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API Listeria Rapid kit for Confirmatory Fenotypic Conventional Biochemical Test of the Prevalence *Listeria monocytogenes* in selected meat and meat products

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Abstract

This study was conducted to confirm the prevalences *Listeria monocytogenes* from the conventional biochemical identification. The prevalences of pathogenic bacteria *Listeria monocytogenes* come from raw and processed meat products. The DIM results of confirmatory identification using the API *Listeria* kit showed that 4 isolates were designated as *L. monocytogenes* with a 'doubtful profile' comment, 98.69%, good identification respectively. On the other hand, 2 isolates were identified as *L.innocua* and *L. seeligeri*.

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Keywords: *Listeria monocytogenes*, API *Listeria* kit, conventional method, raw and processed meat products

INTRODUCTION

The genus *Listeria* is composed of six species namely: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. murrayi* (also called *L. grayi)* [4], [7]. The *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. innocua* are more closely related to each other while *L. grayi* (or *L. murrayi*) is distantly related [4]. However, only *L. monocytogenes* is commonly associated with human listeriosis. Listeriosis caused by *L. ivanovii*, *L. welshimeri,* or

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even *L. seeligeri*, is extremely rare in humans. The other two, *L. innocua* and *L. grayi* are non-pathogenic. The universal occurrence of *L. monocytogenes* in food [11] and the risk of contracting food-borne listeriosis from *L. monocytogenes* have been thoroughly reviewed recently [13]. *Listeria monocytogenenes* (originally named *Bacterium monocytogenes)* is a gram-positive, non-sporeforming, highly mobile, rod-type, and facultative anaerobic bacterium species. It can grow under temperatures between -1.5 °C to 45 °C [8], and at pH range between 4.4 and 9.4, with the optimum pH of 7.

The classical approach to the identification of bacteria is cultural. This approach involves subjecting suspected samples to a series of tests designed to isolate and identify microorganisms possessing a profile of designation belonging to *Listeria* species at the strain and species levels. The common agar media for studying characterization of *L. monocytogenes* are tryptose agar, nutrient agar, and blood agar. Conventional testing methods for the detection of *L. monocytogenes* in food involve growth in pre-enrichment medium, followed by growth on selective medium and confirmatory biochemical and serological tests [5]. These methods are time-consuming and labor-intensive. Furthermore, the prolonged incubation allows growth of other microorganisms. Although this method has contributed much to the present-day knowledge concerning the epidemiology of listeriosis, the prolonged incubation period necessary to obtain positive results is a serious disadvantage. Major improvements in selective enrichment and plating media have since decreased the time needed for analysis from several months to less than 1 week [12]. A variety of morphological, physiological and biochemical tests are also used for identification of microorganisms in conventional methods. In recent years several commercially available kits have been developed to simplify and automate the identification of individual organisms, the result of which is comparable to that of conventional identification systems [10].

The API *Listeria* kit ((BioMerieux, La Balme-les-Grottes, France), a rapid kit used to detect *L. monocytogenes* based on morphological and biochemical characteristics, has recently been introduced. The working principal of API commercial kit is the comparison of sugar fermentation. It consists of 10 strips to test the presence or absence of the following: arylamidase (DIM test), hydrolysis of esculin, presence of α-mannosidase, and acid production from D-arabitol, D-xylose, L-rhamnose, α-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-agatose [3].

The main objective of the present study was to evaluate the reliability of different techniques for detection and identification of *L. monocytogenes* in selected raw and processed meat products. The expected output and contribution of this study is to provide information regarding the prevalence of *L. monocytogenes* in selected raw and processed meat products. This is necessary in order to meet USFDA's regulation of zero tolerance to *L. monocytogenes* in ready-to-eat and processed foods. The outcome of this study is also expected to provide some laboratories with recommendations regarding applicable, effective, and reliable methods for detecting *L. monocytogenes* in selected raw and processed meat products. Recommended methods shall be minimizing the recalling products and the impact cost additional products distribution.

