

Prevalence of *Listeria* spp. with PRS Genes in Fresh Vegetables from Traditional Markets

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ABSTRACT

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Listeriosis is a foodborne infectious disease caused by *Listeria monocytogenes* and is considered a serious health problem, due to the severity of symptoms and a high mortality rate in worldwide. This study aims to identify and determine the prevalence of *Listeria* species through prs gene screening of fresh vegetables distributed in several markets of Makassar City. A total of 57 fresh vegetable samples were collected from February to May 2019 in four traditional markets Makassar. The isolates are examined to phenotypically and genotypically Vitek and Multiplex PCR with prs and lmo1030 primer. Phenotype analysis did not show the presence of *Listeria* species, but the results of genotypic were found 8 positive prs gene samples (14.03%), consisting of 31.2% long beans, 18.2% cabbage, and 9.1% cucumber and. All *Listeria* species found in this study is *Listeria monocytogenes*. This study also provides information and additional data that prs genes can be used as screening genotype for identifying *Listeria* species in fresh vegetables.

Keywords:
fresh vegetables; *Listeria* spp.; multiplex PCR; prs gene

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INTRODUCTION

Listeriosis is a foodborne infectious disease caused by *Listeria monocytogenes* and is considered a serious health problem, due to the severity of symptoms and a high mortality rate in worldwide. Recently, other *Listeria* species have been linked to diseases in humans and animals (Mazza R., et al, 2015). Most cases of human *Listeria monocytogenes* are associated with consumption of raw foods (European Food Safety Authority, 2013). *Listeria monocytogenes* is a pathogen that is transmitted through food, widely distributed in food ingredients such as vegetables, fruits, dairy products, and processed foods. Consumption of contaminated raw food and cooked half-cooked can cause listeriosis (Dandapani S., et al, 2011).

The results of the study of Jamali H. et al. (2013), showed that from the samples examined, 17.9% of ready-to-eat food samples contained *Listeria spp.* where 11.4% of the results were identified as *Listeria monocytogenes*. Although the incidence of listeriosis in humans in Indonesia has not been found, but the presence of *Listeria monocytogenes* in food has been reported such as fresh milk in South Sulawesi (Prahesti K.I. et al, 2017) and fresh chicken meat in West Java (Sugiri Y.D. et al., 2014). Approximately 1600 cases caused by are reported, including 255 cases of death each year in the United States (Scallan, et al., 2011). Therefore, the outbreak associated *Listeria spp.* is a public health disease of concern.

The standard method widely used for determining *Listeria spp.* is the culture method, enrichment in liquid media and then isolation on solid selective media (Barocci, et al., 2008 but this method is considered to require a long time and some of the media used are fairly expensive. According to Aznar R. and Alarcón B. (2003), detection of *Listeria monocytogenes* in food samples is difficult because of the low bacterial count., so the genotype method using PCR can be a more effective and faster in diagnosis than others. The *prs* gene that encodes the *putative phosphoribosyl pyrophosphate synthetase protein* is a gene specific to *Listeria spp.* (Doumith M, et al, 2004)

Mortality rate of *Listeria monocytogenes* has been reported in high percentation (30%), the presence of this bacterium in food consumption is an one of the major health problem such as meat, dairy, fish and vegetable products (Pesavento G., et al, 2010). The objective of our study was to determine the prevalence of *Listeria spp.* and rapid identification through *prs* gene screening of fresh vegetables.

MATERIALS AND METHODS

Population and Sample

The population in this study is food in the form of vegetables sold in Makassar traditional markets as a distribution point of vegetables from farmers with minimal decontamination processes. The samples used in this study were vegetables, namely cabbage (*Brassica oleracea*), lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*), basil (*Ocimum citriodorum*), and long beans (*Vigna unguiculata*). The number of samples used was 57 samples with a sampling technique based on *purposive sampling*, where the materials that meet the inclusion criteria is from traditional markets and the exclusion is non-decaying materials will be used as samples.

