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EFFECT OF CONDITIONED MEDIUM ON THE RECOVERY OF DORMANT MYCOBACTERIA IN CULTURE

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (Mpt) is the etiologic agent of Johne's disease, a chronic intestinal disease in cattle that threatens the economic viability of dairy farming. Diagnostic culture is typically unrewarding until several years after infection when clinical signs can be observed. This leads to widespread infection within the herd. Difficulty in culturing Mpt may be a result of oxidative damage due to the increased metabolic rate when dormant organisms are recovered in a nutrient rich medium. In order to improve recovery it is believed that some organisms secrete a growth factor in times of environmental stress which enables them to grow more quickly when conditions improve. The purpose of the project was to test conditioned medium (CM) and its components as a method of improving the recovery of Mpt. The conditioned medium from Mpt was separated into 4 fractions based on molecular weight using centrifuge filters. Unsupplemented media (Middlebrook 7H9C or Luria-Bertani broth) and those containing serial dilutions of fractionated or unfractionated Mpt-conditioned medium were inoculated with Mpt or *Mycobacterium smegatis* (MS); a non-pathogenic species. Growth was monitored by measuring the optical density of the cultures for up to 30 days. Unfractionated Mpt-conditioned medium promoted a two fold or greater enhancement of Mpt growth, but had no effect on that of MS. Results of treatment with conditioned medium fractions are pending. Recovery of dormant Mpt was increased by the use of conditioned medium.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (Mpt) is a Gram positive, acid-fast bacillus (Figure 1) that is the causative agent of Johne's disease (3). Johne's disease is a chronic intestinal disease that occurs in domestic and wild ruminant species. Signs of Johne's disease include weight loss (even though the animal is eating normally) and diarrhea. In the final weeks, the jaw may also swell due to a loss of protein, causing a condition known as bottle jaw (Figure 2). Johne's disease threatens the economic viability of dairy farming, as nearly 23% of the dairy herds in the U.S. are infected with Mpt (3). The cattle are usually infected shortly after birth and generally do not display signs of the disease until two or more years following infection (5). It is estimated that by the time the first animal develops clinical signs of Johne's disease, it has infected as many as 20 other animals in the herd (5). This occurs because the organism, which is shed in feces, cannot be detected reliably by diagnostic tests until infected animals enter the latter stages of the disease. The disease can be spread from herd to herd through the purchase of animals with undetectable infections.

Following its establishment in the host, pathogenic mycobacteria are sequestered in granulomas in immunocompetent hosts (4). Within these granulomas, it is believed that Mpt, an aerobic organism, enters a dormant state as a result of nutrient and oxygen limitation. When these dormant organisms are cultured in nutrient-rich media, 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 (4). Our previous work showed that reducing the oxygen in the culturing environment allowed dormant bacteria to be recovered in a shorter period of time. Conditioned medium (medium in

which cells have grown, and thus contains factors secreted by the cells) from *Micrococcus luteus* has been found to contain resuscitation-promoting factor (Rpf), which enables the recovery of many Gram positive species from a dormant state (2). The genomes of several mycobacterial species, including Mpt, contain open reading frames that share identity with that of Rpf. Such a growth factor, if produced by Mpt, could ease the transition from dormancy to actively growing when cultured in nutrient rich medium. Increasing the number of organisms recovered and decreasing the recovery time would allow for earlier detection of Mpt in fecal samples from infected cattle. This could help to reduce the spread of Johne's disease through and between herds.

