A Rapid Microbiological Method for Enumeration of Pseudomonas fluorescens from Broiler Chicken Carcasses

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ABSTRACT

A method to selectively enumerate Pseudomonas fluorescens from fresh chicken carcasses in less than 24 h using capacitance microbiology was developed. Capacitance assays were conducted on whole-carcass rinses at 25°C using brain heart infusion broth (BHI) containing 25 μg of Irgasan per ml to obtain a detection time. The capacitance samples were spread plated on plate count agar for isolation and identification. From plates with the highest dilution, from each carcass, 4 colonies were randomly selected and identified. Seven species of bacteria including Pseudomonas fluorescens were responsible for capacitance detection times. Various antibiotics and chemicals were added to basal media or brain heart infusion broth with Irgasan and were evaluated to select for the growth of P. fluorescens. BHI broth containing 4 μg of nitrofurantoin, 120 μg of carbenicillin, and 25 μg of Irgasan, all per ml, was found to be optimal and was termed Pseudomonas fluorescens selective additive (PSA) (patent pending). In a second study, 12 carcasses were collected in each of three replicate trials. For each trial, 2 carcasses were sampled immediately and 2 were sampled after storage at 3°C on days 3, 6, 9, 12, and 15. The BHI-PSA broth was found to be excellent for enumeration of P. fluorescens from broiler chicken carcass rinses in assays using capacitance microbiology at 25°C. The time required to enumerate P. fluorescens for all samples (day 0 to 15) was <22.4 h. This method is rapid and would be a useful tool for determining the number of spoilage bacteria on fresh chicken and thus may possibly be used to predict the potential shelf life of fresh chicken and other foods of animal origin.

Key words: Capacitance, shelf-life prediction, Pseudomonas fluorescens, chicken carcasses

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their initial number may vary greatly depending on processing parameters. Hence, enumeration of psychrotrophic bacteria such as Pseudomonas fluorescens on fresh broilers may be used to indicate the effect of processing on bacteria that contribute to spoilage. Halleck et al. (13) reported that immediately after processing, a common spoiler of fresh chicken such as P. fluorescens was found but seldom exceeded 5% of the bacterial population on fresh meats. Barnes and Thornley (3) observed that Pseudomonas spp. made up 2% of the initial flora of broiler chicken carcasses.

Another factor that affects spoilage rate is the holding temperature of carcasses during storage, transport, and retail display. Following processing, throughout distribution and marketing, opportunities exist for fresh broiler carcasses to be exposed to temperature mishandling (21). Fresh chicken that does not meet or exceed its expected potential shelf life may be assumed to have been exposed to temperature abuse. Hence, a potential shelf-life prediction method may also be useful for identifying cases of temperature abuse.

Psychrotrophic plate counts have been used as a general indicator of the potential shelf life of fresh chicken, but this method requires an incubation time of 10 days at 7°C (5). Because most fresh chicken is purchased and consumed within 7 days, this method would not be suitable for predicting potential shelf life.

Bishop et al. (4) reported high correlation coefficients (R² = 0.87 and 0.88) between the shelf life of milk and impedance readings taken at 18 and 21°C, respectively. Ogden (18) observed that conductance readings on fish samples diluted in brain heart infusion broth and incubated at 20°C correlated well (R² = 0.92 to 0.97) to H₂S-producing bacterial counts. However, according to Firstenberg-Eden and Tricarico (9), mesophilic bacterial populations, not Pseudomonas, would be enumerated using electrical measurements on samples of fresh chicken unless a selective medium is used at incubation temperatures of 18 to 21°C.

