery of SD MAP (Figure 3), but as was the case for MS, the concentration of OB that effected maximal recovery differed between experiments. Intermediate-to-low concentrations of OB were observed to improve the resuscitation of MAP in the first experiment, with significant differences between the control and 1:512 OB-treated cultures on days 4 and 5 (P<0.05 for Bonferroni *post hoc* tests) (Figure 3A). Time to detection was reduced and an accompanying increase in the number of positive wells was seen in cultures treated with 1:256 and 1:512 OB (Figure 4A). OB significantly enhanced the rate of recovery of SD MAP in cultures treated with 1:2, 1:4, and 1:8 OB in the second experiment for days 2-20 (P<0.01 for Bonferroni *post hoc* tests) (Figure 3B). Medium containing these dilutions of OB also increased the number of SD MAP cultures that were resuscitated and reduced the time needed to reach positivity (Figure 4B).

OB also enhanced the resuscitation of AD MAP cultures (Figure 5). These observations are discussed in detail below.

Seed culture viability influences the concentration of Oxyrase® for Broth needed for improved resuscitation

While OB improved the recovery of dormant

MS and MAP, the concentration of OB required for maximal resuscitation varied from experiment to experiment. Because the concentrations of MS and MAP were estimated by turbidimetry, which does not distinguish between dead and live organisms, it was suspected that differences in OB-promoted resuscitation reflected differences in seed culture viability. To further investigate this, seed culture viability was estimated in parallel with the setup of experiments using AD MAP.

High viability AD MAP cultures responded best to high concentrations (low dilutions) of OB (Figure 5A). In this experiment, medium containing 1:2 OB exhibited improved resuscitation on days 12 and 14 as compared to the control, although this effect was not significant (P>0.05 for Bonferroni *post hoc* tests). Similar to what was described above for Figure 1B, the mean optical density dropped to or remained at 0.0 on day 7 for all cultures. In contrast, low viability cultures yielded enhanced recovery only in medium containing low concentrations of OB (Figure 5B). Here,

Bonferroni post hoc tests indicated a significant (P<0.01) improvement in recovery for AD MAP grown in the presence of 1:2048 OB on day 11 as compared to the control.

Discussion

Fecal culture is regarded as the gold standard for *ante mortem* diagnosis of MAP infection. However, the overall sensitivity of MAP diagnostic culture is 33%.[3] This is a consequence of the inability to consistently detect infection in asymptomatically infected cattle. This limitation must be overcome if the chances for success of Johne's disease control programs are to be improved. To this end, it was the purpose of this study to evaluate the potential for the inclusion of OB in culture media to improve MAP diagnostic culture sensitivity. Because MS has been previously used in dormancy studies,[18-20] it was used in this investigation for proof of principle. Indeed, OB

increased the rate of recovery and growth yield for dormant MS and dormant MAP. To our knowledge, this is the first report in peer-reviewed literature to document the ability of OB to improve the resuscitation of dormant mycobacteria.

Resuscitation-promoting factors (Rpf), which were originally identified in *Micrococcus luteus*,[21] have been shown to improve the recovery of dormant mycobacteria in several investigations.[22-24] Several open reading frames corresponding to Rpf homologues observed in *M. tuberculosis* have been identified in the genome sequence of MAP.[25] One of the events seen in the transition of mycobacteria to dormancy is the thickening of the cell wall.[26] Lamont and colleagues described the presence of spore-like morphotypes in dormant cultures of MAP, which would be consistent with the appearance of cell wall thickening.[27] As Rpf proteins are believed to have the ability to hydrolyze peptidoglycan,[28] it is tempting to speculate that these proteins also play a role in the resuscitation of MAP.
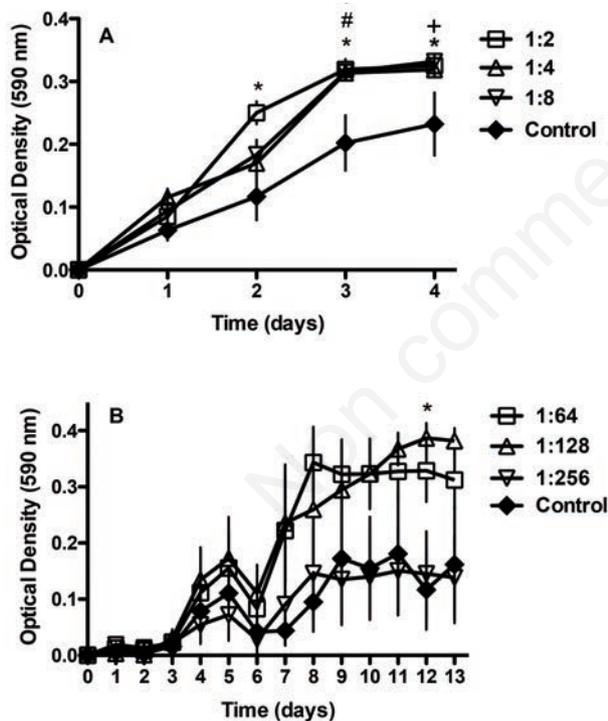