MATERIAL AND METHODS

MATERIALS

The samples investigated for the presence of *L. monocytogenes* were selected from meat and meat products obtained from Supermarkets at Los Baños, Laguna, Philippines. Raw beef samples were obtained from minced beef, beef cubes, and beef liver while processed beef samples were obtained from beef burger, cheese dog and corned beef. Chicken meat samples were obtained

from chicken liver, ground chicken, and chicken breast fillet. On the other hand, the processed chicken meat samples were obtained from chicken luncheon meat, chicken frank sausages, and chicken nuggets.

For spiked samples, the materials were artificially inoculated by the BIOTECH UPLB collection's isolate. The test strain used to inoculate was *L. monocytogenes* #3 (Lm_3). Cell suspension was prepared by mixing Lm_3 cells in 0.85% saline solution. The desired homogeneity of the solution and its target concentration of 3 x 10^8 CFU/mL Lm_3 organisms were achieved by comparing its turbidity with McFarland 0.5 #1.

The conventional method, for isolation and purification as well as the fenotypic methods using API *Listeria* of *L. monocytogenes* were done at the Food, Feed and Specialty Products Laboratory (FFSPL) in The National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). All the isolation and identification steps were strictly done under biosafety II y cabinet, considering *L. monocytogenes* as pathogenic.

Agar mediums were used in this study as followed: Tryptic soy broth (TSB) and tryptic soy agar (TSA) for subculturing medium of *L. monocytogenes* isolates prior to conducting gram reaction, catalase reaction, motility test, β-haemolysis test medium mixture with fresh sheep blood, and for cell lysis purposes. Listeria enrichment broth was used in the enrichment step. PALCAM selective agar base was used for conventional culture medium. SIM medium was prepared for motility tests. Some antibiotic supplements were used for enriched Listeria enrichment broth as followed nalidixic acid, acriflavin and cycloheximide. The enzyme catalase was used for catalase test. 3% hydrogen peroxide solution was used for catalase test. The following reagents were used for gram reaction: crystal violet stain, Lugol's iodine, 95% acetone-alcohol used for decolorizer and safranin O as counterstain. 0.5% purple carbohydrate basal broth of rhamnose, xylose, and mannitol were prepared for sugar fermentation test. A set of API Listeria surely prepared and used for the study.

METHODS

Conventional Methods for Detection of *Listeria monocytogenes*

The conventional method done in this study followed the FDA protocol on the Bacteriological Analytical Manual [2] for *L. monocytogenes*. The protocol included enrichment, isolation, and identification procedures. The macroscopic and microscopic morphological features, and biochemical and physiological examinations of the cultures were followed by further confirmatory tests to detect; catalase reaction, KOH reaction, gram reaction, motility at room temperature, β-haemolysis reaction on blood agar, and sugar fermentation [14].

The API *Listeria* system (BioMerieux, La Balme-les-Grottes, France) is a standardized system for identification of *Listeria* which uses miniaturized tests, as well as a database. The API *Listeria* strip consists of 10 microtubes containing dehydrated substrates which enable the performance of enzymatic tests or sugar fermentations. During incubation, metabolism produces color changes that can either be spontaneously detected or need to be revealed by the addition of reagents. The reactions were read according to the reading table and the identification was obtained by consulting the profile list using the identification software. A 24-hour culture of bacteria on TSA slant were suspended in 2 mL of sterile distilled water (BioMerieux) to reach the same opacity as the MacFarland 0.5 Standard No. 1 on the scale. The suspension was simply homogenized using vortex mixture (Vortex Genie 2, Fisher Scientific, USA). Three milliliters

(3mL) of sterile distilled water was poured into the tray to create a moist atmosphere, and then the reaction strip was removed from its packaging and placed in the tray. The bacterial suspension was then distributed into ten (10) microtubes (100 µL for the DIM test and 50 µL for other cupules, ESC to TAG). Afterwards, the strip box was closed and incubated at 37˚C for 18 to 24 hours. After incubation, one drop of ZYM B (supplied by the manufacturer) was added to the DIM microtube and allowed to react for 3 minutes, and then all of the reactions were noted. Results of biochemical profile obtained for the isolates was entered and analyzed using the Apiweb Identification Software (V5.0) with database, which used the phenotypic data to predict the species identity for each isolate. Interpretations of the fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer-aided database, in which the identification of microorganism is accompanied by the following information: i) the percentage of identification (% ID), which is an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the database; ii) the T-index, which represents an estimate of how closely the profile corresponds to most typical set of reactions for each taxon with its value variying between 0 and 1, and is inversely proportional to the number of a typical tests; and iii) comments on the quality of identification derived from the % ID and the T-index of the selected taxon. An excellent identification should have % ID more than 99.9 and T-index more than 0.75).

