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Source: Journal of Wildlife Diseases, 26(2) : 214-218

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-26.2.214

Journal of Wildlife Diseases, 26(2), 1990, pp. 214–218

A MEDIUM FOR THE SELECTIVE ISOLATION OF *EDWARDSIELLA ICTALURI*

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ABSTRACT: A selective medium, called *Edwardsiella ictaluri* medium (EIM), has been formulated for the isolation of *Edwardsiella ictaluri*. The medium inhibits the growth of most gram-negative bacteria, except *Proteus* sp., *Serratia marcescens* and some isolates of *Aeromonas hydrophila* and *Yersinia ruckeri*. The bacteria that grow on the EIM are easily differentiated from *E. ictaluri* based on colony morphology. The EIM inhibits gram-positive bacteria with the exception of enterococci. The addition of fungizone to EIM suppressed the growth of most fungi. The EIM allows the evaluation of environmental reservoirs, levels of contamination and carrier states of *E. ictaluri*.

Key words: *Edwardsiella ictaluri*, selective medium, fish disease, enteric septicemia of catfish, isolation, experimental study.

INTRODUCTION

Edwardsiella ictaluri is the major cause of mortality by bacteria in cultured channel catfish (*Ictalurus punctatus*). Recent studies have focused on the biochemical characteristics and potential virulence factors of *E. ictaluri* (Waltman et al., 1986b), the histopathology (Blazer et al., 1985) and the pathogenesis of the disease (Shotts et al., 1986), as well as possible vaccination and chemotherapy methods (Plumb et al., 1986; Waltman and Shotts, 1986b). The environmental reservoir of *E. ictaluri* is unknown, as is the level of *E. ictaluri* in the environment and the presence and site of carriage in the catfish or other animals. The main hindrance to these studies has been the absence of a selective medium for isolating *E. ictaluri*. This organism is relatively slow growing, and may be overgrown by normal flora found in the same environments. This study was undertaken to formulate a selective medium which could be used for studying the presence of *E. ictaluri* in the environment. The potential usefulness of this selective medium for the isolation of *E. tarda* also was evaluated.

MATERIALS AND METHODS

Bacteria

Over 100 isolates of *E. ictaluri* and *E. tarda* were collected and characterized biochemically (Waltman et al., 1986a, b) and antimicrobially (Waltman and Shotts, 1986a, b). Other gram-positive and gram-negative bacteria for media evaluations were availbale from laboratory stock collections and were identified by standard biochemical procedures (Krieg and Holt, 1984).

Media formulation

A medium (EIM) was formulated based on the biochemical and antimicrobial susceptibilities of *E. ictaluri*. The medium consisted of 10 g Bacto-tryptone (Difco Laboratories, Detroit, Michigan 48232, USA), 10 g yeast extract (Difco), 1.25 g phenylalanine (Fisher Scientific, Norcross, Georgia 30091, USA), 1.20 g ferric ammonium citrate (Fisher), 5 g sodium chloride (Fisher), 0.03 g brom thymol blue (Fisher), 17 g agar (Difco), and 990 ml distilled water. The components were dissolved, the pH was adjusted to 7.0 to 7.2, and the medium was autoclaved at 121 C for 15 min. A 10 ml solution containing 3.5 g mannitol (Difco) and 10 mg colistin (Sigma Chemical Co., St. Louis, Missouri 63178, USA) was filter-sterilized and added to the medium prior to pouring the plates. Other additives, evaluated for their effectiveness, included bile salts (Difco) (0.1% and 1.0%), crystal violet (Fisher) (10 μg/ml and 100 μg/ml), bacitracin (Sigma) (10 IU/ml), and fungizone (Sigma) (0.5 μg/ml).

Other media used included MacConkey (MAC) agar, trypticase soy agar (TSA), brain heart infusion (BHI) broth and thioglycollate (THIO) broth. These media were obtained commercially (Difco) and prepared as recommended by the manufacturer.

Media evaluations

The EIM was tested for its sensitivity in growing *E. ictaluri*. Gram-poitive and gram-negative

bacteria were grown overnight in BHI broth and then inoculated onto EIM. The plates were incubated at 30 C and growth was determined at 24 and 48 hours. If bacteria grew, the size, shape and color of the colony was recorded.

