IN VITRO GROWTH OF THE BACTERIAL KIDNEY DISEASE ORGANISM RENIBACTERIUM SALMONINARUM ON A NONSERUM, NONCHARCOAL-BASED "HOMOSPECIES-METABOLITE" MEDIUM

Author: Teska, Jeffrey D.

Source: Journal of Wildlife Diseases, 30(3) : 383-388

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-30.3.383

Journal of Wildlife Diseases, 30(3), 1994, pp. 383–388

IN VITRO GROWTH OF THE BACTERIAL KIDNEY DISEASE ORGANISM *RENIBACTERIUM SALMONINARUM* ON A NONSERUM, NONCHARCOAL-BASED "HOMOSPECIES-METABOLITE" MEDIUM

Jeffrey D. Teska
National Biological Survey, National Fish Health Research Laboratory,
1700 Leetown Rd., Kearneysville, West Virginia 25430, USA

ABSTRACT: Laboratory and field trials were conducted to evaluate in vitro growth of *Renibacterium salmoninarum* in media without serum or charcoal. Growth of this bacterium, the cause of bacterial kidney disease (BKD) in salmonids, is accelerated by addition of a growth enhancing "metabolite" of unknown composition to KDM2 medium, the medium commonly used for isolation of *R. salmoninarum*. KDM2 medium supplemented with greater than 1% (v/v) metabolite enhanced growth even without addition of either serum or charcoal. Medium containing 5% metabolite (denoted Five-M) allowed optimal growth in laboratory studies and was further evaluated as a primary plating medium for recovery of the bacterium isolated from chinook salmon (*Oncorhynchus tshawytscha*) exhibiting clinical BKD. Recovery rates of *R. salmoninarum* using Five-M medium were 4% and 36% higher, respectively, than comparable rates using a serum-based medium for the two salmon populations evaluated. Five-M medium is an effective, inexpensive alternative to serum-based or charcoal-based media.

Key words: *Renibacterium salmoninarum*, KDM2 medium, metabolite supplementation, in vitro growth, culture medium.

INTRODUCTION

Successful in vitro isolation and cultivation of fastidious bacteria depends on the nutrient formulation of the medium used. The in vitro growth of *Renibacterium salmoninarum*, a fastidious Gram-positive organism causing bacterial kidney disease (BKD) in salmonid fish, was significantly advanced by the introduction of KDM2 medium (Evelyn, 1977). The medium contains L-cysteine, required by *R. salmoninarum* for growth, along with a protein source, yeast extract, and serum. The medium supports growth of the microorganism; visible colonies generally appear 20 to 45 days after incubation at 15 C. Any isolation medium requiring bovine serum supplementation is potentially cost prohibitive, especially for smaller diagnostic laboratories operating on limited budgets. In an attempt to eliminate the need for serum, a charcoal-based medium was developed (Daly and Stevenson, 1985). Activated charcoal, however, has a relatively short shelf-life after exposure to air, and charcoal agar offers no significant improvement over KDM2 with respect to incubation time or colony size (Daly and Stevenson, 1985). Either medium can be used for the confirmatory diagnosis of BKD. Little or no growth of *R. salmoninarum* occurs on medium lacking either serum or charcoal, such as cysteine-supplemented Mueller-Hinton agar (Daly and Stevenson, 1985).

Growth of *R. salmoninarum* on serum-containing media is improved by addition of a growth-enhancing "metabolite" developed by T. P. T. Evelyn; metabolite is of unknown composition (Teska, 1993). The metabolite (possibly a soluble surface antigen), prepared from spent broth in which *R. salmoninarum* was grown, is used as a 1% (volume/volume) supplementation. It is not known if the metabolite at this concentration functions as a nutrient source or a medium detoxifier, as occurs in charcoal agar. Growth of the bacterium on this supplemented medium is significantly accelerated and is independent of metabolite source (Teska, 1993).

In this study, I further evaluated the growth-enhancing and growth-supporting properties of this metabolite of unknown composition. My objective was to determine if metabolite produced by *R. salmoninarum* could be used as a serum sub-

TABLE 1. List of National Fish Health Research Laboratory (NFHRL) *Renibacterium salmoninarum* isolates used in this study, including primary isolation data.