Ideally, to predict the potential shelf life of fresh broiler chicken, the bacteria responsible for producing spoilage defects should be enumerated. Halleck et al. (13) reported that, in the latter part of storage, Pseudomonas fluorescens constituted approximately 80% of the bacterial species on meat. Russell et al. (22) reported that bacteria isolated from spoiled broiler chicken carcasses that produced sulfur off-
odors in chicken skin medium were *Shewanella putrefaciens* A, B, and D, and the pseudomonads (*Pseudomonas fluorescens* A, B, and D, *P. fragi*, and *P. putida*). Others have implicated *P. fluorescens* as an important spoiler of milk (10, 11, 14, 16, 19, 25). Therefore, any potential shelf-life prediction method should involve selective enumeration of the species of *Pseudomonas*, namely *P. fluorescens*, that produces objectionable spoilage defects in many types of fresh foods of animal origin.

An accurate method for determining the potential shelf life of fresh chicken would be useful for determining (i) the potential shelf life under ideal holding conditions, (ii) the effect of processing on bacteria that contribute to spoilage, and (iii) exposure of the product to temperature abuse. The objectives of this study were to develop a medium to selectively enumerate *Pseudomonas fluorescens* using capacitance microbiology and to develop an assay to enumerate this spoilage bacterium in less than 24 h.

**MATERIALS AND METHODS**

**Study 1**

*Sample collection and preparation.* Ten whole ready-to-cook broiler chicken carcasses were obtained from the chiller exit of a commercial processing plant. The carcasses were placed on ice, transported to the laboratory, individually bagged in sterile polyethylene bags (permeability 3,000 cm$^3$ of O$_2$ at 22.8°C/m$^2$/24 h at 1 atm [101.29 kPa]) within 1 h of collection, and maintained at 3°C. The carcasses were sampled on the day of collection by rinsing in 100 ml of sterile deionized water according to the procedure described by Cox et al. (6).

*Brain heart infusion broth with Irgasan.* A 2.5% (wt/vol) stock solution of 2, 4, 4'-trichloro-2'-hydroxydiphenyl ether (Irgasan, DP300, CAS# 33-80345; Ciba-Geigy, Greensboro, N.C.) was made in absolute ethanol. To 100 ml of brain heart infusion broth (BHI), (Difco Laboratories, Detroit, MI), 0.1 ml of Irgasan stock solution was added to achieve a final concentration of 25 μg of Irgasan per ml (BHI-I).

One milliliter of the carcass rinse fluid was added to 9 ml of BHI-I. Samples (1 ml) were assayed in duplicate in Bactometer module wells. Capacitance assays were conducted at 25°C using test code 6 on the Bactometer Microbial Monitoring System M128 (bioMérieux Vitek, Inc., Hazelwood, MO). As soon as capacitance changes produced by metabolically active bacteria. Three replicate tubes of each medium combination were inoculated with each bacterial species isolated with BHI-I. These tubes were incubated at 25°C for 48 h. Visible turbidity was used to determine microbial growth. If turbidity was observed, the sample was considered positive for growth and if no turbidity was observed, the sample was considered negative for growth. Three replicate trials were conducted.

**C. General purpose medium plus (GPMP, bioMérieux Vitek, Inc.)** was also investigated as a basal medium with the addition of various antibiotics and chemicals. GPMP is commonly used for conducting impedance determinations because it enhances electrical changes produced by metabolically active bacteria. Three replicate tubes of each medium combination were inoculated with each bacterial species isolated with BHI-I.

*Additives used to select for the growth of Pseudomonas fluorescens.* Combinations of BHI, antibiotics, and chemicals were used at the following concentrations, all per ml: 10 to 100 μg of ampicillin (Sigma), 200 to 600 μg of carbenicillin (Sigma), 200 to 1,000 μg of cephalothin (Sigma), 10 to 30 μg of chloramphenicol (Sigma), 5 to 25 μg of Irgasan (Ciba-Geigy), 1 to 150 μg of nitrofurantoin (Sigma), 10 to 15 mg of NaCl (J. T. Baker), and 1 to 100 mg of TMAO (Aldrich).

