

# Johne's Disease: *Mycobacterium paratuberculosis* Super-shedders: Detection and Contribution to Passive Shedding (False-positive Fecal Cultures)

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## Introduction

Johne's disease continues to emerge as an important infectious disease of many ruminant species, having its greatest impact on dairy cattle. Both the 1996 and 2002 Dairy NAHMS studies reported Johne's as an important economic disease of dairy cattle. Isolation of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) bacteria by fecal culture is widely recognized as the ante-mortem "gold-standard" to identify cattle with Johne's disease (Collins *et al*, 2005). Current laboratory practice standards in the US do not include enumeration of MAP colony-forming unit (CFU) counts beyond 50 visible CFU per tube on solid media, such as Herrold's egg yolk medium (HEYM). Culture tubes with CFU exceeding this level are commonly designated as too numerous to count (TNTC), and the sample is reported as being a "high" or "heavy" shedder. Serial dilution of samples collected in a study has established that samples classified as TNTC can have a wide range of CFU counts (100,000 to more than 4 million cfu/gram). We propose the term "super-shedders" to describe a subset of shedding cows based on further enumeration of MAP CFU counts. This so-called super-shedder phenomenon has been reported for other bacteria, including verotoxigenic *Escherichia coli* O157 in cattle (Bach *et al*, 2005; Cray and Moon, 1995) and MAP infection in sheep (Whittington *et al*, 2000). We believe that super-shedder dairy cows have always existed, that this phenomenon has not been adequately recognized or accounted for in control programs such as the Voluntary Bovine Johne's Disease Control Program (VBJDPCP).

Preliminary observations suggest that most super-

shedder cows do not have clinical signs of Johne's disease (weight loss and diarrhea), although they shed as much, or significantly more, MAP into the environment as a typical cow with clinical Johne's disease. Since these cows are not readily recognized by dairymen, super-shedder cows may be responsible for massive environmental contamination that results in a disproportionate percentage of new MAP infections that occur in a herd. In addition, they may contribute to the fecal "pass-through" phenomenon previously described by Sweeney *et al* (1992), where positive fecal culture results are obtained from non-infected cows for several days after they have consumed feces from infected cattle.

Traditionally, cattle were classified as either positive or negative for Johne's disease based on growth of MAP from a fecal culture. Culture-positive cattle were judged to be infected, shedding MAP and potential sources of infection for herd mates. The standard practice was to recommend all positive animals be culled as quickly as possible because a positive fecal culture was considered the gold standard reference test, with 100% specificity (Collins *et al*, 2005). Over time, however, it has been recognized that differences exist in MAP shedding levels among culture-positive cattle, and the higher the number of visible colony forming units (CFU) on the surface of solid media, typically Herrold's egg yolk medium (HEYM), the greater the risk of spreading disease. Cattle are classified as low (1 to 9 CFU/tube), moderate (10 to 50 CFU/tube) or high/heavy (>50 CFU/tube) shedders. One recent study (Crossley *et al*, 2005) showed that most infected cows were in the low (51.4%) or high (30.8%) CFU categories. Although categorization of CFU may vary slightly among laboratories, other published studies (Whitlock *et al.*, 2000) have yielded similar findings.

Most US laboratories now report the number of visible MAP colonies on each tube of HEYM. However, most laboratories do not enumerate above 50 to 70 CFU/tube. Samples with CFU > 50 are classified as TNTC or heavy shedders. During 2004, the laboratory committee of the National Johne's Working Group (NJWG) discussed the need to enumerate heavy shedders. Based on an overwhelming consensus opinion of experienced Johne's investigators, little justification could be provided to count > 50 CFU/tube. As a result, national policy was developed whereby Johne's testing laboratories doing solid media culture are required to report any fecal sample with > 50 CFU of MAP/tube as a heavy shedder. Most members of the laboratory committee concurred at that time that some cattle classified as heavy shedders had >100 CFU/tube, but there was no biological reason to justify enumerating MAP CFU above 50 CFU/tube.

We now realize this decision was flawed, because there is a vast range of shedding levels greater than 50 CFU. Some cows with TNTC culture results are super-shedders, and if they are not identified, the consequences could be disastrous. Current recommendations have generally evolved to identification of the most infectious cows by culture or ELISA, with subsequent culling of the most heavily infected animals first. Theoretically, removal of all TNTC cows should prevent any further contamination of the dairy environment with MAP. However, not all dairy producers expeditiously cull cows that have fecal MAP counts reported as TNTC. If a super-shedder cow is producing well and has no clinical signs of Johne's disease, the animal is likely to stay in the herd even though it is shedding massive numbers of MAP. Prioritization of cows for culling on the basis of more categories of shedding levels (with less emphasis on low and moderate CFU) would have practical utility just as ELISA interval-based likelihood ratios (Collins *et al*, 2005) have had for serologic results.