Materials and Methods

To test the effect of CM, Mpt and *Mycobacterium smegmatis* (MS) were grown in rich media until cultures were anaerobic. Mpt took several months to become anaerobic while MS only took several weeks to become anaerobic. The cultures were centrifuged until a cell pellet formed and the supernant was collected. The conditioned medium was filtered and stored. Half of the conditioned medium from Mpt was split into four fractions based on molecular weight using centrifuge filters. A 96 well microplate was inoculated with unsupplemented nutrient media. Serial dilutions of conditioned medium were also added to each plate (Table 1). The microplates were set up such that columns 1-11 had serial dilutions of the conditioned medium added to the nutrient medium. Column 12 was reserved as a control with no supplemental media included. The cell pellet obtained earlier in the experimental set up was resuspended in a volume of nutrient medium that would yield 200 cfu/well. MS or Mpt was dispensed into rows A-F, columns 1-12, leaving rows G and H without any bacteria (Figure 3). Growth was monitored by

measuring the optical density (595 nm) of the cultures using a microplate reader. The cultures were monitored for up to 30 days. The optical density data were entered into Microsoft Excel for analysis.

Data were analyzed in two ways. First, the optical densities of cultures were normalized by averaging the optical density of all wells immediately after experimental setup (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. The number of positive wells within a treatment was also determined. Values higher than 3 standard deviations above the average Day 0 reading was considered positive.

Results and Discussion

Trial 1 was set up using MS with Mpt conditioned medium. It was observed that CM from Mpt does not seem to affect MS growth (Figure 4). Both the treated and untreated wells grew at the approximately the same rate and to the same optical density. If there was an effect it would have been expected that a specific treatment would have grown to a higher optical density more quickly then the other treatments.

Trial 2 was set up using MS with MS conditioned medium. No difference was observed in the MS-CM treated wells when compared to the untreated wells (Figure 5). Superficially, it would appear that cultures containing the 1:8 dilution of CM grew more quickly and to a greater optical density than the other treatments; however, given the large standard error, the difference was not significant. This observation is further confirmed by the comparing the number of positive wells from this treatment with all other treatments.

Trial 3 was set up using Mpt with Mpt conditioned medium. Mpt treated with Mpt-CM had more organisms recover more quickly than the untreated Mpt (Figure 6). While Mpt treated with CM started to recover from dormancy almost immediately, it took untreated Mpt until day 6 to begin growing. The number of positive wells observed for treatments containing 1:2, 1:4 and 1:8 dilutions of Mpt-CM had all wells become positive on day 2 (all three treatments overlap), while the untreated wells did not become positive for growth until after day 4.

Trial 4 was set up the same as Trial 3. Trial 4 had similar results except the cells took longer to emerge from dormancy (Figure 7). Organisms treated with CM still grew better and more rapidly than the untreated organisms. There was no apparent difference between the number of positive wells observed CM-treated and untreated Mpt. However, the standard deviation of the first day average for this trial was very low, resulting in more wells being called positive.

To summarize, components released into the environment by Mpt during times of stress may aid in their recovery when conditions improve. When grown in the presence of Mpt-CM, Mpt had a growth yield 2 times that of untreated Mpt. Mpt treated with CM also grew more quickly than untreated Mpt. MS does not appear to have the ability to produce or respond to the components of CM that aid in recovery from dormancy. This difference may have to do with the different lifestyles of the two organisms. Mpt is a pathogenic organism, whereas MS is a non-pathogenic organism. Results are still pending on fractionated Mpt-CM. Several trials of Mpt and MS with fractionated Mpt-CM were set up; however, all but one were contaminated. Inadequate sealing of culture microplates may have been responsible for the contamination. The method of incubation

was changed to minimize sealing failure, and experimentation using fractionated Mpt- CM are in progress.

Conclusion

Unfractionated Mpt-CM resulted in a two-fold or greater enhancement of growth by previously dormant Mpt. Unfractionated CM did not have an effect on the recovery of dormant MS. We plan to further characterize and eventually isolate the factor that enhances recovery of Mpt from dormancy. Once this factor has been identified, it may be added to culture media to evaluate its efficacy in fecal culture.

References

1. **Collins, M., Manning, E**. Johne's Information Center. http://www.johnes.org/general/_Holstein_front.html. University of Wisconsin. 2001.

2. **Mukamolova, G.V., et. al.** A bacterial cytokine. *Microbiology* **1998:**8916-8921.

3. **NAHMS.** 1997. Johne's disease on U.S. dairy operations #N245.1097. USDA:APHIS:CS,CEAH, National Animal Health Monitoring System.