For all media preparations, brain heart infusion broth was mixed with deionized water, sterilized, and tempered to 25°C. Stock solutions of ampicillin, carbenicillin, cephalothin, and nitrofurantoin were prepared in deionized water to achieve final concentrations of 50,000, 40,000, 70,000, and 100 μg/ml, respectively. Stock solutions of chloramphenicol and Irgasan were dissolved in 100% ethanol to achieve final concentrations of 10,000 and 25,000 μg/ml, respectively. A stock solution of TMAO was prepared in deionized water to achieve a final concentration of 100,000 μg/ml. All stock solutions were filter sterilized using a 0.2-μm-pore-size Supor® Acrodisc® 25 syringe filter (Gelman Sciences, Ann Arbor, MI). The NaCl was added directly to BHI before sterilization.

To determine the ability of the bacteria isolated to proliferate in BHI-I containing the various basal media, antibiotics, and chemicals, one loopful (10 μl) of a 24-h-old actively growing culture of each isolate was placed in triplicate test tubes containing 5 ml of BHI-I or GPMP. Tubes were incubated at 25°C for 48 h. After incubation, the tubes were placed into a colorimeter and the percent transmittance was determined and reported as (+), bacteria produced visible turbidity in medium and (−), bacteria produced no visible turbidity, to take into account differences in baseline transmittance. All trials were replicated at least three times. Enumeration of *P. fluorescens* using capacitance was determined for those media which showed selectivity.
**Study 2**

Sample collection and preparation. Twelve whole ready-to-cook broiler chicken carcasses were obtained from the chiller exit of a commercial broiler-processing plant and prepared as described above. Two carcasses were sampled on the day of collection. The remaining 10 carcasses were refrigerated at 3°C and 2 carcasses each were sampled at 3, 6, 9, 12, and 15 days. Carcasses were rinsed in 100 ml of sterile deionized water according to the procedure described by Cox et al. (6). Three replicate trials were conducted.

The combination of ingredients that were added to BHI and used to enumerate *P. fluorescens* was named *Pseudomonas fluorescens* selective additive (PSA) (patent pending) and contained the following ingredients per ml of sterile deionized water: 4 µg of nitrofurantoin, 120 µg of carbenicillin, and 25 µg of Irgasan.

Diluent (1 ml) from each carcass was added to 9 ml of brain heart infusion-*Pseudomonas fluorescens* selective additive (BHI-PSA). Samples (1 ml) were assayed in duplicate in Bactometer module wells. Capacitance assays were conducted at 25°C using test code 6 on the Bactometer Microbial Monitoring System M128. As soon as capacitance detection times were recorded, 1 ml of the sample was immediately taken from the module well, placed into 9 ml of PW, diluted to 10^-6, 10^-7, or 10^-8, spread (0.1 ml) onto duplicate plate count agar plates, and incubated at 25°C for 48 h according to the procedure described by Russell et al. (23). Plates at the highest dilution with 4 or more colonies per plate were used for selection of isolates for identification. Four colonies per carcass were randomly selected, restreaked for isolation and purity on PCA, and incubated at 25°C for 48 h.

Colonies isolated for identification were characterized for Gram reaction, cytochrome oxidase activity, and ability to produce the enzyme catalase. Bacteria were subsequently identified using the Nonfermentor card with the Vitek AutoMicrobic System and API NFT strips.

**RESULTS AND DISCUSSION**

**Study 1**

Irgasan was added to BHI because Furia and Schenkel (12) and Power and McCuen (20) demonstrated that it selects for the growth of *Pseudomonas* spp. while suppressing the growth of other bacteria. Irgasan, at a concentration of 25 µg/ml, is also used as the selective agent in pseudomonas isolation agar, which is used for *Pseudomonas* spp. plate counts.

The bacterial species and number of isolates that reached the threshold level of 10^6 cells per ml in BHI-I at the time of detection were as follows: *Pseudomonas fluorescens*, 14; *Pseudomonas putida*, 4; *Aeromonas sobria*, 2; *Aeromonas salmonicida salmonicida*, 4; *Aeromonas hydrophila caviae*, 2; *Vibrio alginolyticus*, 2; and *Serratia liquefaciens*, 2. Six of the 36 bacterial isolates did not survive to be identified.

