CHAPTER 7. ISOLATION AND IDENTIFICATION OF *AEROMONAS* SPECIES FROM MEAT AND POULTRY PRODUCTS

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7.1 Introduction

Members of the genus *Aeromonas* typically are aquatic bacteria and sometime pathogens of fish and cold-blooded vertebrates that inhabit wet environments. Nevertheless, aeromonads are isolated (often in considerable numbers) from various foods of animal origin. These include seafood, raw milk, beef, pork, lamb, and poultry. They grow readily at refrigeration temperatures. Production of enterotoxins can be demonstrated using various laboratory assays, and indirect epidemiological evidence suggests that members of the genus *Aeromonas* have been involved in sporadic human gastroenteritis outbreaks involving seafood. However, no fully confirmed foodborne outbreak has been described in the scientific literature.

The method presented describes procedures for isolation and identification of species of the *Aeromonas hydrophila* group which consists of *A. hydrophila*, *A. sobria* and *A. caviae*. A procedure for detection of hemolysin(s) is also provided. Burke et al., 1983, reported a 97% correlation between hemolysin production and enterotoxin production among *Aeromonas* species.

7.2 Equipment, Reagents and Media

7.21 Equipment

(isolation/identification)

a. Incubator, static 28 ± 1°C
b. Osterizer-type blender with sterilized cutting assemblies and adapters for use with Mason jars, or Stomacher™ (Tekmar) with sterile Stomacher™ bags
c. Sterile bent glass rods ("hockey sticks")

(hemolysin test)

d. Incubator, static 37°C
e. Microtiter plate reader equipped to read at 540 nm
f. Centrifuge capable of 12,000 RPM

g. Shaker incubator (30°C; 210 RPM)
h. Screw-cap Erlenmeyer flasks, 125 ml
i. Sterile screw-cap centrifuge tubes: 15 ml conical and 50 ml round bottom
j. 96-well microtiter plates
k. Membrane filters, 0.2 µm
l. Bench top clinical centrifuge

7.22 Reagents

(isolation/identification)

a. Butterfield's phosphate diluent (BPD)
b. Mineral oil, sterile
c. N,N-dimethyl-p-phenylenediamine monohydrochloride (1% aqueous solution)

(hemolysin test)

d. Rabbit blood, defibrinated
e. Phosphate buffered saline (PBS)
f. Distilled water, sterile

7.23 Media

(isolation/identification)

a. Tryptic soy broth plus 10 µg/ml ampicillin (TSBA)
b. Starch-ampicillin (SA) agar
c. Triple sugar iron (TSI) agar
d. Nutrient agar
e. Mannitol fermentation broth with Andrade's indicator
f. Arginine decarboxylase broth (Moeller)
g. Ornithine decarboxylase broth (Moeller)
h. Decarboxylase broth base (Moeller)
i. Glucose fermentation broth with Andrade's indicator
j. Bile esculin agar

(hemolysin test)

k. Brain heart infusion (BHI) broth

7.3 Isolation Procedure

Serial dilutions of meat samples may be surface-spread directly on SA agar. However better recovery of *Aeromonas* will be achieved by

using enrichment procedures, particularly when the aeromonads have been freeze-injured or are low in number.

a. Blend 25 g of meat in 225 ml TSBA with a blender or Stomacher™ for 2 minutes. Incubate at 28°C for 18 to 24 h.

b. After incubation prepare serial dilutions of the enrichment cultures in BPD. Transfer 0.1 ml of the 10^{-4} to 10^{-6} dilutions onto the surface of SA plates. Evenly spread the inoculum with sterile bent glass rods. The plates must be free of surface moisture if single colonies are to be obtained. Incubate the plates at 28°C for 18 to 24 h.

c. Pick three typical colonies per sample from the SA agar plates to TSI agar and nutrient agar slants. Incubate overnight at 28°C. *Aeromonas* colonies are typically 3 to 5 mm in diameter and appear yellow to honey-colored on SA agar.