4. **Wayne L. G., and C. D. Sohaskey.** 2001. Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu Rev Microbiol **55:**139-63.

5. **Whitlock, R. H., and C. Buergelt.** 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract **12:**345-56.

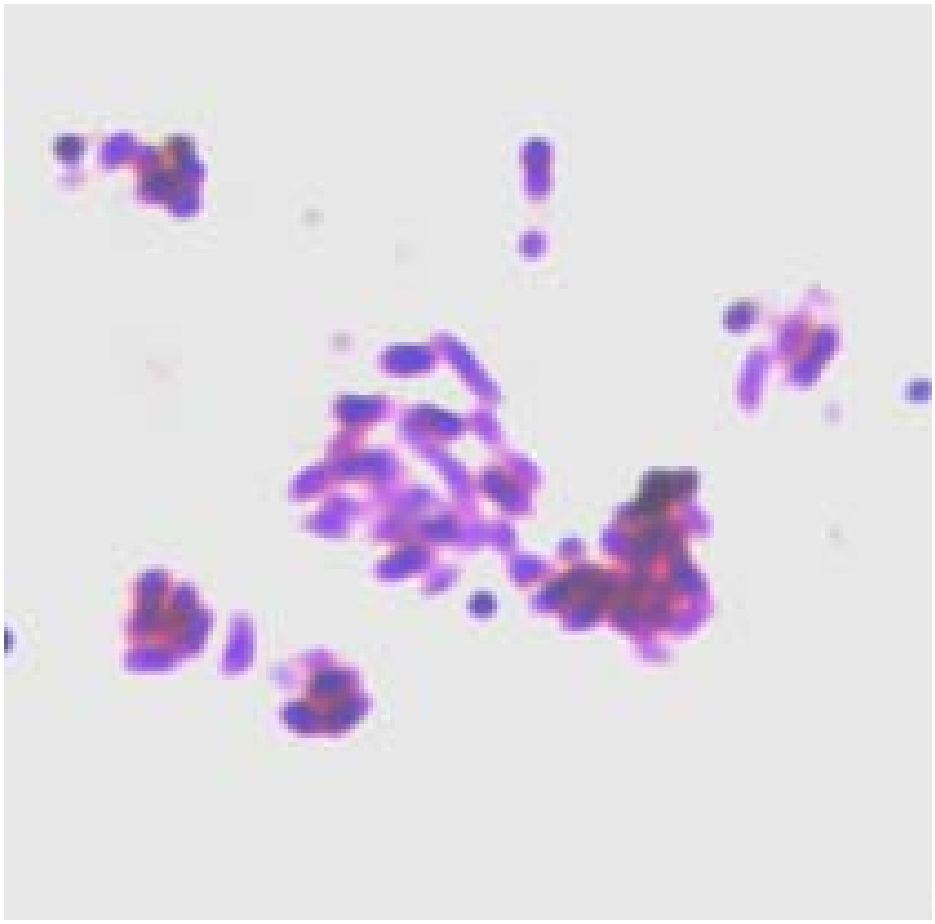

Figure 1A: *Mycobacterium avium* subsp. *paratuberculosis.* An acid-fast stain of Mpt examined at 1000x magnification with a light microscope reveals acid-fast bacilli.

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Figure 2A: Cow with Johne's disease. This is a picture of a cow in the latter stages of Johne's disease (1). The cow is severely emaciated and will likely die.

Figure 2B: An example of Bottle Jaw. As shown by the arrow in the picture this is what bottle jaw looks like (1). The jaw swells due to a loss of protein caused by profuse diarrhea.

Table 1: Microplate Testing Matrix. The following table shows each component added to a microplate in a specific experiment. Along with the organism and medium supplement nutrient medium was also added. For Mpt Middlebrook 7H9C was used and for MS Luria-Bertani broth was used.