*Pseudomonas fluorescens* is one of the three most often isolated spoilage bacteria associated with fresh poultry (22, 24). Viehweg et al. (24) reported that the pseudomonads and *Shewanella* produced more sulfur compounds than other spoilage bacteria. These sulfur compounds were able to mask other less odoriferous volatile compounds in subjective odor evaluations. These authors also reported that *P. fluorescens* and *P. putida* made up 50% of the spoilage flora by the 8th day of storage and remained detectable at all stages of spoilage.

*Pseudomonas fluorescens* was one of the seven species selectively enumerated using BHI-I. Therefore, three other basal media and BHI-I containing various antimicrobial compounds and chemicals were analyzed to determine which combination could be used to suppress the growth of other bacteria and allow only *Pseudomonas fluorescens* to reach the threshold level.

Cephaloridine–fucidin–cetrimide (CFC) medium was developed by Mead and Adams (17) to rapidly isolate pigmented and nonpigmented pseudomonads. These authors reported that CFC medium supported the growth of *Pseudomonas aeruginosa*, which is not involved in poultry spoilage. Forty-one percent of the bacteria isolated from swabs taken from poultry carcasses using CFC medium were pseudomonads, including *Acinetobacter/Moraxella sp.*, 3%; species of *Enterobacteriaceae*, 8%; *Alcaligenes* sp., 2%; *Flavobacterium* sp., 8%; gram-positive, unclassified, 4%; and gram-negative, unclassified, 16% (17). Because interfering species of several genera were able to grow on CFC agar, it was not investigated in this study as a possible medium for rapidly enumerating *Pseudomonas fluorescens*.

The maleate medium for *Pseudomonas fluorescens* without agar added described by Atlas (1) was analyzed to determine its suitability for use in capacitance assays. Pure cultures of *P. fluorescens* were not able to multiply in this medium; no detection times could be recorded (data not shown). When broiler carcass rinses were analyzed with this medium, the detection times were erratic, so that we could not interpret the capacitance curves consistently. Maleate medium was considered unsuitable for conducting capacitance assays for *P. fluorescens*.

PIM-AGAR was no more selective than BHI-I for the growth of *P. fluorescens*. All competing flora isolated with BHI-I grew. PIM-AGAR would not be suitable for enumerating *P. fluorescens* using capacitance.

Ampicillin, carbenicillin, cephalothin, chloramphenicol, and nitrofurantoin were chosen as inhibitors to select for the growth of *P. fluorescens* because Lorian (15) reported that *P. fluorescens* cells were resistant to these compounds and six of the other species of bacteria isolated with BHI-I were susceptible to them. NaCl and TMAO were used because previous research by Easter et al. (7) indicated that these compounds were excellent for enhancing impedance readings in samples containing spoilage bacteria such as the pseudomonads.

All the bacterial isolates used in the growth studies to test the combination of components were isolated by using BHI-I and are listed in Table 1. The growth was evaluated of bacteria in BHI with (per ml) 0 to 25 µg of Irgasan, 10 to 30 µg of chloramphenicol, 700 µg of cephalothin, 400 µg of carbenicillin, 15 mg of NaCl, 1 mg of TMAO, and in TMAO medium. None of the combinations of components sufficiently selected for the growth of *P. fluorescens* while suppressing the growth of the other six species isolated using BHI-I.

The growth was studied of bacteria in BHI with (per ml) 10 to 100 µg of ampicillin, 100 to 400 µg of cephalothin, 100
to 300 μg of carbenicillin, 10 mg of NaCl, 1 mg of TMAO. Although *Pseudomonas fluorescens* grew well, *A. sobria*, *A. salmonicida salmonicida*, *A. hydrophila caviae*, *V. alginolyticus*, and *S. liquefaciens* were also able to grow. None of these media were appropriate for selecting for *P. fluorescens*.