RESULTS AND DISCUSSION

Detection of *Listeria monocytogenes* using Conventional Method (PALCAM Plate)

Using the conventional method, the presence of *L. monocytogenes* in meat samples was detected after at least 90 to 96 hours of incubation at 35˚C to 37˚C. These time durations already include the time taken for enrichment necessary to identify naturally contaminated food samples. A presumptive positive result is indicated by black color on the medium surrounding the colonies, and a pin point in the middle of the colony. The number of colonies was then counted and the data were used to derive the number of colonies in CFU/mL.

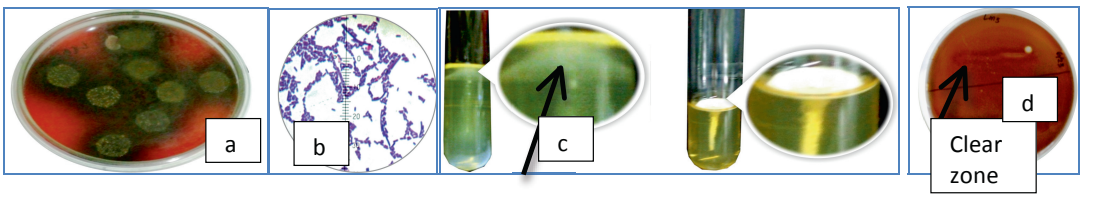

Fig. 1. Appearance of positive presumptive colony of *L. monocytogenes* in Palcam Media (a), microscopic gram positvie appearance (b), motility (c) and haemolysis test (d).

Purification was done to confirm the presumptive result of *L. monocytogenes* in samples. At least one of the separate colonies of each presumptive result was represented, which was picked and streaked on to PALCAM medium agar for purification purposes.

Some of the unaccountable colonies which were noted as TNTC (Too Numerous to Count) were also streaked for further confirmation of presumptive *L. monocytogenes*. Some colonies from the negative plates were also transferred by streaking on to PALCAM medium to confirm. A series of purification could help eliminate the growth of bacteria other than *Listeria* spp in meat and meat products. From the 55 chosen plates of enriched samples, only 37 isolates were

obtained. All of the 37 isolates were not confirmed yet as *L. monocytogenes* but could be *Listeria* spp based on the appearance of growing colony. It is very difficult to distinguish *L. monocytogenes* based only on the appearance. [1] cited that, the main disadvantage of the direct plate method using PALCAM agar is that it cannot differentiate between pathogenic and non-pathogenic *Listeria* spp. There is no difference on the initiation of growing appearance on PALCAM plate within *Listeria* spp. Therefore, presumptive colonies must be further characterized as to whether they are, in fact *L. monocytogenes* or another *Listeria* spp.

After series of purification were done by repeated streaking on PALCAM media, all of the representative colonies were transferred to TSA slants for further characterization tests. Generally, *L. monocytogenes* were phenotypically different from other *Listeria* species. Therefore, morphological and biochemical test for phenotypic identification can differentiate between *Listeria* spp and *L. monocytogenes*. Though time-consuming, tests were conducted individually for each isolate. Confirmatory tests which included catalase test, KOH 3% test, staining test, motility test, haemolysis test and sugar (rhamnose, mannitol, and xylose) utilization were done. To accomplish reliable results, fresh cultures from TSA slant were used to avoid false negative results due to using dead microorganisms. The first step in the identification test started with common and quick tests such as catalase, KOH 3%, staining, and motility test.