Organism	Medium Supplement
Mycobacterium smegmatis	**MS Unfractionated Conditioned Medium**
Mycobacterium smegmatis	**Mpt Unfractionated Conditioned Medium**
Mycobacterium smegmatis	**Mpt Fractionated Conditioned Media**
Mycobacterium paratuberculosis	**Mpt Unfractionated Conditioned Medium**
Mycobacterium paratuberculosis	**Mpt Fractionated Conditioned Media**

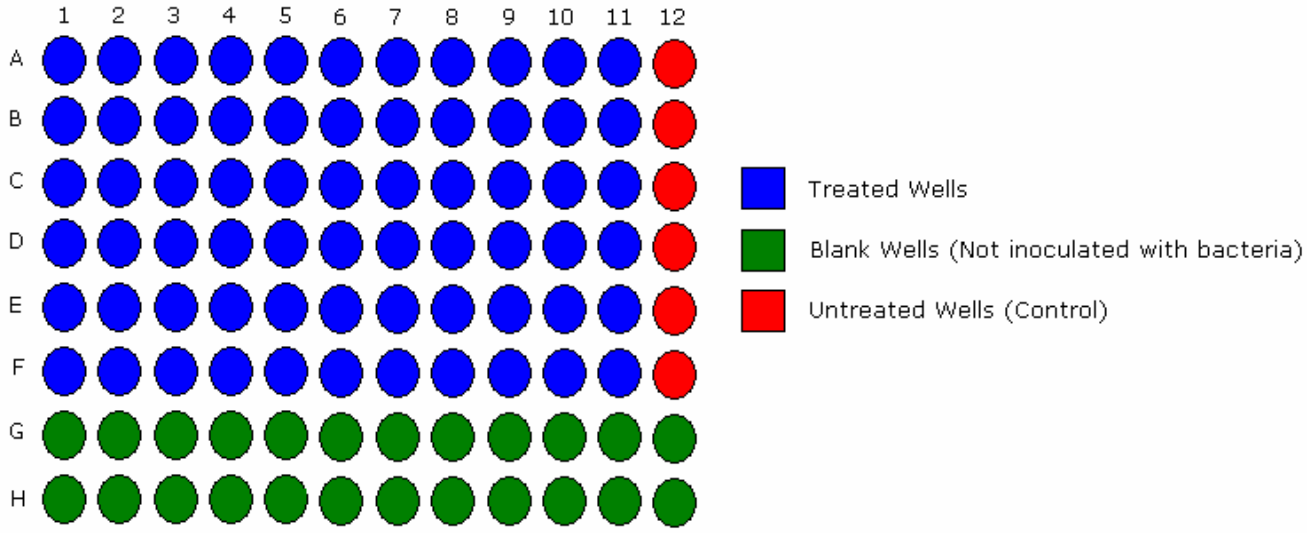

Figure 3A: Components of a microplate CM experiment. Each microplate is divided into three sections. The treated wells contain a dilution of conditioned medium inoculated with the test organism. The blank wells contain unsupplemented medium only. Untreated wells contain the test organism inoculated into unsupplemented medium.