The sensitivity of EIM was determined relative to MAC and TSA. Isolates of *E. ictaluri* and *E. tarda* were grown in BHI broth overnight and diluted in sterile saline solution to give approximately 200 to 2,000 colonies/ml; 100 μl of this solution was added to each plating medium in triplicate. After incubation at 30 C for 24 hr, the bacteria were counted on each plate and the mean numbers of bacteria were determined for each plating medium. The differences in the number of bacteria growing on these three media were analyzed using an analysis of variance. Significant differences in the stastistics were located using a Tukey's test.

The ability of the media to differentiate *E. ictaluri* and *E. tarda* from other bacteria was determined by mixing two or more bacterial species and plating onto EIM. After incubation, the plates were observed for differences in colony morphology.

The usefulness of the plating media for environmental sampling was determined by culturing samples of catfish pond water and mud and also intestinal contents of catfish. These samples were 10-fold serially diluted in sterile saline solution and plated onto MAC, TSA, and EIM. After incubation at 30 C for 24 hr, the total numbers of bacteria were determined on each medium.

RESULTS

With the exception of *Proteus* sp., *Serratia marcescens*, some *Yersinia ruckeri* isolates, some *Aeromonas hydrophila* isolates, and some *Pseudomonas* sp. isolates, EIM effectively inhibited the growth of gram-negative bacteria (Table 1). All *E. ictaluri* grew on EIM, but only 90% of *E. tarda* were able to grow. This was expected since the percentage of *E. tarda* isolates susceptible to colistin had been found to be 10% (Waltman and Shotts, 1986a). The medium did not inhibit gram-positive bacteria. Therefore, crystal violet (10 μg/ml and 100 μg/ml) bile salts (0.1% and 1.0%) and bacitracin (10 IU/ml) were tested as additives. Crystal violet and bacitracin were not effective in selectively inhibiting the gram-positive bacteria. However, 0.1% bile salts was found to be effective (Table 1). At this concentration, bile salts inhibited all the gram-poitive bacteria tested exept for enterococci, and also inhibited many *Pseudomonas* sp. isolates without having a detrimental effect on *Edwardsiella* spp. isolates.

TABLE 1. The ability of selected gram positive and gram negative bacteria to grow on *Edwardsiella ictaluri* medium (EIM).

Bacterial species	No. strains tested	Survival (%) EIM	Survival (%) EIM with 0.1% bile salts
Bacillus sp.	5	100	0
Corynebacterium sp.	3	100	0
Listeria monocytogenes	1	100	0
Micrococcus sp.	3	100	0
Staphylococcus sp.	5	100	0
Streptococcus sp.	6	100	50
Pseudomonas sp.	20	100	0
Edwardsiella tarda	50	90	90
Edwardsiella ictaluri	50	100	100
Proteus (H_2S+)	2	100	100
Proteus (H_2S-)	3	100	100
Serratia marcescens	3	100	100
Yersinia ruckeri	15	33	33
Aeromonas hydrophila	20	75	75
Escherichia coli	5	0	NA*
Enterobacter sp.	3	0	NA
Klebsiella sp.	5	0	NA
Citrobacter sp.	6	0	NA
Salmonella sp.	3	0	NA
Arizona sp.	1	0	NA
Shigella sp.	2	0	NA
Plesiomonas shigelloides	5	0	NA
Pseudomonas aeruginosa	3	0	NA
Pseudomonas putida	1	0	NA
Pseudomonas putrefaciens	2	0	NA
Acinetobacter sp.	1	0	NA
Alcaligenes sp.	1	0	NA
Bordetella bronchiseptica	1	0	NA

* Not applicable since no growth was obtained on EIM alone.

Since EIM might be important for environmental samples such as pond water and mud, we investigated the possibility of adding fungizone to EIM in order to inhibit the growth of fungi. The addition of 0.5 μg/ml of fungizone to the medium did not affect the growth of *Edwardsiella* spp. isolates. Furthermore, in comparison studies with MAC and TSA, it greatly inhibited

TABLE 2. Comparison of the sensitivity of MacConkey (MAC), Trypticase Soy Agar (TSA) and *Edwardsiella ictaluri* medium (EIM) for growing *Edwardsiella ictaluri* and *E. tarda*.