Tools and Materials

The tools used in this research are in the form of tools used in the microbiology laboratory; glassware, sample containers (sterile plastic), ose, pipette, micropipette, scale, eppendorf tube, centrifuge, vortex, glass object, incubator, autoclave, refrigerator, laminary air flow, microscope, Vitek 2 Compact, PCR, electrophoresis and gel doc.

Materials used in research; vegetables, namely cabbage, lettuce, cucumber, basil and long beans. Buffered Listeria Enrichment Broth (BLEB), Blood Agar Plate (BAP), N.AN (Naladixic Acid / Natamycin) supplement, PALCAM Agar solid selective culture medium, Oxford Selective CNCAF supplement, goat blood, SIM agar base,

GP Card, PCR extraction kit, primer, PCR (Go Taq Green) enzyme, RNase Free water, Agarose, Ethidium Bromide, TBE 0.5, Loading Dye, DNA Leader / Marker (100 bp), and Gram stain reagent.

Culture on BLEB Media

The obtained sample was then crushed using a mortar, then weighed as much as 25 grams and then inoculated on BLEB media as much as 225 ml and incubated for 2 x 24 hours at 30 °C. Turbidity on BLEB media indicates bacterial growth.

Culture on Selective Media

The results of growth on the BLEB media are then directly scratched using ose on the PALCAM agar media. Then incubated at 37°C for 2 x 24 hours. Colonies were observed, after incubation for 1-2 days, the colony was grayish green and arose, the colony from PALCAM agar was inoculated to the BAP media for hemolysis tests. on the blood media so that the colonies look like creams and mucoid (hemolysis).

Identification of *Listeria* spp. Fenotype with Vitek

The results of culture that has been incubated, and there is a target colony, then biochemical tests were carried out using a GP card on the Vitek® 2 Compact. The automatic Vitek® 2 Compact system from Biomerieux works on the calorimetric principle for identification, through biochemical and antibiotic sensitivity testing. The

accuracy of the Vitek® 2 Compact automatic system ranges from 97.8% (O'Hara, 2005) to 98.02% (Duggal, *et al.*, 2012).

Identification of *Listeria* spp. Genotype by Multiplex PCR

DNA extraction using TIANampp Genomic DNA Kit. Table 1 shows the primers used to detect *Listeria* spp. (*prs*) and *Listeria monocytogenes* (*lmo1030*). The reaction mixture for PCR is 25 µl each set, in which there are 5 µl DNA templates, 12.5 µl Go Taq Master Mix, 1 µl forward primer and 1 µl reverse primer, and the addition of nuclease free water to suffice the mixture to 25 µl. The PCR cycle refers to Ryu J, *et al* (2012) using conventional PCR, one cycle for initial denaturation at 94 °C for 5 minutes, then 35 cycles for denaturation at 94 °C for 30 seconds, annealing 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. and one cycle for the final extension at 72°C for 5 minutes. PCR products were analyzed using 0.5 TBE buffer electrophoresis gel at 100 V voltage for 120 minutes, with a composition of 2% agarose and 10 µl Ethidium Bromide. Use 100bp DNA Marker ladder to see the PCR product band sizes.

Data Analysis

Analysis using MS Excel 2010. Percentage of *Listeria* spp. analyzed by descriptive analysis.

Tabel 1. Primer pairs sequence and target gene

Species	Gene	Primer	Sequens (5'-3')	PCR product (bp)
<i>Genus Listeria</i>	<i>Prs</i>	prs-F	GCTGAAGAGATTGCGAAAGAAG	370
		prs-R	CAAAGAAACCTTGGATTTGCGG	
<i>Listeria monocytogenes</i>	<i>Lmo1030</i>	Lmo1030-F	GCTTGTATTCACCTTGGATTTGTCTGG	509
		Lmo1030-R	ACCATCCGCATATCTCAGCCAAC	

RESULT AND DISCUSSION

During the February-June 2019 period, 57 fresh vegetable samples were obtained from traditional market of Makassar City, Indonesia. Table 2 shows the prevalence of *Listeria spp.* from fresh vegetables samples in Traditional Market, Makassar, Indonesia is 14.03% (8 of 57 samples). Long bean samples is the highest prevalence of 31% where there are 4/16 positive samples. Lettuce from 11 samples, both phenotypically and genotypically showed no positive results.

