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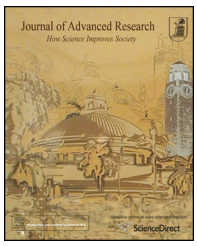

ORIGINAL ARTICLE

Recovery of *Renibacterium salmoninarum* from naturally infected salmonine stocks in Michigan using a modified culture protocol

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KEYWORDS

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Culture

Abstract *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), is a fastidious and slow-growing bacterium that is extremely difficult to grow *in vitro*. Herein, we describe a modified primary culture protocol that encompasses a modified bacteriological culture medium and a tissue processing procedure. In order to facilitate the release of *R. salmoninarum* from granulomatous tissues, kidneys of infected fish were homogenized in a high speed stomacher. The kidney disease medium (KDM2), routinely used for primary culture of *R. salmoninarum* was modified by the addition of antibiotics and metabolites. When a relatively large inoculum of diluted kidney homogenate was streak-plate inoculated onto the modified KDM2, colonial growth of *R. salmoninarum* was achieved within 5–7 days, compared to the standard of two weeks or more. The modified procedure was then used to determine the prevalence of *R. salmoninarum* among representative captive and feral salmonid stocks in Michigan. Prevalence and clinical manifestations varied among species, strains of fish, and locations; however, *R. salmoninarum* isolates were biochemically homogenous. The improved primary culture procedure described in this study enabled

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selective and quick isolation of *R. salmoninarum*. Also, the isolates retrieved in this study constitute a unique biological resource for future studies of *R. salmoninarum* in the Laurentian Great Lakes.

Introduction

Bacterial kidney disease (BKD), caused by the gram positive bacterium *Renibacterium salmoninarum*, is a serious disease threatening salmonids all over the northern hemisphere [1]. *R. salmoninarum* elicits the formation of granulomatous tissues, primarily in hematopoietic tissues of the kidneys, thereby impairing the vital functions of this important organ [2]. The bacterium is transmitted both horizontally and vertically, through gamete to offspring, a matter that poses a great challenge for BKD control. In the Laurentian Great Lakes, BKD was first described in the early 1950s [3] and has since spread and became endemic in the entire basin [4,5]. In the late 1980s, BKD prevalence reached untold levels in wild and hatchery-propagated salmonids, and was associated with widespread mortality of wild salmonids in Lake Michigan [6,7]. In the absence of an effective vaccine, BKD control relies primarily on culling infected wild or captive spawning adults and resulting egg lots, and treating the broodstock fish or fertilized eggs with a drug such as erythromycin. Regulatory agencies in States and Canadian provinces bordering the Great Lakes enacted continuous monitoring and surveillance of susceptible fish species in order to determine the extent of this disease in the different water bodies [4,5].

Obtaining accurate prevalence data of *R. salmoninarum* infections in carrier, apparently healthy fish is difficult due to the inconsistent successes to isolate the bacterium in culture. *R. salmoninarum* is fastidious in it's requirement for *l*-cysteine and is very slow growing, a matter that allows the overgrowth of bacterial and fungal contaminants upon primary isolation. Moreover, the layers of granulomatous tissues that the host forms around infection foci make the bacterium difficult to attain for isolation using standard bacterial recovery methods. This problem is further complicated by the uneven distribution of *R. salmoninarum* aggregations within affected kidney tissues, especially in asymptomatic fish [8,9], and the presence of inflammatory mediators that inhibit *R. salmoninarum in vitro* growth [10,11]. The culture medium routinely used for the isolation of *R. salmoninarum* is the kidney disease medium (KDM2) developed by Evelyn [12], which allows bacterial growth within 12 weeks. Later, Evelyn et al. [13,14] noted that when filter-sterilized broth that had previously been used to grow *R. salmoninarum* (spent medium) was used to supplement fresh KDM2, *R. salmoninarum* colonial growth was improved and the incubation time to visualize colonies was reduced. The improved growth was attributed to metabolites secreted by the initial culture. Using the same concept, Teska [15] and Starliper et al. [16] incorporated *R. salmoninarum* spent medium (1% v/v) into KDM2 for related BKD studies. While these modifications have supported the subculturing of previously isolated *R. salmoninarum* strains, direct isolation from infected tissues, particularly from carrier fish, yielded inconsistent results.

Despite the widespread distribution of BKD in the Great Lakes, only a few number of *R. salmoninarum* isolates were retrieved from resident fish [17,18]. Among the retrieved isolates, those from the Michigan side of Lake Michigan were the most genetically diverse [18] and were of higher virulence [19] when compared to isolates obtained from other locations in the USA and the world. To better understand the biological and genetic diversity of Great Lakes *R. salmoninarum*, there is a need to retrieve a greater number of isolates. To this end, we present a combination of a modified tissue processing protocol and culture medium to enhance the primary isolation of *R. salmoninarum*. The modified protocol was then used to determine *R. salmoninarum* prevalence in representative captive and feral salmonine stocks in Michigan.

Material and methods

Modification of the kidney disease medium (KDM2)

Standard KDM2 was modified by supplementing its components with 10% fetal calf serum, four antimicrobials, and filtered (0.45 μM) 1% *R. salmoninarum* spent medium (metabolite). These modifications combined the observations that each of these supplements enhance and/or select *R. salmoninarum* growth [14–16,20]. The modified medium will be referred to as the modified KDM2 (MKDM). Briefly, MKDM consists of peptone (1% w/v), yeast extract (0.05% w/v), *l*-cysteine HCl (0.1% w/v), cycloheximide (0.005% w/v), new born calf serum (10% v/v), filter-sterilized *R. salmoninarum* spent broth (1% v/v), oxolinic acid (0.00025% w/v), polymyxin B sulfate (0.0025% w/v), D-cycloserine (0.00125% w/v), and agar (1.5% w/v). The pH was adjusted to 6.8 using 1 N NaOH. All MKDM ingredients were purchased from Sigma (Sigma Chemical Co, St. Louis, MO, USA) with the exception of agar, which was from Remel (Remel, Lenexa, Kansas, USA).

Modified protocol of fish tissue processing and plating to enhance R. salmoninarum recovery

Kidney tissues were collected from 515 wild adult salmon returning to spawn in Michigan's gamete collecting stations (weirs). Fish included 150 returning chinook salmon (*Oncorhynchus tshawytscha*) collected from the Little Manistee River Weir (LMRW) at Manistee, Michigan (Lake Michigan watershed), the Swan River Weir (SRW) at Rogers City, Michigan (Lake Huron watershed) and the Platte River Weir (PRW) at Beulah, Michigan (Lake Michigan watershed). An additional 165 Michigan-adapted coho salmon (*Oncorhynchus kisutch*) strain and 56 Hinchenbrook coho salmon strain were collected from the Platte River Weir (PRW). Kidney tissues were also collected from captive broodstock collected from Michigan state fish hatcheries including 60 brook trout (*Salvelinus fontinalis*) of the Iron River strain, 60 lake trout (*Salvelinus namaycush*) that were kept in raceways receiving surface water from the Cherry Creek (Lake Superior watershed) at the Marquette State Fish Hatchery (MSFH), Marquette, MI. Additionally, kidneys were collected from 12 brown trout (*Salmo trutta*) of the Wild Rose strain and 12 rainbow trout