Bacterial strain	Number of colonies[a]		
	MAC	TSA	EIM
Edwardsiella ictaluri			
79-21	246.0	242.0	240.7
19	96.7	150.0	150.3
84-225	219.3	204.0	187.3
884	191.3	230.7	171.3
915	30.7	38.3	32.7
916	ND[b]	38.0	37.0
Edwardsiella tarda			
21	28.0	43.0	40.3
23	17.3	18.0	20.0
75	94.3	112.7	115.3
87	123.7	105.0	118.3
88	41.0	39.7	33.3

[a] Mean of triplicate plates.
[b] Not determined.

TABLE 3. A comparison of the total number of bacteria growing on MacConkey (MAC), Trypticase Soy Agar (TSA), and *Edwardsiella ictaluri* medium (EIM) from environmental samples.

Sample	Total bacterial counts (CFU/ml)		
	MAC	TSA	EIM
Pond water A	6×10^2	1×10^4	6×10^1
Pond water B	2×10^3	2×10^4	6×10^1
Pond mud	7×10^5	3×10^7	8×10^4
Fish intestine A	8×10^6	3×10^6	$<1 \times 10^3$
Fish intestine B	6×10^7	3×10^7	$<1 \times 10^3$

the growth of fungi associated with pond water and mud, allowing the recovery of *Edwardsiella* spp.

The EIM was formulated so that species of bacteria growing on it could be differentiated from *Edwardsiella* spp. isolates based on colony morphology. Both *E. ictaluri* and *E. tarda* produced 0.5 to 1.0 mm green translucent colonies after 48 hr. *Proteus* sp. produced 2 to 3 mm brownish-green colonies that might swarm. *Serratia marcescens* produced 2 to 3 mm reddish colonies; *A. hydrophila* produced 2 to 5 mm yellowish-green opaque colonies; and *Y. ruckeri* produced 1 to 2 mm yellowish-green colonies. Enterococci capable of growing on EIM produced tiny (approx. 0.5 mm) yellowish colonies.

The relative ability of EIM to support the growth of *Edwardsiella* spp. isolates was assessed relative to MAC and TSA (Table 2). There were no significant differences (at a 95% confidence level) in the growth capabilities of EIM.

Mixed cultures of *Edwardsiella* spp., and isolates of *Proteus* sp., *A. hydrophila* and *Y. ruckeri* were prepared. These were plated onto MAC, TSA, and EIM. *Edwardsiella* spp. isolates could be easily differentiated on EIM based on colony morphology. Swarming *Proteus* sp. isolates overgrew the plate, resulting in reduced recovery, but this also occurred with MAC and TSA.

Samples of pond water, mud and intestinal contents of catfish were quantitatively plated onto MAC, TSA and EIM for comparing the total number of bacteria present. On EIM, the total numbers of bacteria were significantly reduced (Table 3). There was a 10 to 100,000-fold decrease in bacterial numbers relative to MAC and a 100 to 100,000-fold decrease in bacterial numbers relative to TSA. The same samples also were inoculated with *E. ictaluri* and *E. tarda* and plated. The recovery of these organisms was greater on EIM than on MAC or TSA.

DISCUSSION

The EIM takes advantage of the unusual resistance of *Edwardsiella* spp. to colistin relative to other gram-negative bacteria. The gram-negative bacteria that are capable of growing on EIM (namely *Proteus* sp., *S. marcescens*, some *A. hydrophila* and some *Y. ruckeri* isolates) may be differentiated by reactivity with the other EIM components. Phenylalanine and ferric ammonium citrate were added because *Proteus* sp. have the enzyme phenylalanine deaminase, which results in a brownish-green coloration of their colonies. Mannitol and the pH indicator brom thymol blue were added to discriminate *A. hy-*

drophila and *Y. ruckeri* isolates from *Edwardsiella* spp. isolates; the former ferment mannitol, producing acid which results in yellow colonies. Both *A. hydrophila* and *Y. ruckeri* are proteolytic. Thus, after 24 hr incubation their colonies begin to turn greenish due to proteolytic degradation and an increase in pH. However, even after prolonged incubation and the reversion of their colonies to green, there is no difficulty in distinguishing these colonies from *Edwardsiella* spp. colonies because the former are opaque and much larger. *Serratia marcescens* colonies were typically reddish because these bacteria produce a red pigment when incubated at temperatures below 37 C.