The results are shown in Table 1. All of the 37 isolates were shown positive on catalase, KOH 3% and staining tests. Positive results are indicated by the presence of bubbles for the catalase tests, no changes of viscosity on a drop of KOH and a colony suspension within 60 seconds for KOH 3% tests, and exhibited gram-positive rods under microscopic examination for staining test as seen at Figure 1 (b). *L. monocytogenes* is a Gram-positive, facultatively anaerobic, non-spore-forming, short, rodshaped bacterium [9]. Positive isolates for motility test were confirmed by the umbrella shape and growth radiating out from the central of stab line in the SIM medium as seen at Figure 1 (c). Observation was made after one week of incubation at room temperature [9]. Motility test confirmed that there were two isolates that gave negative results. These were the beef burger sample enriched on LEB base and artificially contaminated with *L. monocytogenes* and cheese dog sample that was enriched on Fraser VIDAS broth without artificially contaminated.

Beyond the simple tests outlined above, the more important biochemical tests are the assessments of hemolytic activity and carbohydrate utilization patterns. Haemolysis activity was done by streaking a horizontal line of the individual isolate on the surface of sheep blood agar. Observation was done very carefully after 48 hours incubation at 37˚C. As cited from [9], assessing β- haemolysis with presumptive *L. monocytogenes* can be problematic due to the very small clearing zone as seen at Figure 1 (d) and some isolates are more difficult to judge. Sometimes it is easier to see clearing of the blood if the colony is gently removed from the surface of the plate with a loop. As shown in Table 1, twenty-nine out of thirty-seven isolates were presumptively *L. monocytogenes*. Some presumptive of *Listeria monocytogenes* also gave positive results on the β-haemolysis test. Three isolates (NB 43, NB 26, and NB 7) had the characteristics of positive *L. monocytogenes* but gave negative results on the β- haemolysis test.

Further confirmation of *L. monocytogenes* was conducted through sugar utilization tests. Sugar (carbohydrate) utilization tests were done using three kinds of carbohydrate broth: rhamnose, xylose, and mannitol. Sugar utilization patterns were recommended by various official methods for distinguishing *Listeria* and for differentiating between *Listeria* spp. Sugar tests were

done by inoculating individual isolate into carbohydrate broths, each containing a sugar with a specific pH indicator, and then incubated up to seven days at 37°C. A positive reaction was exhibited by changes in the sugar broth color from purple into red-orange tinge. *L. monocytogenes* is rhamnose positive, xylose and mannitol negative, whereas the opposite is true for *L. ivanovii*. Differentiating *L. monocytogenes* from *L. innocua* solely by carbon utilization is difficult. The sugar fermentation patterns of *L. monocytogenes* and *L. innocua* are very similar, with rhamnose used in *L. innocua* also being strain dependent [9].