Figure 1. Inclusion of Oxyrase® for Broth (OB) in culture medium enhances the rate of recovery of *Mycobacterium smegmatis* (MS). The optical density (590 nm) of MS grown in LB broth containing serial dilutions of OB was measured until growth ceased or contamination was observed. The average optical density all inoculated wells on day 0 was subtracted from that of each at all subsequent time points. Only the dilutions of OB that affected MS growth are shown. A) Experiment 1. Asterisks, pound signs, and pluses indicate significant differences between controls and 1:2, 1:4, and 1:8 OB-treated cultures, respectively (P<0.05). B) Experiment 2. Asterisks indicate significant differences between controls and 1:128 OB-treated cultures (P<0.05).
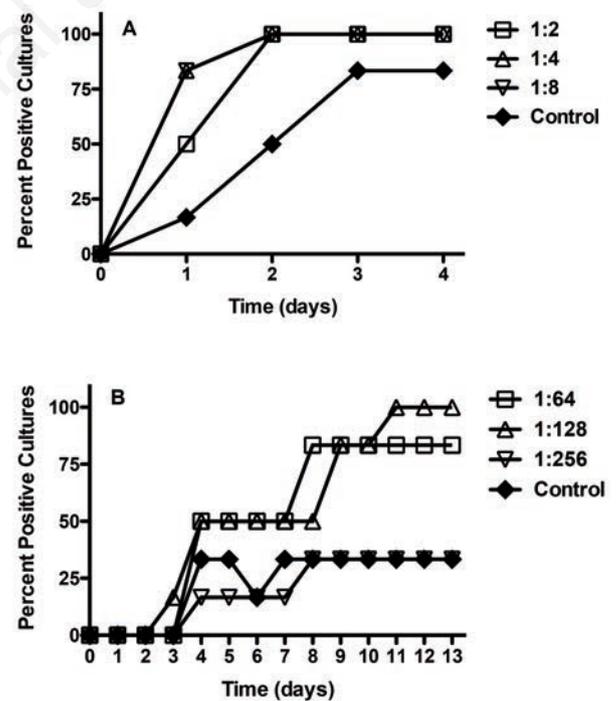
Figure 2. Oxyrase® for Broth (OB) increases the number of *Mycobacterium smegmatis* (MS) cultures that recover from dormancy. The optical density (590 nm) of MS grown in LB broth containing serial dilutions of OB was measured until growth ceased or contamination was observed. Wells were regarded positive if the optical density was greater than 0.085 OD_{590} units greater than the plate average at day 0. Only the dilutions of OB that affected MS growth are shown. A) Experiment 1. B) Experiment 2.

Nevertheless, the observation that OB promoted recovery of dormant MAP indicates that the degree of oxidative damage that occurs when dormant mycobacteria resume growth in rich media, such as those used in diagnostic culture, is an important consideration that may influence the outcome of diagnostic culture, particularly in cases where low numbers of organisms are present in the specimen.

While the recovery of dormant mycobacteria was clearly enhanced by the inclusion of OB in culture media, the concentration that led to this enhancement varied considerably among all trials, and this was likely due to differences in the concentration of viable organisms in dormant cultures. Others have observed considerable differences in dormant MAP culture viability.[29] Votyakova and colleagues[30] observed that in order for resuscitation of dormant *Micrococcus luteus* to occur, that population of cells had to contain a subpopulation of at least 9 actively growing cells, suggesting that these actively growing cells were a source of some factor that acted in a paracrine manner to effect resuscitation. It is possible that high concentrations of OB prevent the oxidative metabolism of the very small number of actively growing MAP present in low viability dormant cultures, and that these resuscitative paracrine factors can be produced in sufficient quantity only under low OB concentrations.

