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Detection of Virulence Genes in *Aeromonas* spp. Isolated from Ready-to-eat Salad

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ABSTRACT

Nowadays, most people are more concerned with their personal health. Salads that are ready to eat are frequently a healthy eating option. It is consumed raw and unheated, which promotes the growth of numerous microorganisms. Furthermore, microbial contamination may occur because of the use of contaminated water for cleaning and packing. *Aeromonas* spp. are bacteria that grow on the water's surface. They can survive in water that has been chlorinated to eliminate bacteria, which is critical for public health. Because bacteria can produce and secrete a variety of enzymes that are toxic to human tissue, there are a number of factors that contribute to violence. In people with low immunity, the majority of them can cause serious disease. As a result, the goal of this research is to look into and identify *Aeromonas* spp. isolated from ready-to-eat salad. The 16s rDNA gene was used to confirm the findings, and a PCR was used to look into the virulence factor genes. In this study, 9 isolates of *Aeromonas* spp. were found in 136 ready-to-eat salad samples, accounting for 6.6 percent of the total. Six virulence genes (*ast*, *fla*, *lip*, *act*, *alt*, and *aph*B) were used to identify each of 9 isolates where *fla* were found 4 isolates (44.44 %), and *ahp*B were also discovered 8 isolates (88.88 %). Therefore, there is the potential that ready to eat salad can be contaminated by *Aeromonas* spp. containing virulence factor which can cause a severe health risk such as diarrhea to consumers.

Key words : *Aeromonas spp., Virulence gene, Ready-to-eat salad*

Introduction

Ready-to-eat salads are frequently used as a weight-controlling food. Salads are also easy to prepare and readily available today. According to the findings of several reports, ready-to-eat salads contained a variety of detectable microorganisms such as, *Escherichia coli*, *Klebsiella, Enterobacter, Proteus*, *Pseudomonas aeruginosa*, *Salmonella, Shigella*, *Staphylococcus aureus*, *Listeria monocytogenes,* as well as aerobic bacteria, yeast and fungi (Itohan *et al*., 2011), indicating that a variety of bacteria that may contaminate in ready-to-eat salad can cause food poisoning or food-borne diseases, which is a problem that always arises.

Aeromonas spp.is a gram-negative, waterborne bacteria that can contaminate food and can be found in the environment. *Aeromonas* spp. are isolated from the patients who have gastrointestinal infection. They have enteropathogenicity and these microorganisms are also found in salads. All isolates that came from salads have an enteropathogenicity marker (Mattick and Donovan, 1998). There have been clinical cases of diarrhea or gastrointestinal inflammatory syndrome in humans. In patients with weakened immune systems, it can lead to wound infections or opportunistic infections. The diarrhea