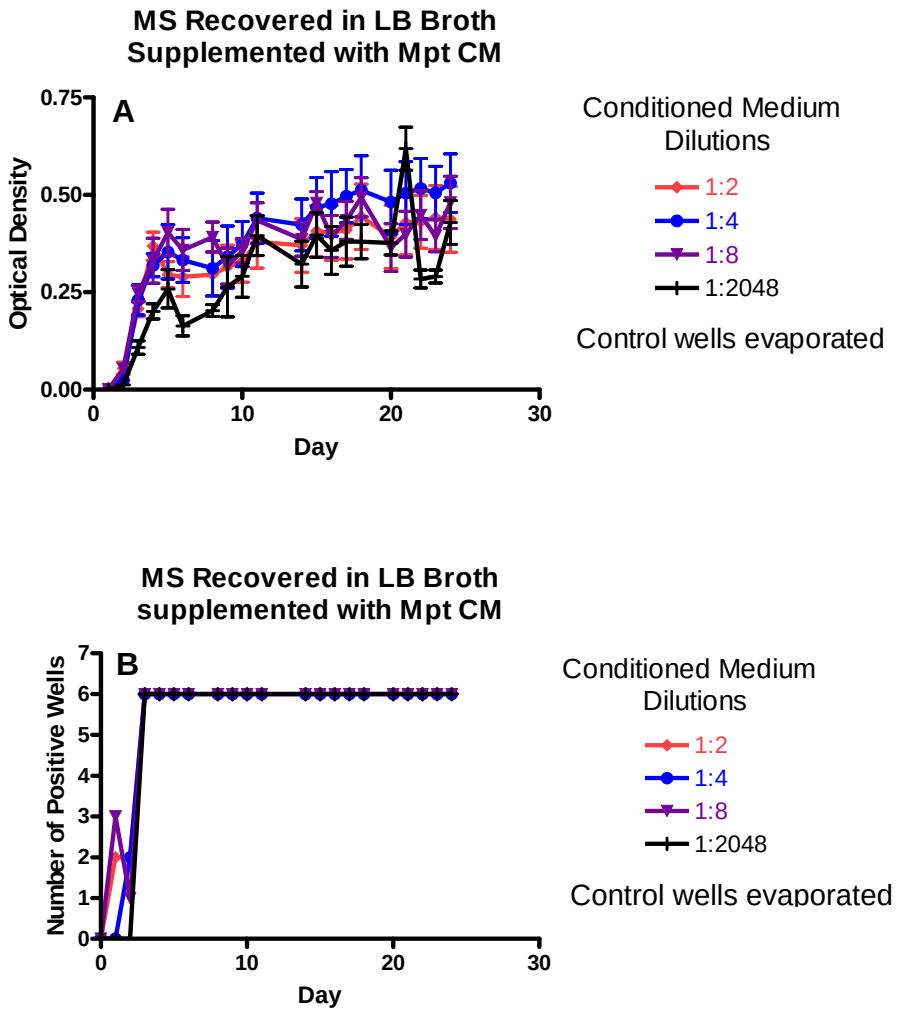

Figure 4: Conditioned medium from Mpt does not affect the growth of MS. Optical densities of MS grown in LB broth containing serial dilutions of Mpt CM were measured for a period of 25 days. Because the control wells evaporated in this trial, the most dilute concentration of CM was used as a comparison to the most concentrated treatments. **A.** The average optical density of the wells containing Mpt on day 0 was subtracted from that of each well containing Mpt at all time points. **B.** Wells were regarded positive if the optical density was greater than 3 standard deviations higher than the plate average at day 0.