The phenotype test was conducted by referring to FDA testing standards with BLEB as a selective media and PALCAM media as a culture media. Genotypic test using multiplex PCR method with the *prs* gene as a marker of *Listeria spp.*

From the results of vitek using a GP card does not show specific bacterial species may be caused by bacteria growing on PALCAM media not contained in the GP card. The culture of *Listeria monocytogenes* was not identified because the number of bacterial cells did not reach the detection sensitivity, which was 104 CFU / ml. Beumer and Wilma (2003) say that most likely most raw food products will hold *Listeria* in low quantities. In the case of a low number of *Listeria* cells, isolation is difficult so inoculation is recommended to test animals, including embryo eggs.

According to Beumer R.R. and Hazeleger W.C. (2003), the success of the culture method depends on several factors. Such as, the number and condition of bacteria in the sample, media selectivity (balance between inhibition of competing bacteria and target bacteria), selection of isolation media (the difference between target bacteria and competitive microflora) and incubation conditions (for example, temperature, time, and oxygen).

Among the 5 types of vegetables, *Listeria spp.* more dominant in long beans with 31.2% (5/16). *Listeria monocytogenes* previously in string beans was isolated by Ponniah J., et al (2010) with

a percentage of 31.3% (10/32). In lettuce either through phenotype or genotype testing, eight samples showed negative results from *Listeria spp.* The results of the study of Jamali H., et al (2013), *Listeria monocytogenes* isolated from lettuce, only 5.6% (1/18). Overall the results of this study are not much different from the previous findings summarized by Zhu Q. et al (2017) which was the percentage of *Listeria spp.* low on lettuce. Figure 1 shows the results on agarose gel electrophoresis using a special primer for the *prs* gene (370bp).

In all 8 samples with positive *prs* genes, then back in the PCR using the *lmo1030* gene as a marker of *Listeria monocytogenes* to determine whether the sample was contaminated with *Listeria monocytogenes* bacteria. Species identification is only done using the *lmo1030* gene as a marker of *Listeria monocytogenes* because the only available control is *Listeria monocytogenes* ATCC 7644. The availability of control of other *Listeria* species and the difficulty to obtain is a limitation of this study. Positive *Listeria monocytogenes* isolates were confirmed by PCR test using the *lmo1030* gene as primer (Table 3).

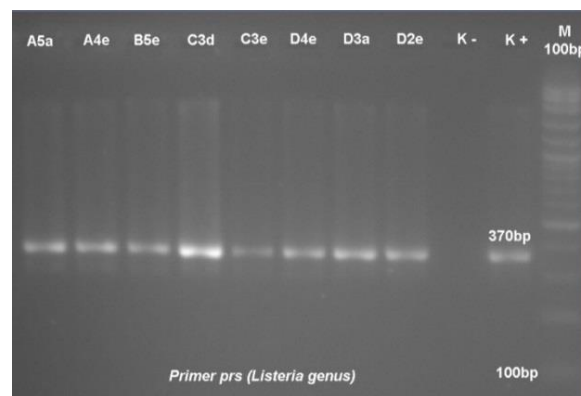
All positive *Listeria spp.* samples found in this study is *Listeria monocytogenes*. Figure 2 shows the result on agarose gel electrophoresis using primers specific to the *lmo1030* gene (509bp) for the isolate, positive and negative control samples. The multiplex PCR method detects *Listeria monocytogenes* by targeting the *prs* gene as a marker of the genus *Listeria* and the *lmo1030* gene as a marker of *Listeria monocytogenes*.

Aznar R. and Alarcón B. (2003) revealed that at least 103 CFU / g must be present to be detected by PCR after DNA extraction methods.