NFHRL number	Site isolated and date of isolation	Host fish species
7.01	Leaburg, Oregon (USA) (ATCC #33209) 1974	*Oncorhynchus tshawytscha*
7.05	Siletz River, Oregon (USA) Date unknown	*O. kisutch*
7.06	Quinault National Fish Hatchery, Washington (USA) Date unknown	unknown
7.07	Salmon Creek, Alaska (USA) Date unknown	unknown
7.08	Margaree River, Prince Edwards Island, Canada Date unknown	*Salmo salar*
7.30	G. Adams State Fish Hatchery, Washington (USA) 1992	*O. tshawytscha*
7.32	G. Adams State Fish Hatchery, Washington (USA) 1992	*O. tshawytscha*
K91-12K	Kewaunee, Wisconsin (USA) 1991	*O. tshawytscha*
K91-53K	Kewaunee, Wisconsin (USA) 1991	*O. tshawytscha*
M91-05H	Manistee, Michigan (USA) 1991	*O. kisutch*
M91-09H	Manistee, Michigan (USA) 1991	*O. kisutch*
S91-13K	Strawberry Creek, Wisconsin (USA) 1991	*O. tshawytscha*
S91-58K	Strawberry Creek, Wisconsin (USA) 1991	*O. tshawytscha*

stitute, thereby eliminating the need for the potentially cost prohibitive purchase of serum. Laboratory and field trials were conducted to determine optimal metabolite concentration and to evaluate the medium for recovery of *R. salmoninarum* among infected salmonid populations. I compared cell counts, colony size, and isolation rates from fish obtained on metabolite-containing media formulations with similar data obtained from the serum-based selective kidney disease medium (SKDM) (Austin et al., 1985) routinely used to isolate the bacterium.

MATERIALS AND METHODS

Isolates of *Renibacterium salmoninarum*, previously obtained from Atlantic and Pacific salmon (Table 1), were subcultured on KDM2 medium (Evelyn, 1977) consisting of 1% peptone (Difco Laboratories, Detroit, Michigan, USA), 0.05% yeast extract (Difco), 0.1% cysteine (Sigma Chemical Company, St. Louis, Missouri, USA), 10% (v/v) newborn calf serum (Intergen Company, Purchase, New York, USA), and 1.5% agar (Difco). The KDM2 medium also was supplemented with 0.005% cycloheximide (Sigma), 0.00125% D-cycloserine (Sigma), 0.0025% polymyxin B sulphate (Sigma), and 0.00025% oxolinic acid (Sigma) as recommended by Austin et al. (1985); their medium was called selective kidney disease medium (SKDM). All cultures were incubated on SKDM at 15 C until visible growth was detected, and colonies were confirmed as *R. salmoninarum* using the Gram-stain and the direct fluorescent antibody technique (DFAT) as described by Bullock et al. (1980). Rabbit anti-*R. salmoninarum* serum labeled with fluorescein isothiocyanate (Kirkegaard and Perry Labs, Gaithersburg, Maryland, USA) was used for the DFAT.

Five media were used in the initial laboratory studies and two media were subsequently evaluated in the field trials. Cell counts and colony size studies were conducted during the summer of 1992 and the isolation rates from fish were completed in September and October 1992 during the fall spawning season. Selective kidney disease medium without serum was used as the basal formulation (BF). Media evaluated included BF plus 10% serum (SKDM), BF plus serum and 1% (v/v) metabolite (denoted as Met), BF plus 10% metabolite (denoted as Ten-M), BF plus 5% metabolite (denoted as Five-M), and BF plus 1% metabolite (denoted as One-M). All plates were stored at 4 C and used within 2 days following preparation.