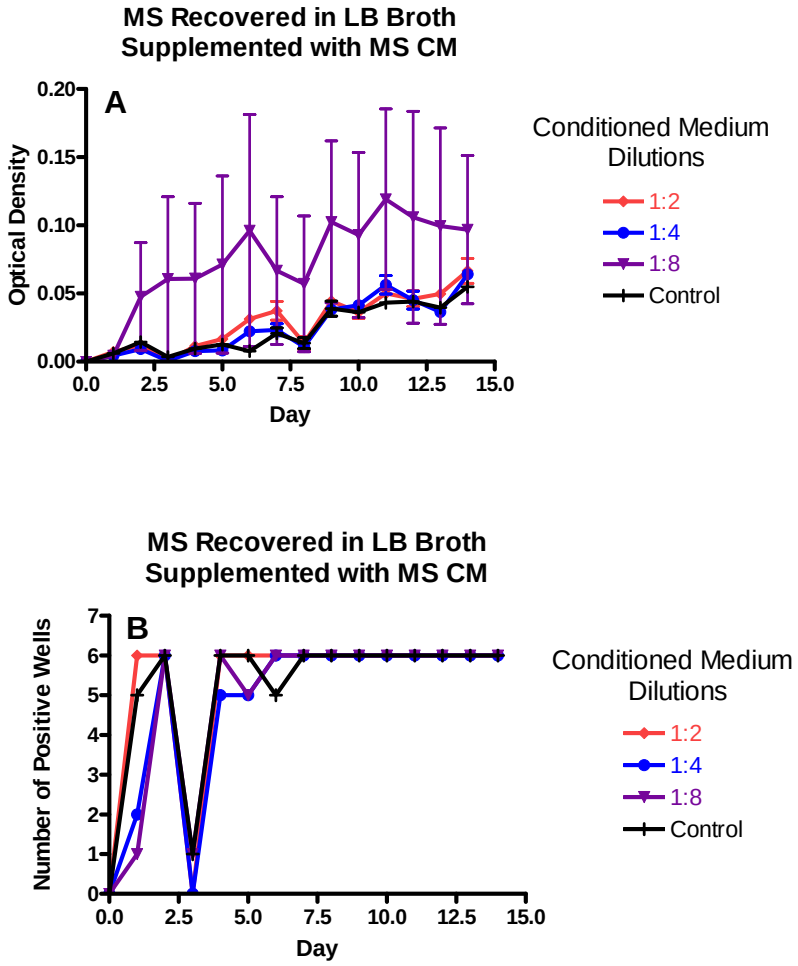

Figure 5: Conditioned medium from MS does not affect the growth of MS. Optical densities of MS grown in LB broth containing serial dilutions of Mpt CM were measured for a period of 14 days. **A.** The average optical density of the wells containing Mpt on day 0 was subtracted from that of each well containing Mpt at all time points. **B.** Wells were regarded positive if the optical density was greater than 3 standard deviations higher than the plate average at day 0.