Organisms such as *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli*, which have been successfully resuscitated from the VNC state through the use of OB,[14,15] are facultative anaerobes. In the presence of OB, these organisms likely ferment or respire anaerobically. Like *Micrococcus*, mycobacteria are considered obligate aerobes; yet, it has been demonstrated that AD mycobacteria survive using a form of nitrate reductase,[20,31] which fuels a gene expression profile distinct from that of actively growing mycobacteria.[32] Nevertheless, a functional nitrate reductase is not present in the genome of MAP.[25] How these organisms are able to metabolize in the presence of OB, particularly at high concentrations, has yet to be elucidated. The *sudden* drop in optical density that was reported herein for two experiments is unlikely to have represented a corresponding death of MAP. Optical density does not distinguish between live and dead cells, and MAP is unlikely to have lysed during that short window of time. Rather, we suspect that the drop in optical density represents the consequence of a technical error, such as reading the microplate with condensation on the plastic film covering the plate, which was magnified during the normalization process.

Because the concentration of OB needed to yield efficient resuscitation of MAP requires foreknowledge of the number of viable organisms present in the sample, it is not anticipated that the application of OB as a medium supplement for routine diagnostic culture will occur in the near future. Still, this approach may prove useful diagnostic testing of animals prior to sale or breeding.
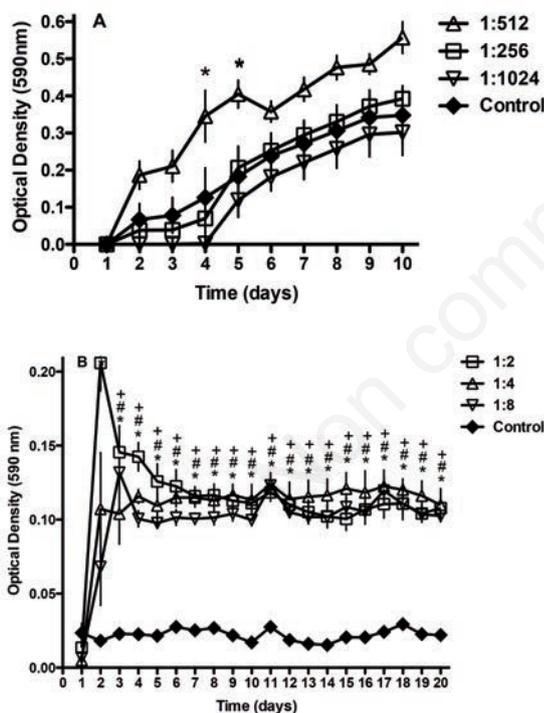