Supernatant from a 1-l KDM2 broth culture of *R. salmoninarum*, National Fish Health Research Laboratory (NFHRL) isolate 7.15, was separated from whole cells, filter-sterilized, and stored as described by Teska (1993). Briefly, a KDM2 broth culture of *R. salmoninarum* was grown to an absorbance of 1.25 at a wavelength of 540 nm using a Spectronic 20D spectrophotometer (Milton Roy Company, Ivyland, Pennsylvania, USA). The cellular component was reduced to a pellet by centrifugating at 5,000 × *g* for 20 min and discarded. The supernatant then was sterilized by passage through a 0.45-micrometer filter and frozen at −70 C until needed. This unconcentrated, cell-free supernatant was used as the source of metabolite for both the laboratory studies and field trials. All metabolite was standardized by growing broth cultures to an absorbance of 1.25 at a wavelength of 540 nm and adjusting the total protein concentration of the cell-free extract to 11 mg/ml. Total protein concentration was 1.1, 0.55, and 0.11 mg/ml for Ten-M, Five-M, and One-M, respectively. Protein concentrations were determined by the method of Shatkin (1969).

Thirteen *R. salmoninarum* isolates were grown on SKDM agar slants for 2 to 4 wk at 15 C. Each slant was washed with cold, sterile peptone-saline (PS) consisting of 0.1% peptone (Difco) and 0.8% sodium chloride. Cells were washed twice with PS by centrifugation for 20 min at 5,000 × *g*. The final pellet was resuspended in a small volume of PS, and each suspension was adjusted to an absorbance of 1.25 at a wavelength of 540 nm using a Spectronic 20D spectrophotometer so as to standardize the subsequent comparisons. Log_{10} dilutions were made in cold PS, and the bacterial cell counts of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were determined on each test medium using the dilution plate count method described by Cipriano et al. (1992). All plates were sealed in plastic to retain moisture and incubated at 15 C. Plate counts and colony size were determined on day 20 for each of the isolates.

Due to the lack of statistical significance between colony counts on Ten-M and Five-M media, the latter was chosen for evaluation in the field trials. Two chinook salmon (*Oncorhynchus tshawytscha*) populations with clinical BKD were selected for the field evaluations. Populations included 1992 fall-spawning adult chinook salmon from western Lake Michigan at Kewaunee, Wisconsin (USA) (87°30′N, 44°30′W) and eastern Lake Michigan at Manistee, Michigan (86°25′N, 44°15′W). Spawning adult salmon were collected at the respective weirs and euthanized by bubbling compressed carbon dioxide into the holding tank water until fish ceased movement. Initially, 60 fish per population were necropsied and their kidneys streaked on SKDM and Five-M media, using sterile inoculating loops. All plates were sent via next-day mail to the NFHRL and incubated at 15 C immediately upon arrival. Due to contamination or shipping damage of one or both SKDM and Five-M plates comparisons were made only with paired samples, and consequently, sample sizes used in the final evaluation were less than 60. Even with the use of antibiotics, some contaminating fungal or bacterial colonies occurred on the agar surface. Plates therefore were examined at 5-day intervals and contaminating growth was removed with a sterile scalpel. Due to the nonquantitative nature of the streak plating technique, distinct colonies of *R. salmoninarum* could not be counted but were recorded as positive or negative for isolation of *R. salmoninarum* after 20 days of incubation.

Data were analyzed by an analysis of variance test (ANOVA) with alpha = 0.05, using five medium (SKDM, Met, Ten-M, Five-M, One-M) treatments at three dilution (10^{-4}, 10^{-5}, 10^{-6}) levels of a standardized cell suspension (Zar, 1974). The intent was to determine if colony forming units per milliliter (CFU/ml) differed significantly among the isolates grown on the media tested. Statistically significant data were further analyzed by the Newman-Keuls multiple range test (Keuls, 1952) to determine which media, if any, supported significantly different colony counts.

RESULTS

All five media formulations used in the laboratory evaluation supported growth of the 13 *R. salmoninarum* isolates to some degree. Plates containing One-M medium were least effective in supporting growth based on viable cell counts and colony size (Tables 2 and 3). Colony size for One-M medium at all three dilutions (data for 10^{-4} and 10^{-6} dilutions not shown) was less than 0.5 mm at day 20. Colony sizes for the remaining four media ranged between 1.0 and 2.0 mm after the same incubation time. There was a significant ($P < 0.05$) difference in cell counts among the five media treatments at all three dilutions tested. Based on the Newman-Keuls multiple range paired comparisons of colony counts on the five media, there was a significant ($P < 0.05$) difference between the lower counts on One-M medium and all other media at all three dilutions. With one ex-

TABLE 2. Mean colony counts (10^{-5} dilution) obtained after 20 days incubation at 15 C for 13 *Renibacterium salmoninarum* isolates grown on selective kidney disease medium (SKDM), SKDM plus 1% (v/v) metabolite (Met), SKDM minus serum plus 10% metabolite (Ten-M), SKDM minus serum plus 5% metabolite (Five-M), and SKDM minus serum plus 1% metabolite (One-M).