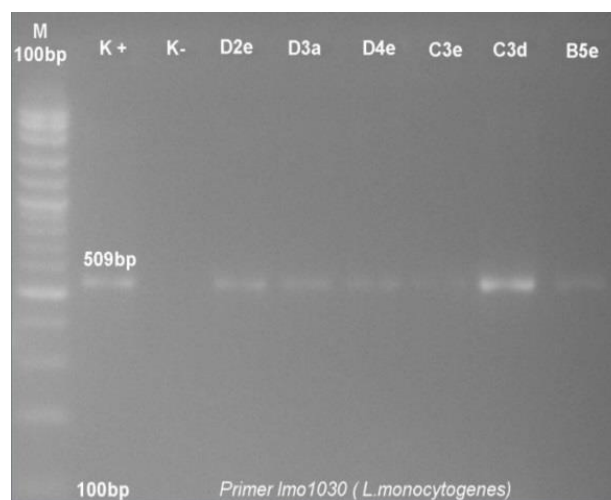
Based on the results of the examination that *Listeria spp.* (*Listeria monocytogenes*) has been detected in fresh vegetables from traditional markets. This indicates that some vegetables that are often consumed in raw conditions may be contaminated by *Listeria spp.* and therefore can pose health risks. The presence of *Listeria monocytogenes* in these vegetables is of concern because these various types of vegetables are often consumed with minimal processing, only through washing.

Table 2. The prevalence of *Listeria* spp. on fresh vegetables from traditional markets

No	Vegetables	Number of Sample	Identification result		Positive samples (%)
			Phenotype	Genotype	
1	Cabbage	11	-	2	18,2
2	Lettuce	8	-	-	0
3	Basil	11	-	-	0
4	Cucumber	11	-	1	9,1
5	Long beans	16	-	5	31,2
Total		57	0	8	14,03

**Figure 1.** Multiplex PCR results of the *prs* gene as a marker for the genus *Listeria* from vegetable samples**Table 3.** Electrophoresis results of PCR gene *lmo1030* in 8 positive samples of *Listeria* spp.

No.	Kode Sampel	<i>Listeria</i> spp.
1	D2e	<i>Listeria monocytogenes</i>
2	D3a	<i>Listeria monocytogenes</i>
3	D4e	<i>Listeria monocytogenes</i>
4	C3e	<i>Listeria monocytogenes</i>
5	C3d	<i>Listeria monocytogenes</i>
6	B5e	<i>Listeria monocytogenes</i>
7	A4e	<i>Listeria monocytogenes</i>
8	A5a	<i>Listeria monocytogenes</i>

**Figure 2.** Multiplex PCR results of the *lmo1030* gene as a marker for *Listeria monocytogenes* from vegetable samples.

According to Salvat G. and Fravallo P. (2004), if *Listeria monocytogenes* in food is at least 1 CFU/100 g it can cause food insecurity in 32 days, whereas 10 CFU / g can cause food insecurity in 8 days. *Listeria monocytogenes* is able to grow in the temperature range of about -0.4 to 45 °C with an optimal temperature of 37 °C. Therefore, this can cause the prevalence of *L. monocytogenes* in food to increase and reach unsafe levels during the storage period. The dose of *L. monocytogenes* infection for healthy or vulnerable individuals has not been established, however, it is estimated that 107-109 CFU in healthy individuals and 105-107 CFU in susceptible individuals such as people with immune disorders or pregnant women.

Supervision of vegetables consumed raw against the threat of pathogenic microbes is important. Moreover, we do not know the path of contamination of *Listeria monocytogenes* bacteria to food to be consumed. One form of prevention is not to use the same cutting board or knife to prepare raw meat and vegetables, wash hands regularly, wash vegetables, fruit and meat under running water

CONCLUSION

Our study has shown that prevalence of *Listeria spp.* with *prx* genes in fresh vegetables from traditional markets of Makassar City, Indonesia is eight of fifty seven (14%). All *Listeria* species found in this study is *Listeria monocytogenes*. We also provide information and additional data that *prx* genes can be used as screening genotype for identify *Listeria* species in fresh vegetables. Therefore, further study is needed with more number and types of vegetables to identify *Listeria spp.*

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