Table 1. Phenotypic identification test for confirmation of positive isolates

Sample Source	Palcam Plate Code	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	API
Chicken frank sausages (LxB-spiked)	NB 3	+	+	+	+	+	+	-	-	
Chicken frank sausages (LxB-spiked)	NB 4	+	+	+	+	-	-	-	-	
Chicken frank sausages (LxB-unspiked)	NB 5	+	+	+	+	+	+	-	-	*L. Innocua*
Chicken frank sausages (LxB-unspiked)	NB 6	+	+	+	+	+	+	-	-	
Chicken luncheon (LxB-spiked)	NB 7	+	+	+	+	-	+	-	-	*L. monocytogenes*
Chicken luncheon (LxB-spiked)	NB 8	+	+	+	+	+	+	-	-	
Chicken luncheon (LxB-spiked)	NB 9	+	+	+	+	+	+	-	-	
Cheese dog (LxB-unspiked)	NB 13	+	+	+	+	+	-	-	+	*L. seeligeri*
Cheese dog (LxB-unspiked)	NB 14	+	+	+	+	+	+	-	-	
Cheese dog (LxB-unspiked)	NB 15	+	+	+	-	+	+	-	-	
Beef burger (LxB-spiked)	NB 16	+	+	+	+	+	+	-	-	
Beef burger (LxB-spiked)	NB 17	+	+	+	+	+	+	-	-	
Beef burger (LxB-spiked)	NB 18	+	+	+	+	+	-	-	+	
Beef burger (LxB-unspiked)	NB 19	+	+	+	+	+	+	-	-	*L. monocytogenes*
Beef burger (LxB-unspiked)	NB 20	+	+	+	+	+	+	-	-	
Beef burger (LxB-unspiked)	NB 21	+	+	+	+	+	+	+	+	
Cheese dog (LxB-spiked)	NB 22	+	+	+	+	+	+	-	-	
Cheese dog (LxB-spiked)	NB 23	+	+	+	+	+	+	-	-	
Cheese dog (LxB-spiked)	NB 24	+	+	+	+	+	+	-	-	
Cheese dog (LxB-spiked)	NB 25	+	+	+	+	+	+	-	-	
Cheese dog (LxB-unspiked)	NB 26	+	+	+	+	-	+	-	-	
Beef burger (LEB-unspiked)	NB 40	+	+	+	+	+	+	-	-	*L. monocytogenes*
Beef burger (LEB-unspiked)	NB 41	+	+	+	+	+	+	-	-	
Beef burger (LEB-unspiked)	NB 42	+	+	+	+	+	+	-	-	
Ground beef (LEB-unspiked)	NB 43	+	+	+	+	-	+	-	-	
Ground beef (LEB-unspiked)	NB 44	+	+	+	+	+	+	-	-	
Ground beef (LEB-unspiked)	NB 45	+	+	+	+	+	+	-	-	
Beef burger (LEB-spiked)	NB 46	+	+	+	+	+	+	-	-	
Beef burger (LEB-spiked)	NB 47	+	+	+	+	+	+	-	-	
Beef burger (LEB-spiked)	NB 48	+	+	+	+	+	+	-	-	
Beef burger (LEB-spiked)	NB 49	+	+	+	-	+	+	-	-	
Corned beef (LEB-spiked)	NB 50	+	+	+	+	+	+	-	-	
Corned beef (LEB-spiked)	NB 51	+	+	+	+	+	+	-	-	
Corned beef (LEB-spiked)	NB 52	+	+	+	+	+	+	-	-	

Note: (1) Catalase Test; (2) KOH Test; (3) Gram's Reaction ; (4) Motility Test; (5) Haemolysis Test (6 Rhamnose Test; (7) Mannitol Test; (8) Xylose Test; (9) API

Confirmatory of *Listeria monocytogenes* Pure Colonies using API *Listeria*

Differentiation of the species within the genus *Listeria* was done using the rapid identification kit API *Listeria* (bioMérieux, Inc., Marcy l'Etoile, France). The API *Listeria* system has been listed as one of the preferred rapid methods for the biochemical identification of *Listeria* species in the routine microbiology food laboratory [6]. Only five isolates were tested using the API *Listeria* kit. These are NB 5, NB 7, NB 13, NB 19, and NB 40. This system takes 24 hours to identify *Listeria* species, based on 10 sugar fermentation reactions and enzymatic reactions in microtubes, usually without the need for additional tests [3]. As cited from [3], the DIM results for all strains tested were unequivocal. The positive results of *Listeria* isolates is indicated by hydrolysis of esculin and acid production from D-arabitol and acid production from D-arabitol and α-methyl-D-glucosidase (except for *L. grayi*). The presence of arylamidase (DIM test) and α-mannosidase and acid production from D-xylose, L-rhamnose, D-ribose, glucose-1-phosphate, and D-tagatose were used for species and subspecies identification. The positive-negative color fermentation for each microtubes were then analyzed using API web system software. Confirmatory identification using the API *Listeria* kit showed that isolates NB 7, NB 19, and NB 40 were designated as *L. monocytogenes* with a 'doubtful profile' comment, 98.69%, good identification respectively. On the other hand, isolates NB 5 was identified as *L.innocua* with 99.6% very good identification comment. The last isolate, NB 13 was excellent identification to the species *L. seeligeri* with 94.2% ID.

Conclusion

Prevalence of *Listeria monocytogenes* was obtained from the beef buger sample. While from cheese dog and chicken frank sausages samples were obtained the species of *Listeria innocua* and *Listeria seeligeri.* The differentiation wihin species successfully confirmed by API Listeria method after several time purification and confirmatory step on conventional method. Based on the time of efficiency, conevntional method is more time consuming than API Listeria method. Using the conventional method, the presence of *L. monocytogenes* in meat samples was detected after at least 90 to 96 hours of incubation at 35˚C to 37˚C. While, using the API Listeria takes only 24 hours to identify *Listeria* species.

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