Survival of Mycobacterium avium subsp. paratuberculosis in Synthetic Human Gastric Juice and Acidified Porcine Bile

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The bactericidal activities of synthetic gastric juice and acidified porcine bile on Mycobacterium avium subsp. paratuberculosis were assessed using propidium monoazide (PMA)-mediated quantitative reverse transcription-PCR, which allowed rapid relative quantitative analysis of viable M. avium subsp. paratuberculosis cells.

M. avium subsp. paratuberculosis is a pathogen that infects many domestic animals farmed for meat and milk (1). It is the etiological agent of Johne’s disease (JD) and has been controversially associated with Crohn’s disease (2). M. avium subsp. paratuberculosis has been isolated from a variety of food sources, including retail dairy products, potable water supplies, and beef carcasses (3–5). This indicates that human populations may be exposed to M. avium subsp. paratuberculosis. Upon exposure to M. avium subsp. paratuberculosis, the body has an array of defenses against such food-borne organisms, including gastric juice and acidified bile. Here, we examined the bactericidal activities of both synthetic gastric juice and porcine bile toward M. avium subsp. paratuberculosis through propidium monoazide (PMA)-mediated quantitative reverse transcription-PCR (qRT-PCR) to examine the effectiveness of these defenses against M. avium subsp. paratuberculosis, as the conditions M. avium subsp. paratuberculosis encounters in the human gut vary from those of its usual hosts.

M. avium subsp. paratuberculosis K-10 was used as a representative strain, albeit we accept this study will have to be extended to other strains to allow a more general conclusion. Cells were grown in 7H9 Middlebrook medium (Becton, Dickinson, Oxford, England) supplemented with 10% oleic acid-albumin-dextrose-catalase (Becton, Dickinson), 2 mg/liter Mycobactin J (Symbiotics Europe, France), 0.2% glycerol (Sigma-Aldrich, Germany), 0.05% Tween 80, naladixic acid (17.5 mg/liter), vancomycin (17.5 mg/liter), and amphotericin B (12.5 mg/liter). M. avium subsp. paratuberculosis was grown to mid-exponential phase (optical density at 600 nm, 0.7; ca. 2 × 10^8 CFU/ml), washed, and thoroughly resuspended. One volume of M. avium subsp. paratuberculosis was added to nine volumes of sterile synthetic gastric juice (6) or acidified porcine bile (0.3%, 1%, or 2%; pH 5.5, achieved using 1 M HCl) made up in 7H9 Middlebrook medium. M. avium subsp. paratuberculosis aliquots were exposed to these mixtures for 30, 60, 90, and 120 min. At each time point, samples were centrifuged, resuspended, and thoroughly washed in sterile phosphate-buffered saline (PBS). After washing, samples were resuspended in 50 µl of sterile PBS. Propidium monoazide (PMA; Biotium) treatment was carried out as previously described (7) with one minor change. A 400-W lamp was used instead of a 600-W lamp. The clarified sample was then used as the template for M. avium subsp. paratuberculosis F57 qRT-PCR.

Samples of various ratios of live to dead M. avium subsp. paratuberculosis cells were used as standards to which results could be compared. M. avium subsp. paratuberculosis cells were heat killed to indicate 0% viable cells, and nontreated cells were used to represent 100% viable cells (ca. 2 × 10^8 CFU/ml). The results with the standards were plotted on a graph, and a standard curve for that M. avium subsp. paratuberculosis concentration was determined. Viable cell numbers for each time point in both assays were extrapolated from the relevant standard curve. M. avium subsp. paratuberculosis qRT-PCR was carried out using the LightCycler 480 real-time PCR system (Roche). M. avium subsp. paratuberculosis detection was carried out using a previously described assay (8). To determine the effectiveness of the PMA treatment used in this study, the change in crossing points between PMA-treated live and dead cells was examined. In the gastric juice assay this difference was 5.07 cycles, and in the bile survival assay this difference was 4.9 cycles; these results are similar to those from previously reported experiments (7). The median values and ranges for each standard are plotted in Fig. 1.

After 30 min of exposure to the synthetic gastric juice, the amount of viable M. avium subsp. paratuberculosis had dropped approximately 2-fold. M. avium subsp. paratuberculosis was seen to consistently drop over the 120-min course of the experiment. After 120 min, approximately 5 × 10^3 M. avium subsp. paratuberculosis cells remained viable, representing a 4-fold decrease (Fig. 2). M. avium subsp. paratuberculosis was also examined for its ability to survive in increasing levels of acidified porcine bile. Aliquots of M. avium subsp. paratuberculosis were exposed to 0.3%, 1%, and 2% porcine bile at pH 5.5 for 30 to 120 min. With all bile concentrations tested, a killing effect was seen within the first 30 min. An increased killing effect was observed with increasing bile concentration (Fig. 3). This trend continued throughout the course of the experiment. All samples displayed reductions after 120 min.

The ability of a large number of the M. avium subsp. paratuberculosis cells to survive conditions similar to those tested here suggests that M. avium subsp. paratuberculosis could survive the...
initial human bactericidal barriers. The time frame for gastric emptying varies considerably; one study found that this can be as quick as 40.3/10 min (mean ± standard deviation) for a liquid meal and between 65.5 and 102 min for a solid meal (9), indicating that a large number of M. avium subsp. paratuberculosis cells could survive under physiological conditions. Cells were observed to aggregate quickly upon exposure to the acidified solution, and this aggregation could account for this survival effect. When food initially enters the stomach, it takes time for the acidification process to occur. It is possible that the bacteria would not be subjected to the same pH in the stomach as the low pH used in this experiment. M. avium subsp. paratuberculosis could also enter the stomach sequestered within a foodstuff, which would further protect it from the killing effects of the gastric juice. When the M. avium subsp. paratuberculosis cells encountered the acidified bile, a reduction of approximately 2- to 5-fold (0.3% to 2% bile exposure, respectively) was observed within the first 30 min of exposure to bile. The 0.3% bile concentration was chosen as the lower level, as this was thought to approximate what bacteria encounter in vivo (10). Over the 120-min course of the experiment, this concentration of bile was seen to lead to an approximate 5-fold reduction. Taking these results in combination, it can be concluded that M. avium subsp. paratuberculosis could survive conditions present in the small intestine and the colon. If M. avium subsp. paratuberculosis were shown to have an effect on the development or exacerbation of Crohn’s disease, then the presence of M. avium subsp. paratuberculosis in food would become an added concern, in light of our findings for the inability of the synthetic gastric juice or acidified bile to kill the bacteria. It must be borne in mind that this assay does not strictly measure cell viability, but rather is an indicator of membrane integrity. As long as the limits of the technique are understood, viable data can be obtained with speed. In conclusion, this study has demonstrated the extent and speed of the killing effects of both gastric juice and acidified bile for M. avium subsp. paratuberculosis.

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REFERENCES