	Mean colony counts for 10^{-5} dilution				
Isolate	SKDM	Met	Ten-M	Five-M	One-M
7.01	0.0	2.3	9.7	19.3	0.0
7.05	0.3	0.3	2.3	3.7	0.0
7.06	0.0	1.7	1.0	1.3	0.0
7.07	0.0	4.3	19.0	28.0	0.0
7.08	17.7	28.3	35.0	43.7	0.0
7.30	0.0	75.7	108.3	107.7	0.0
7.32	11.0	28.0	40.7	68.3	0.0
K91-12	117.0	96.3	77.3	114.3	0.0
K91-53	TNTC*	TNTC	TNTC	TNTC	50.0
M91-05	193.0	164.0	139.7	174.0	0.0
M91-09	TNTC	TNTC	TNTC	TNTC	0.0
S91-13	TNTC	TNTC	TNTC	TNTC	90.0
S91-58	TNTC	TNTC	TNTC	TNTC	TNTC

* TNTC = Too numerous to count (>300 colony-forming-units).

ception of higher counts on Five-M medium than Met medium at the 10^{-5} dilution, there were no significant differences in colony counts for SKDM, Met, Ten-M, and Five-M media.

Field trials were designed to evaluate the recovery of *R. salmoninarum* using SKDM and Five-M media for primary isolation of the microorganism from kidney tissue. Recovery rates on Five-M medium for both populations of chinook salmon were better than on SKDM. The percentage of *R. salmoninarum* positive samples on SKDM and Five-M were 80% (20 of 25) and 84% (21 of 25) for the Kewaunee fish, and 53% (24 of 45) and 89% (40 of 45) for the Manistee fish, respectively.

DISCUSSION

Based on the laboratory studies, metabolite added to the basal formulation (i.e., KDM2 minus serum) at concentrations greater than 1% supported the growth of *R. salmoninarum*. The optimal concentration for metabolite supplementation was 5%.

Both Ten-M and Five-M media supported growth of the bacterium at least as well as the two serum-based media, SKDM and Met, at all three dilutions tested. At very low cell counts, Ten-M and Five-M media supported growth, even in the absence of growth on SKDM for isolates 7.01, 7.06, 7.07, 7.30 (Table 3). Similar results occurred for 10^{-4} and 10^{-6} dilutions (data not shown). Based on these results, Ten-M and Five-M media not only supported the growth of the bacterium, but also enhanced the detection of low cell numbers, as has been described for Met (Teska, 1993).

Based on the data from the two field trials, prevalences of *R. salmoninarum*, as determined on Five-M medium, were greater than those determined using SKDM. Recovery rates using Five-M medium were 4% higher at Kewaunee, Wisconsin, and 36% higher at Manistee, Michigan. These results agree with data obtained from the laboratory studies, illustrating that Five-M medium is an effective substitute for the growth and study of pure cultures in the laboratory or for the confirmatory isolation of the bacterium from clinical specimens.

Metabolite-supplemented medium in the absence of serum or charcoal can support and enhance the growth of *R. salmoninarum*. The Five-M medium presented in this study provides an inexpensive alternative to serum or charcoal-based me-

TABLE 3. Range of colony sizes (10^{-5} dilution) obtained after 20 days incubation at 15 C for 13 *Renibacterium salmoninarum* isolates grown on selective kidney disease medium (SKDM), SKDM plus 1% metabolite (Met), SKDM minus serum plus 10% metabolite (Ten-M), SKDM minus serum plus 5% metabolite (Five-M), and SKDM minus serum plus 1% metabolite (One-M).