### Materials and Methods

Serial dilutions (1:1, 1:5, 1:10, 1:50, 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000 and 1:50,000) of five well characterized repository fecal samples provided estimates of the range of MAP CFU in cows initially designated as TNTC or heavy shedders. These data then provided guidelines for the range of dilutions that would be most appropriate for routine dilutions of fecal samples characterized as heavy shedders. At each dilution, 100  $\mu$ l of inoculum was placed on the surface of HEYM in three 75-ml tissue culture flasks. Previous experience in this laboratory has indicated that HEYM in 70 ml tissue culture flasks has yielded higher MAP CFU compared to traditional culture slants in glass tubes. Some of the

difference for improved recovery of MAP is attributed to a more uniform thickness of the media and a larger surface area in the flasks compared to tube slants.

Fecal samples for RT-PCR (qRT-PCR) were evaluated using the Tetracore quantitative real-time PCR, according to manufacturer directions. The target sequence of the MAP PCR is the *hspX* gene, which contains MAP-specific sequences capable of distinguishing MAP from other organisms, including other mycobacteria. Use of the *hspX* gene for diagnostic detection of MAP is covered by US Patent No. 5,985,576, under exclusive license to Tetracore, Inc., Rockville, MD from the USDA. The extraction procedure involves bead-beater and chaotropic separation of DNA.

Retrospectively, fecal samples classified as heavy shedders (>70 cfu/tube) from three demonstration herds (Herd-A, 312 cows; Herd B, 103 cows and Herd C, 125 cows, respectively) were serially diluted at 1:100 and 1:1,000 to determine MAP cfu/gm of manure. Many of these samples were also tested by RT-PCR to determine cycles to position (Ct). Lower CFU samples (both low and moderate shedders) were cultured a second time to determine which culture-positive fecal samples could be the result of passive shedding due to manure contamination of the environment. Cattle that were both ELISA-negative and culture-negative on two or more sampling times were classified as "passive shedders".

### Results

The initial pilot dilutions from 1:1 to 1:50,000 suggested that a 1:1 dilution would be valuable to re-confirm the original MAP CFU prior to freezing at -1,228° F (-700° C) for a period of time. Dilutions of 1:100 and 1:1,000, for routine processing of samples suspected to be super-shedders, provided an acceptable range to determine final MAP CFU/gm of manure. Estimated range of mycobacteria in these five samples previously recorded as heavy shedders was 63,000 to 1,470,000 MAP CFU/gram. In a similar manner the cycles to positive (Ct) ranged from 33.3 for the lowest cfu/gm to 19.4.

The RT-PCR cycles to positive (Ct) was closely related to the MAP CFU/gm in the original five serially diluted samples. The Ct range was 33.3 to 19.4 for those fecal samples with 63,000 to 1,470,000 CFU MAP/gm. Subsequent comparisons of RT-PCR to serial dilutions of fecal samples has provided an excellent correlation.

Two of ten culture-positive fecal samples from Herd A were classified as heavy shedders (TNTC). Upon serial dilution, one sample had 7,000 CFU MAP/gm while the other had 462,000 CFU MAP/gm. Two low shedders among the ten culture cows were classified as transient shedders and two other cows were probable passive shedders, or 40% of the culture-positive cattle. Two of seven culture-positive fecal samples from Herd B were classi-

fied as heavy shedders. One serially diluted resulted in 210,000 CFU MAP/gm, which increased to 742,000 CFU MAP/gm when sampled seven months later. Four of the seven (57%) cows (low and moderate shedders) were classified as passive shedders. The moderate shedder has MAP CFU of 5, 3, 22, 5 on the four tubes of HEYM. This cow was ELISA-negative three times and culture-negative six months later. One of 17 culture-positive samples was classified as a heavy shedder and, upon serial dilution, had 1,260,000 CFU MAP/gm. A minimum of nine in 17 (53%) of the culture-positive cattle were classified as pass-through positive, and likely 16 of the 17 (94%) were transiently culture-positive attributable to one super-shedder.

### Significance

This report further documents the critical importance of identifying super-shedders, since they contribute billions of MAP CFU daily to the environment, serving to disseminate MAP to susceptible cattle. In many herds, this results in significant numbers of culture-positive fecal samples through passive shedding. The frequency of super-shedders among culture-positive cattle in infected herds is unknown. However, our preliminary investigations have shown that each of three current herds has at least one super-shedder cow, with five super-shedder cows having been identified among the 7.1% (41 of 575) of culture-positive cattle in the herds. Our preliminary estimates are that 10% of heavy shedders (or about 2 to 3% of all culture-positive cattle at a

single time-point) may be super-shedders, excreting >10 billion CFU MAP per cow per day.

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