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Effects of Cell Diluents on Enumeration of Viable *Renibacterium salmoninarum*

Renibacterium salmoninarum is the etiological agent of bacterial kidney disease of salmonid fishes. This slow-growing bacterium is a Gram-positive coccobacillus with an absolute requirement for a sulfur-containing amino acid supplement in primary isolation and culture media. One of the earliest media used to cultivate *R. salmoninarum* was Mueller Hinton supplemented with 0.1% l-cysteine HCl. In 1977, an improved kidney disease medium (KDM2) which incorporated serum was developed. More recently, a phenomenon was described whereby autonomously secreted metabolic products of *R. salmoninarum* enhanced its own growth. This phenomenon evolved into a medium supplement (metabolite) that results in much more luxuriant bacterial growth in broth and agar media.

More Reliable Determination of Viable Cell Counts Needed

In many laboratory experiments, the researcher's ability to achieve valid results depends upon accurately knowing the number of viable bacterial cells in a culture. The characteristic slow-growing nature of *R. salmoninarum* makes viable cell determination unavailable for at least 2 weeks after inoculation. Without a method to more reliably produce viable cell counts, results of studies conducted within this 2-week interim could be invalid. Peptone-saline (0.1%-0.85%; PS) is a frequently used cell diluent for studies of *R. salmoninarum*. At this laboratory, inconsistent results have occasionally been seen with the use of PS diluent in studies requiring viable cell count data, in the form of lack of growth and extremely small colonies on the plating medium. Other researchers have quantified *R. salmoninarum* cells using spectrophotometer absorbance values; however, dead cells also produce an absorbance reading with this method, which could affect count accuracy.

The present study was initiated to evaluate cell diluent media that could be used to determine the number of viable cells in *R. salmoninarum* cultures. Because metabolite has been shown to greatly enhance bacterium growth, we thought it would increase performance of diluents as well, and we incorporated metabolite into some diluents tested.

Culture Growth and Cell Count Procedure

Seven strains of *R. salmoninarum* isolated from kidney tissues of clinically diseased chinook (*Oncorhynchus tshawytscha)* and coho salmon (*O. kisutch*) were used. To produce a culture for viable cell enumeration, we used a frozen 1-mL aliquot of the strain to seed 50 to 100 mL of a kidney disease medium (KDM2) broth supplemented with 1% filter-sterilized ATCC 33209 metabolite. Incubation was on a shaker (100 rpm) at 15C until good growth was achieved or for about 2-3 weeks. Larger volume cultures (500 or 1,000 mL) were inoculated using the 50- to 100-mL culture as the seed inoculum (1% v/v). Cell counts were made from the larger volume cultures. We quantified

cells by preparing serial tenfold dilutions in the various diluents, then dropping 0.025-mL volumes onto the surface of KDM2 + metabolite agar plates. The plates were incubated at 15C, and after 2-3 weeks the resulting colonies were visually enumerated. Cell numbers are expressed as colony-forming units per milliliter (cfu/mL). Diluents evaluated were peptone-saline (PS), PS supplemented with 1% ATCC 33209 metabolite (PSM), KDM2 broth supplemented with 1% ATCC 33209 metabolite (KDM2+M), and KDM2+M without l-cysteine (KDM2+M-C). We performed an analysis of variance on the cell enumeration data.

Two different diluents were used to determine virulence in brook trout (*Salvelinus fontinalis*) of one *R. salmoninarum* culture, resulting in low (PS) and high (KDM2+M) colony-forming units per milliliter. Groups of 15 fish (27 g each) were challenged by intraperitoneal injection with 0.1 mL of each tenfold dilution, the same dilution sets used for the viable cell counts. Mortalities were recorded, and bacterial kidney disease was diagnosed using the fluorescent antibody test of kidney tissues. Virulence was expressed by LD_{50} values.

Best Results Obtained Using KDM2+M Without l-Cysteine

The highest mean counts for the 14 *R. salmoninarum* cultures enumerated were obtained with KDM2+M-C cell diluent (2.56×10^8 cfu/mL). The mean counts with KDM2+M (2.09×10^8cfu/mL) and PSM (2.46×10^8 cfu/mL) diluents were similar, but lower. Lower still was the mean count using PS (1.27×10^8 cfu/mL). Analysis of variance of the viable cell counts determined no statistically significant difference between the four diluents ($P = 0.84$). The mean count using KDM2+M was similar to the highest; however, we did encounter one major problem using this diluent. Colonies could not be enumerated for 9 of 14 replicates because of interference by l-cysteine precipitation on the surface of the plating medium. When l-cysteine was deleted from the diluent, the precipitate was not formed. Although l-cysteine is a required growth medium component, it is not necessary to incorporate it into the cell diluent.

No statistically significant difference was noted among the diluents evaluated, but the most consistent results were obtained using KDM2+M-C, which yielded the highest average colony-forming units per milliliter. More importantly, no counts were lost due to precipitation of l-cysteine. All three diluents that resulted in the highest mean cell counts contained metabolite; for 10 of 14 individual comparisons, the highest cell count was recorded with a diluent that contained metabolite. In some comparisons, cell counts were similar regardless of the diluent used, but with others at least a 100-fold variation was noted. Incorporation of metabolite into the cell diluent often resulted in far greater growth in the colony, making counting much easier.

In the virulence assay, PS-diluted cells yielded a viable cell count of 8.96×10^5 cfu/mL, compared to 1.83×10^7 cfu/mL for cells diluted in KDM2+M. All fish that died following challenge were diagnosed with bacterial kidney disease, with numerous cells in kidney tissues. Calculated LD_{50} values were 2.61×10^5 cfu/mL for fish challenged with cells diluted in PS and were $5.29\ 10^5$cfu/mL for KDM2+M-diluted cells. Although calculated LD_{50} values were more similar than the viable cell counts, mortality of the two groups challenged with respective 1×10^{-1} dilutions was quite different. Cells diluted in KDM2+M produced greater mortality (86.7%) than did cells diluted in PS (6.7%), perhaps indicating that some cells in the PS diluent lost viability, also indicated in reduced viable cell counts.

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