Gram-positive bacteria were not inhibited by colistin. However, bile salts proved to be an effective inhibitor of all gram-positive bacteria tested except enterococci. Enterococci could be differentiated since they appear as tiny yellow colonies. An additional benefit of using bile salts was the inhibition of several colistin-resistant *Pseudomonas* spp. commonly isolated from environmental samples. The incorporation of bile salts into the EIM had no detrimental effect on *Edwardsiella* spp., therefore they were added to the medium formulation.

A potential use of the selective medium would be the isolation of *E. ictaluri* from environmental samples such as pond water or mud. Typically, these sites have been difficult to culture for *E. ictaluri* due to overgrowth by other microorganisms, especially *Bacillus* spp. and fungi. The EIM successfully inhibits most other environmental bacteria, including *Bacillus* spp. The addition of fungizone did not detrimentally affect the growth of *Edwardsiella* spp. isolates but effectively inhibited the growth of most fungi, especially when compared to parallel cultures on MAC and TSA.

Suspect *Edwardsiella* spp. colonies growing on EIM must be tested further to differentiate *E. ictaluri* from *E. tarda* or possibly some *Pseudomonas* sp. A rapid method would be to pick colonies to triple sugar iron agar (TSI). After incubation *E. ictaluri* produces an alkaline over acid reaction (K/A) with no H_2S and little gas; *E. tarda* produces an alkaline over acid reaction with H_2S and gas; *Pseudomonas* sp. produces a neutral or alkaline reaction in both the slant and butt. Further biochemical characterization may be done as described in Waltman et al. (1986b).

The sensitivity of EIM, or the ability to recover *Edwardsiella* spp. isolates, was compared to that of MAC and TSA, two conventional media currently in use. The EIM grew *Edwardsiella* spp. isolates as well or better than either MAC or TSA, indicating that the selective agents in EIM did not suppress the growth of *Edwardsiella* spp. isolates relative to MAC or TSA.

Results with mixed cultures of *Edwardsiella* spp. isolates and other bacteria, including *Proteus* sp., *S. marcescens, A. hydrophila* and *Y. ruckeri* were as we had expected from observing the colonial growth of each of these bacteria in pure culture. *Edwardsiella* spp. colonies were easily distinguished based on colony morphology. One potential problem that may be encountered is the presence of swarming *Proteus* spp., which may overgrow the plate, but this is also a problem with MAC and TSA. To minimize this difficulty, the plates should be dry prior to inoculation and checked for growth after 16 to 20 hr. Fortunately, swarming *Proteus* spp. are not very common in the water environment or in the catfish intestines.

A liquid version of EIM was prepared by omitting the agar. This was tested for use as an enrichment medium. It was found to be effective in recovering as few as two *Edwardsiella* spp./ml in 24 hr, whereas the same number of bacteria failed to grow in THIO even after 48 hr. A problem with this broth may be the presence of *Proteus* spp., since this organism is not inhibited and when plated will overgrow *E. ictaluri*. Therefore, the use of EIM as an enrichment broth should be attempted with caution.

Samples of pond water, pond mud and catfish intestinal contents were cultured for total bacterial counts on EIM, MAC and TSA. With EIM, the total bacterial counts were much less, especially for intestinal contents, indicating the selective power of EIM and its potential for recovery of *E. ictaluri* in such samples.

The EIM was found also to have potential for isolating *E. tarda* from environmental sites. However, EIM may be less useful for this purpose, since several studies have already characterized various environmental reservoirs and levels of *E. tarda* (White et al., 1973; Wyatt et al., 1979; Minnagawa et al., 1983).

The results of this study indicate the potential usefulness of the EIM in determination of environmetnal reservoirs of *E. ictaluri* and in diagnostic isolation of this bacterium. The medium allows a qualitative evaluation of the presence of *E. ictaluri* and also may provide a quantitative assessment. The EIM also will facilitate the study of carriage of *E. ictaluri* in catfish or other animals.

ACKNOWLEDGMENTS

This study was supported in part by USDA grant 10-21-RR211-048. We thank Ta Chaun Hsu for his encouragement and assistance with the technical aspects of this paper. We also thank John Brown for his stastistical analysis of the data.

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Received for publication 17 November 1988.