in children is often severe, but in adults there will be more chronic symptoms. Komathi *et al*., published data in 1998 that identified *Aeromonas* spp. in diarrhoea-affected children below 10 years of age. This study's *Aeromonas* spp. were resistant to trimethoprim, sulphdiazine, chloramphenicol and tetracycline. *Aeromonas* spp. drug resistance is critical when treating a patient infected with this organism. *Aeromonas* spp. virulence factors have also been reported in patients with acute diarrheal disease; the study found that the isolates strain had at least one of virulence factors associated with enteropathogenicity, such as enterotoxin, cytotoxin, hemolytic (Longa *et al*., 2005). The important thing is that the infection can spread from the gastrointestinal tract into the circulatory system due to the presence of a relatively wide range of virulence factors such as flagella. The flagella consist of two subunits, flagellin A and flagellin B, encoded by the *fla*A and *fla*B genes (Umelo and Trust, 1997; Rabaan *et al*., 2001), which are important for adhesion and invasion of the tissue layer of the host cell (Ramos *et al*., 2004). In addition, there are their virulence factors,such as the presence of cytotoxic enterotoxin produced from the*act*gene containing effects on villi on the small intestine that cause diarrhea. Diarrhea and cytotoxic enterotoxin can induce hemolysis (Chopra and Houston,1999). *Aeromonas* spp. has another virulence factor called cytotonic enterotoxin, which has a similar mechanism of action to cholera toxin. They were divided into two groups: heat-labile cytotonic enterotoxin (from *alt* gene) (56 °C, 10 min) and heat-stable cytotonic enterotoxin (from *ast* gene) (100 °C, 30 min) (Chopra *et al*., 1994; 1996; 1999). Lipase and elastase are two more pathogenic enzymes. Elastase is an enzyme that breaks down elastin, a type of protein found in the tight junctions between tissues of various organs in the intestines when exposed. Damaged tissues are tightly bonded thanks to elastase digestion. It is possible for fluid to be secreted from tissues. Because *Aeromonas* spp. are resistant to free chlorine (Havelaar *et al*., 1990), they could be a major source of contamination in water supplies if these sources are used in the preparation of ready-to-eat salads. The presence of *Aeromonas* in drinking water and river water has been reported in several countries, including Netherlands, Indiana (U.S.A), and also the northern part of Thailand (Van der Kooij 1988; Havelaar *et al*., 1990; Chauret *et al*., 2001; Tasanapak *et al*., 2018). *Aeromonas* spp. were found to produce beta-haemolysin and have cytotoxic activity against vero cells, and all of these isolates were resistant to various antibiotics such as tetracyclin, nalidixic acid, and approximately 83.35 percent of isolates were resistant to erythromycin (Alavandi *et al*., 1999). *Aeromonas* spp. can also grow at 4 °C. This behavior of *Aeromonas* spp. can grow in refrigerators and produce and express the virulence factor at 37 °C and the temperature in the refrigerator. These characteristics indicate that this pathogen may pose a risk to food stored in a refrigerator (Beuchat, 1991; Kirov *et al*., 1993). Most importantly, ready-to-eat salad is a food that has not been heated to kill microorganisms prior to consumption, so it is prone to microbial contamination (Rodríguez-Caturla *et al*., 2012).

This research is interested in detecting *Aeromonas* spp. from ready-to-eat salad as well as virulence factorsin *Aeromonas* spp. to understand the severity of virulence factors that pose a risk to human health, as well as tosafely consume.

Materials and Methods

Isolation and identification of *Aeromonas* spp.

The 136 ready-to-eat salad samples were collected from a market in Thailand's north. The method was followed by Tasanapak *et al*., (2018). Pour 100 ml alkaline peptone water into each 100 g sample and gently mix. Allow 10 minutes for the microorganisms on the vegetables' outer surface to be washed away. Filtered through a 0.45 µm membrane and incubated at 35 °C for 24 hours with a vancomycin agar plate containing 20 mg/l ampicillin and 2 mg/l vancomycin (ADA-V, Hi Media Laboratories Pvt. Ltd., Mumbai, India), observing yellow colonies. Gram staining, oxidase, trehalose fermentation test, and Indole test were the biochemical tests used to identify *Aeromonas* spp.

Molecular confirmation of *Aeromonas* spp. and their virulence gene detection

The gram-negative rod and positive results in biochemical tests were confirmed using a primer specific to the 16srDNA gene for *Aeromonas* spp. For the 16s rDNA gene, forward and reverse primers were used as 16s rDNA–AERR and 16s rDNA–AERR, respectively, where the PCR product was 935 bp. The sequences of this primer set were followed by Lee *et al*. (2002) and Tasanapak *et al*. (2018). DNA extraction was performed on each isolate and used as a

DNA template. PCR amplifications were carried out in a final volume of 25 µl with 2 µl of DNA extract, 20 pmol of each primer, one PCR (GeneDireX, Inc., Taoyuan, Taiwan), 12.5 µl, and filled up with sterile distilled water. The amplification started with one step of pre-denature step (94 °C, 4 min), followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 51.5 °C for 30 sec, extension at 72 °C for 90 sec, and final extension at 72 °C for 10 min. Agarose gel electrophoresis was used to analyze the PCR products.