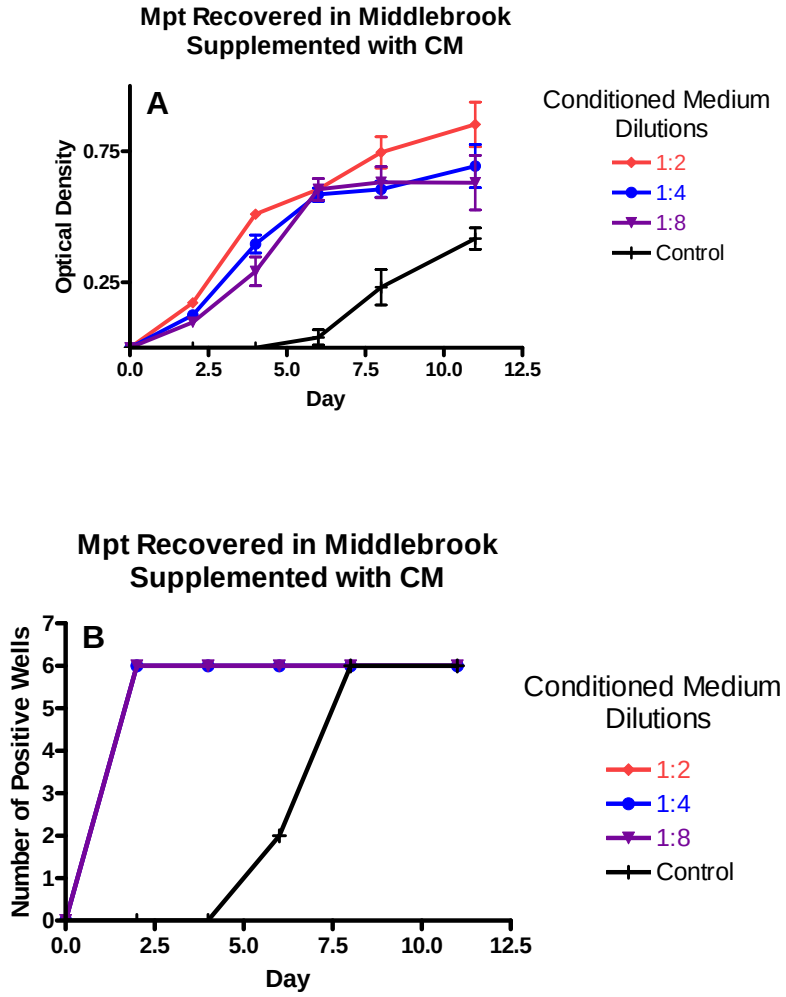

Figure 6: Mpt conditioned medium increased the number of cultures that recover from dormancy as well as the rate of recovery. Optical density of Mpt grown in Middlebrook 7H9C containing various Mpt CM concentrations was measured for a period of 11 days. **A**. The average optical density of the wells containing Mpt on day 0 was subtracted from that of each well containing Mpt at all time points. **B.** Wells were regarded positive if the optical density was greater than 3 standard deviations higher than the plate average at day 0.

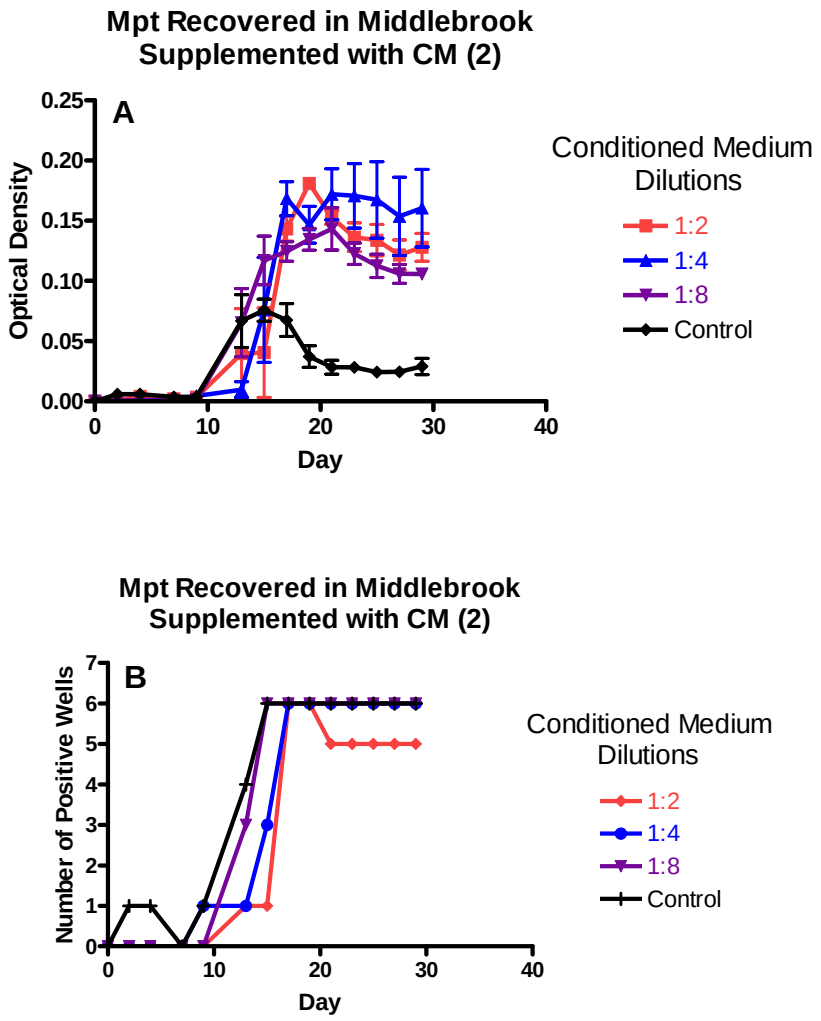

Figure 7: Mpt conditioned medium enhances the growth of recovering Mpt. Optical densities of Mpt grown in Middlebrook 7H9C containing serial dilutions of Mpt CM were measured over a period of 30 days. **A**. The average optical density of the wells containing Mpt on day 0 was subtracted from that of each well containing Mpt at all time points. **B.** Wells were regarded positive if the optical density was greater than 3 standard deviations higher than the plate average at day 0. This figure does not show a difference between treatments because the standard deviation of the day 0 average was very small.