	Range of colony sizes[a] (mm) for 10^{-5} dilution				
Isolate	DKSM	Met	Ten-M	Five-M	One-M
7.01	NG	1.0–2.0	>2.0	>2.0	NG
7.05	0.25–0.5	>2.0	>2.0	>2.0	NG
7.06	NG	0.5–1.0	0.5–1.0	0.5–1.0	NG
7.07	NG	>2.0	>2.0	1.0–2.0	NG
7.08	0.25–0.5	>2.0	>2.0	>2.0	NG
7.30	NG	1.0–2.0	1.0–2.0	1.0–2.0	NG
7.32	0.25–0.5	1.0–2.0	>2.0	1.0–2.0	NG
K91-12	>2.0	>2.0	>2.0	>2.0	NG
K91-53	>2.0	>2.0	>2.0	>2.0	<0.25
M91-05	0.5–1.0	1.0–2.0	>2.0	>2.0	NG
M91-09	>2.0	>2.0	>2.0	>2.0	NG
S91-13	1.0–2.0	>2.0	>2.0	>2.0	0.25–0.5
S91-58	1.0–2.0	1.0–2.0	1.0–2.0	1.0–2.0	0.25–0.5

[a] NG = no growth.

dia. Metabolite can be prepared in advance and stored frozen at −70 C until needed. When added to the basal medium, a 5% supplement of metabolite will provide a medium with growth-supporting properties equal to or exceeding those of SKDM or KDM2. Five-M medium will also exhibit growth-enhancing properties at very low levels of cells not obtained with SKDM or KDM2.

A second advantage gained by the elimination of serum from *R. salmoninarum* growth medium is the reduction of interfering proteins in studies designed to evaluate the nature of the metabolite itself. When Met medium is used to prepare additional metabolite for use in studies designed to characterize the metabolite, the presence of serum can potentially interfere with extraction and purification methods. The use of Five-M medium eliminates potentially interfering proteins by eliminating the need for serum in broth used to prepare the metabolite. Future studies directed at characterizing the metabolite will incorporate the use of Five-M medium.

ACKNOWLEDGMENTS

I thank John Hnath (Michigan Department of Natural Resources) and Sue Marquenski (Wisconsin Department of Natural Resources) for assistance in arranging the field trials. Thanks to Cliff Starliper for assisting with sample collection during the field trials. Thanks also to Vicki Blazer, Rocco Cipriano, Roger Herman, and Bane Schill for their critical review of this manuscript.

LITERATURE CITED

AUSTIN, B., EMBLEY, T. M., AND M. GOODFELLOW. 1985. Selective isolation of *Renibacterium salmoninarum*. Federation of European Microbiological Societies (FEMS) Letters 17: 111–114.

BULLOCK, G. L., B. R. GRIFFIN, AND H. M. STUCKEY. 1980. Detection of *Corynebacterium salmoninus* by direct fluorescent antibody test. Canadian Journal of Fisheries and Aquatic Sciences 37: 719–721.

CIPRIANO, R. C., L. A. FORD, J. D. TESKA, AND L. E. HALE. 1992. Detection of *Aeromonas salmonicida* in the mucus of salmonid fishes. Journal of Aquatic Animal Health 4: 114–118.

DALY, J. G., AND R. M. W. STEVENSON. 1985. Charcoal agar, a new growth medium for the fish disease bacterium *Renibacterium salmoninarum*. Applied and Environmental Microbiology 50: 868–871.

EVELYN, T. P. T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bulletin de Office International Epizooties 87: 511–513.

KEULS, M. 1952. The use of the "studentized range" in connection with an analysis of variance. Euphytica 1: 112–122.

SHATKIN, A. J. 1969. Estimation of RNA, DNA, and

protein by the use of isotopic precursors followed by chemical fractionation. *In* Fundamental techniques in virology, K. Habel and N. P. Salzman (eds.). Academic Press, New York, New York, pp. 238–241.

TESKA, J. D. 1993. In-vitro growth enhancement of *Renibacterium salmoninarum*: The aetiology of bacterial kidney disease. Biomedical Letters 48: 27–33.

ZAR, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 620 pp.

Received for publication 23 November 1993.