Figure 3. Oxyrase® for Broth (OB) enhances the recovery of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The optical density (590 nm) of MAP grown in Middlebrook 7H9C containing serial dilutions of OB was measured until growth ceased or contamination was observed. The average optical density of all inoculated wells on day 0 was subtracted from that of each well at all subsequent time points. Only the dilutions of OB that affected MAP growth are shown. A) Experiment 1. Asterisks indicate significant differences between controls and 1:512 OB-treated cultures (P<0.05). B) Experiment 2. Asterisks, pound signs, and plusses indicate significant differences between controls and 1:2, 1:4, and 1:8 OB-treated cultures, respectively (P<0.0001).
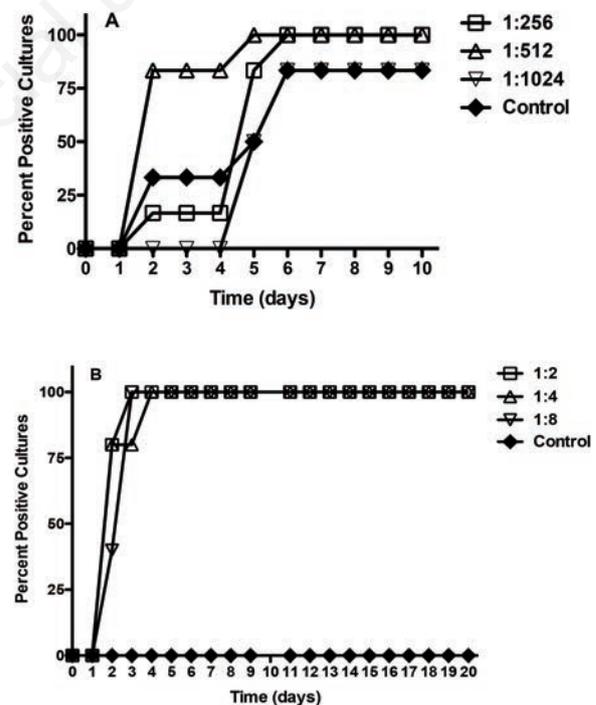
Figure 4. Oxyrase® for Broth (OB) increases the number of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cultures that recover from dormancy. The optical density (590 nm) of MAP grown in Middlebrook 7H9C containing serial dilutions of OB was measured daily until growth ceased or contamination was observed. Wells were regarded positive if the optical density was greater than 0.085 OD$_{590}$ units greater than the plate average at day 0. Only the dilutions of OB that affected MAP growth are shown. A) Experiment 1. B) Experiment 2.
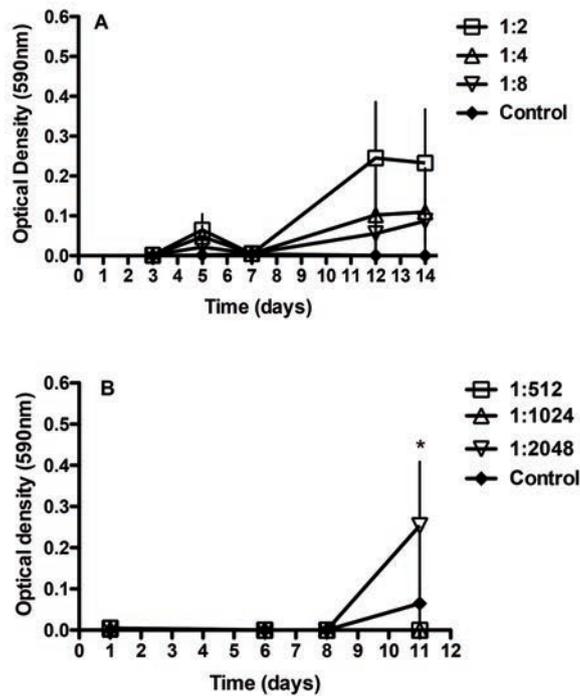
Figure 5. The concentration of Oxyrase® for Broth (OB) that enhances the recovery of dormant *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is influenced by the number of viable organisms present. The optical density (590 nm) of MAP grown in Middlebrook 7H9C containing serial dilutions of OB was measured every 2-4 days until growth ceased or contamination was observed. Only the dilutions of OB that affected MAP growth are shown. A) Growth of organisms in plates prepared from a high viability dormant culture. B) Growth of organisms in plates prepared from a low viability dormant culture. The asterisk indicates a significant difference between the control cultures and those treated with 1:2048 OB (P<0.05).

Conclusions

Efforts to control the spread of Johne's disease are compromised by the poor sensitivity of diagnostic fecal culture for MAP. Modification of fecal culture media and protocols to enable the consistent recovery of dormant MAP must be developed in order to improve fecal culture sensitivity. While OB can potentially be used to resuscitate dormant MAP, the inclusion of this reagent in culture protocols is presently hampered by the observation that the number of viable MAP in feces determines the concentration of OB needed to promote the recovery of dormant MAP in culture. Further, it remains to be established whether the resuscitative effect reported herein for OB also applies to other MAP strains. The MAP PAMSUM-8 strain has phenotypic characteristics most consistent with those of MAP cattle (C, or type II) strains (Secott 2003, not published). Whether this strain is representative of the response of C strains, sheep (S, or type I and type III) strains, or all strains will require further testing.[33] OB may prove useful for improving culture sensitivity once

mycobacterial dormancy is more thoroughly characterized. However, the use of OB to promote the resuscitation of MAP should be evaluated by testing fecal samples from animals with known MAP infection status, employing all of the culture media currently used for diagnostic culture. Whether OB acts directly on recovering cells to promote resuscitation by preventing oxidative damage, or acts in concert with other factors has yet to be determined.

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