The growth was tested of bacteria in BHI with (per ml) 500 to 1,000 μg of cephalothin, 200 to 600 μg of carbenicillin, 10 mg of NaCl, and 1 mg of TMAO. Although *P. fluorescens* grew well in three of the media tested, *Aeromonas sobria* was able to produce higher turbidity than *P. fluorescens* in those media. These media would not be suitable for enumerating *P. fluorescens*.

The growth was studied of bacteria in general purpose medium plus (GPMP) with (per ml) 500 to 1,000 μg of cephalothin, 200 to 600 μg of carbenicillin, 10 mg of NaCl, and 1 mg of TMAO. None of the media tested supported the growth of *P. fluorescens* enough to be used in a capacitance assay.

The growth was evaluated of bacteria in BHI with (per ml) 1 to 20 μg of nitrofurantoin, 20 to 300 μg of carbenicillin, 5 to 20 μg of Irgasan, and 1 mg of TMAO. Seven of the eight media analyzed using these ingredients allowed *P. fluorescens* to multiply while suppressing the growth of the other bacterial isolates. BHI with 25 μg of nitrofurantoin, 80 μg of carbenicillin, 25 μg of Irgasan, and 1 mg of TMAO per ml of medium was most selective. The tubes containing this medium and *P. fluorescens* allowed a maximum of 20% less light transmittance than cultures of the other bacterial isolates analyzed.

The growth was studied of bacteria in BHI with (per ml) 1 to 20 μg of nitrofurantoin, 20 to 300 μg of carbenicillin, 5 to 20 μg of Irgasan, and 1 mg of TMAO. BHI containing 3 μg of nitrofurantoin, 80 μg of carbenicillin, and 5 μg of Irgasan per ml of medium was most selective. The tubes containing this medium and *P. fluorescens* allowed a maximum of 24% less light transmittance than cultures of the other bacterial isolates analyzed. This medium did not contain TMAO. Hence, TMAO was not included in the medium to be used for capacitance assays. Although this medium was excellent for selecting for the growth of *P. fluorescens* while suppressing the growth of the other isolates analyzed, the amount of Irgasan in the medium was probably not sufficient to suppress the growth of other gram-negative bacteria commonly associated with broiler chicken rinses. Therefore, a similar medium containing the amount of Irgasan that was used to obtain the seven isolates recovered in BHI-I was investigated. Also, the nitrofurantoin and carbenicillin concentrations were increased to ensure the ability of the medium to control competing flora.

The growth was tested of bacteria in BHI broth with 4 μg of nitrofurantoin, 25 μg of Irgasan, and decreasing concentrations, 25 to 100 μg of carbenicillin, all per ml. As carbenicillin concentration was reduced to 100, 75, 50, and 25 μg/ml, of the spoilage bacteria tested, only *Aeromonas sobria* was able to proliferate at levels below 120 μg/ml. Therefore, reduction of carbenicillin below 120 μg/ml was not considered acceptable for selecting for the multiplication of *P. fluorescens*.

The growth of bacteria in BHI with (per ml of medium) 4 μg of nitrofurantoin, 120 μg of carbenicillin, and 25 μg of Irgasan, (patent pending) is presented in Table 1. This medium was considered ideal because it contained high levels of antibiotic to inhibit other species while allowing for rapid proliferation of *P. fluorescens*. This medium was named BHI-PSA and was used for capacitance assays. BHI was used as the basal medium because it is readily available and commonly used; PIM-AGAR and TMAO medium did not enhance the growth of *P. fluorescens* over the other bacterial isolates isolated using BHI-I, and GPMP did not produce acceptable impedance readings (data not shown).

**Study 2**

The number of isolates recovered from broiler chicken carcasses analyzed by measuring capacitance at day 0 or after holding at 3°C for 3, 6, 9, 12, or 15 days with BHI-PSA as the culture medium is presented in Table 2. Samples were diluted and plated because studies have shown that bacteria must multiply to 10⁶ cells per ml or greater before they are able to change the impedance, conductance, or capacitance of the medium sufficiently to be detected by the monitoring device (8). By culturing the sample immediately after detection, the bacteria found on the plate at the highest dilution are assumed to be those responsible for changing the capacitance of the medium (23).