The six virulence genes (*ast*, *fla*, *lip*, *act*, *alt*, and *aph*B) of *Aeromonas* spp. were analyzed by PCR following Sen and Rodgers (2004) and Tasanapak *et al*. (2018) with slight modifications. One PCR reaction is compose of 2 µl of DNA extract, 20 pmol of each primer, one PCR (GeneDireX, Inc., Taoyuan, Taiwan), 12.5 µl, and filled up with sterile distilled water to make a final volume of 25 µl. The PCR cycles begin with pre-denature at 95 °C for 5 min, followed by 30 cycles of denature at 95 °C for 25 sec, annealing at 55 °C (for *ast*, *fla*, and *lip*); 50 °C (for *act* gene); 58 °C (for *alt* and *aph*B gene) for 30 sec, extension at 72 °C for 1 min, and 1 cycle of final extension at 72 °C for 5 min. Agarose gel electrophoresis was used to analyze PCR products.

Results and Discussion

A total of 136 samples of ready-to-eat salad were cultured on a selective medium, ampicillin dextrin agar with vancomycin, to detect bacteria in genus *Aeromonas* spp. The yellow colonies, suspected to be *Aeromonas* spp., were found in 113 (83%) samples. All positive result were analyzed for their biochemical testing. Fifty-seven isolates had positive results in oxidase test, trehalose fermentation test, and indole test, whichwere confirmed by 16srDNA gene detection.Nine *Aeromonas* spp., representing 6.66%, were found, and all nine were studied for the virulence gene. Indicating that ready-to-eat salads were a potential source for *Aeromonas* spp. that can survive and grow in salads. Krovacek *et al*. in 1992 reported that the cause that promotes the contamination of *Aeromonas* spp. in ready-to-eat salads may be from the use of tap water, water from irrigation or water supply systems with contamination from the production process, washing or trimming salads. *Aeromonas* spp. can be isolated from a variety of contaminated sources such as drinking water, fish, food, fresh vegetables, and water (Krovacek *et al*., 1992; Tasanapak *et al*., 2018). *Aeromonas* spp. is capable of forming biofilm and thus is resistant to tap water or various water supply systems that add chlorine. (Havelaar *et al*., 1990). In addition, Saad *et al*. (1995) found that *Aeromonas* spp. can be detected in fresh vegetables. It has been shown to be a risk factor for human health (Saad *et al*., 1995).

The nine isolates of *Aeromonas* spp. from ready-to-eat salads were detected for their virulence factor genes. Of the nine isolates, we found 44.44% were positive for *fla* gene (Fig. 1) and 88.88% were positive for the*ahp*B genes (Fig. 2). Cascón *et al*., (2000) also found the *ahp*B gene (translating to Elastase) as the dominant virulence factor in bacterial strains. Pathogenicity or virulence factors were discovered

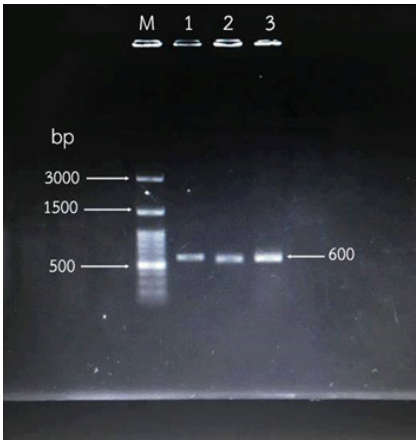

Fig. 1. Agarose gel electrophoresis of *fla* genePCR product. Lane M, DNA marker; Lane 1 –3 are PCR amplicon of 608 bp of *fla* gene from *Aeromonas* spp.

Fig. 2. Agarose gel electrophoresis of *aphB* genePCR product. Lane M, DNA marker; Lane 1 –3 are PCR amplicon of 513 bp of *aphB* gene from *Aeromonas* spp.

to contain important components such as elastase, lipase, and flagella. Four of the nine isolates had two genes, which would have resulted in pathogenicity, according to Sen and Rodgers' report in 2004. In *Aeromonas* isolates from drinking water, they discovered more than one virulence gene. Our findings suggest that *Aeromonas* spp. are likely to be present in ready-to-eat salads, and that 2 virulence factors (*fla*, *ahp*B) promotes pathogenic factors may pose a health risk to those who consume them.

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