7.4 Identification

a. Read the TSI reactions. *Aeromonas* reactions on TSI are as follows: acid butt, acid or alkaline slant, H_2S negative, positive or negative gas production.

b. Perform the oxidase test on the nutrient agar slants. Add a few drops of a N,N-dimethyl-p-phenylenediamine monohydrochloride solution (prepared fresh daily) to the growth on the nutrient agar slant. Oxidase positive cultures develop a pink color which successively becomes maroon, dark red, and black in 10 to 30 min. All aeromonads are oxidase-positive and fermentative.

c. Transfer all oxidase-positive fermenters from the TSI agar slants to the following media for biochemical confirmation: mannitol fermentation broth, arginine decarboxylase broth, ornithine decarboxylase broth, glucose fermentation broth, and bile esculin agar. After inoculation, layer the decarboxylase media with sterile mineral oil and incubate at 28°C for 48 h. Incubate the remainder of the confirmation media at 28°C for 24 h.

d. Record the biochemical characteristics of each isolate. All aeromonads produce acid from mannitol and are arginine positive, ornithine negative. Species of the *A. hydrophila* group can be differentiated according to the biochemical characteristics shown below:

Test (Substrate)	*A. hydrophila*	*A. sobria*	*A. caviae*
Gas from Glucose	+	+	-
Esculin hydrolysis	+	-	+

NOTE: Esculin hydrolysis imparts a dark brown color to the medium.

e. Transfer isolates of suspected *Aeromonas* that are to be tested for hemolysin production from TSI agar to nutrient agar slants and incubate overnight at 28°C.

7.5 Hemolysin Test

The hemolysin test described below is based on that of Burke et al., 1983 and 1984.

7.51 Preparation of Culture Filtrate

a. Transfer growth from the nutrient agar slant to BHI broth (25 ml broth in a 125 ml Erlenmeyer flask). Incubate overnight at 30°C on a shaker incubator at 210 RPM.

b. Centrifuge the broth culture at 11,950 RPM (SS-34 Dupont-Sorvall rotor) for 30 minutes. Decant and save the supernatant liquid; discard the cell pellet.

c. Filter sterilize the supernatant through a sterile disposable membrane filter (0.2 µm).

d. Hold the sterile culture filtrate at 4°C until needed, and test it for hemolysin activity within 24 h of preparation.

7.52 Preparation of Rabbit Erythrocyte Suspensions

a. Centrifuge 10 ml of defibrinated rabbit blood in a 15-ml conical centrifuge tube at 2400 RPM in a bench top clinical centrifuge for 5 minutes.

b. Remove the supernatant and white blood cell layer by suction and discard.

c. Add 10 ml of cold PBS to the packed erythrocytes, mix gently, and centrifuge as described above. Discard supernatant.

d. Wash the erythrocytes in PBS two more times, as described above.

e. After the final wash, note the volume of packed erythrocytes in the centrifuge tube. Prepare a 10% and a 1% erythrocyte suspension in PBS. Hold the two suspensions at 4°C until needed (use within 24 h).

7.53 Preparation of Hemoglobin Standard Curve

a. Transfer 1 ml of the 10% erythrocyte suspension into 8 ml of sterile distilled water. Shake the mixture until all cells are lysed. Add 1 ml of 10X PBS to obtain a 1% hemoglobin solution.

b. Add 1% hemoglobin solution and 1% erythrocyte suspension to conical centrifuge tubes in the following volumes:

Volume (ml)	% hemoglobin										
	0	10	20	30	40	50	60	70	80	90	100
Hemoglo -bin	0	.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0
Erythro -cytes	1.0	.9	.8	.7	.6	.5	.4	.3	.2	.1	0

c. Centrifuge tubes at 2400 RPM for 5 minutes in a clinical centrifuge. Transfer 0.5 ml of supernatant from each tube into wells of a 96-well microtiter plate. Hold the plate for the hemolysin test (Section 7.54).

7.54 Hemolysin Test

a. Add 1 ml of sterile culture filtrate (Section 7.51) to 1 ml of the 1% erythrocyte suspension (Section 7.52) in a conical centrifuge tube and mix gently.

b. Incubate at 37°C for 1 h, then incubate for an additional 1 h at 4-5°C.

c. Centrifuge at 2400 RPM for five minutes.

d. Transfer 0.5 ml of supernatant to the 96-well plate containing the standards (Section 7.53).

e. Read the plate on a microtiter plate reader at 540 nm.

f. A positive hemolysin test is defined as the production of an O.D. reading $\geq$ the O.D. of the 20% hemoglobin standard in the standard curve prepared above in Section 7.53.

7.6 Selected References

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