On day 0, for 4 of the 6 carcasses analyzed, *P. fluorescens* was the bacterium that reached the capacitance detection threshold first. For the other 2 carcasses, *P. putida* was responsible for changing the capacitance of the medium to the extent that the instrument could detect the change. Only one other species of bacteria was isolated from the plates at high dilutions. Plates on which bacterial species other than *P. fluorescens* were isolated were examined to determine the bacterium that was overwhelmingly predominant and thus would be the organism that produced the impedance change. *Pseudomonas fluorescens* was the overwhelmingly predominant species on the day 0 plate that contained CDCEF4. Overall, *P. fluorescens* was the bacterium that was selectively enumerated on day 0 carcasses.

**TABLE 1. Bacteria isolated with brain heart infusion broth plus 25 μg of Irgasan per ml: growth in brain heart infusion broth plus (per ml) 4 μg of nitrofurantoin, 120 μg of carbenicillin, and 25 μg of Irgasan**

<table>
<thead>
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<th>Bacterial isolate</th>
<th>Growth of isolate in replicate</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Aeromonas sobria</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida salmonicida</em></td>
<td>-</td>
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<tr>
<td><em>Aeromonas hydrophila caviae</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Serratia liquefaciens</em></td>
<td>-</td>
</tr>
</tbody>
</table>

*Growth was determined using a colorimeter. +, bacteria produced visible turbidity in medium; −, bacteria produced no visible turbidity compared to baseline transmittance.*
TABLE 2. Number of isolates recovered from broiler chicken carcasses obtained from the chiller exit of a commercial processing plant and analyzed at day 0, 3, 6, 9, 12, and 15 by using brain heart infusion broth with Pseudomonas fluorescens selective additive (BHI-PSA) as the culture medium

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Day</th>
<th>Bird 1</th>
<th>Bird 2</th>
<th>Bird 1</th>
<th>Bird 2</th>
<th>Bird 1</th>
<th>Bird 2</th>
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<td>4*</td>
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<td>Pseudomonas putida</td>
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<td>Alcaligenes spp.</td>
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*Predominant bacterium visually observed on plates at the highest dilution.

P. putida was unable to multiply in the BHI-PSA medium when tested individually in study 1. For 5 of the 6 carcasses held for 3 days at 3°C (Table 2), P. fluorescens was the bacterium that reached 10⁶ cells per ml first and, hence, was responsible for the capacitance reading. Flavobacterium meningosepticum was the only species of bacteria other than P. fluorescens that was isolated from the plates at high dilutions; however, the overwhelmingly predominant species on that plate was P. fluorescens. On day 6, 9, 12, and 15 carcasses (Table 2), for all samples, P. fluorescens was responsible for changing the capacitance of the medium to the extent that the instrument could detect the change. Although other species of bacteria (CDCEF4, Flavobacterium meningosepticum, gram-negative bacilli, Aeromonas hydrophila caviae, Alcaligenes spp., Chromobacterium violaceum) were isolated at high dilutions, the predominant species on those plates was P. fluorescens. In most cases, only one colony of these contaminants was visually apparent on plates at these dilutions.

BHI containing 4 μg of nitrofurantoin, 120 μg of carbenicillin and 25 μg of Irgasan per ml was excellent for enumeration of P. fluorescens from broiler chicken carcass rinses by measuring capacitance at 25°C. The average time required to enumerate P. fluorescens from day 0, 3, 6, 9, 12, and 15 samples was 22.4, 17.2, 12.0, 4.6, 2.9, and <1.0 h, respectively (data not shown). This procedure is rapid and would be a useful tool for determining the number of spoilage bacteria on fresh chicken and, thus, may possibly be used to predict the potential shelf life of fresh chicken and other foods of animal origin.

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