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Detection of *Klebsiella pneumoniae* in raw vegetables using Most Probable Number-Polymerase Chain Reaction (MPN-PCR)

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Abstract

Klebsiella pneumoniae (*K. pneumoniae*) is one of the most important members of *Klebsiella* genus in Enterobacteriacae family, which is responsible for pneumonia (the destructive lung inflammation disease). Vegetables are known as source of contamination with *K. pneumonia*. Raw vegetables are usually consumed in salads and other dishes. The aim of this study was to investigate the occurrence of *K. pneumoniae* in raw vegetables marketed in Malaysia. Two hundred commonly used salad vegetables (lettuces, parsley, cucumber, tomato and carrot) from hypermarkets and wet markets were investigated for presence of *K. pneumoniae* using Most Probable Number-Polymerase Chain Reaction (MPN-PCR). *K. pneumoniae* was found to be significantly more frequent (100%) and (82.5%) in lettuce and cucumbers, respectively. *K. pneumoniae* contamination was lowest in carrot samples (30%). All samples were contaminated with *K. pneumonia*e ranging from <3 to 1100 MPN/g. Results showed the high health risk associated with consumption of raw vegetables.

Introduction

Klebsiella pneumoniae (*K. pneumoniae)* is a rod shaped non motile, Gram negative, lactose fermenting and facultative anaerobic bacterium which is usually found in the normal flora of skin, mouth, and intestines. *K. pneumoniae* is one of the most important members of *Klebsiella* genus in Enterobacteriacae family, which is responsible for pneumonia (the destructive lung inflammation disease). Besides *Klebsiella* is found to cause infections in the urinary and lower biliary tract (Lopes *et al*., 2005; Ryan, 2004). *Klebsiella* is an opportunistic pathogens that primarily attacks immunocompromised individuals and hospitalized patients (Podschun and Ullmann, 1998). In 2002, Tsukadaira *et al*. reported four cases of *K. pneumonia*e infection. The cases were acute bronchopneumonia with subclinical aspiration, typical lobar pneumonia (Friedlander pneumonia) and chronic *K. pneumoniae* with typical cavitary lung abscesses.

Due to the high nutritional value, vegetables are considered as important components in every healthy human diet. Raw vegetables are rich in vitamins, minerals and dietary fibre. Regular consumption of vegetables can reduce risk of some important disease such as cancers, stroke and cardiovascukar diseases (Van Duyn and Pivonka, 2000). Vegetables can be contaminated with harmful enteric bacteria in farm, pre-harvest, harvest and post-harvest activities and even in transportation and processing line. Untreated wastewater and animal/human faeces are considered as usual sources of contamination (Beuchat, 2002; Gupta *et al*., 2009). Raw vegetables are widely consumed in the form of salads in most countries, including Malaysia. Consumption of raw or slightly cooked vegetables can increase the risk of food-borne disease.

A number of reports showed that there was an increase in the number of outbreaks of food-borne diseases associated with consumption of fresh produce (Beuchat, 1995; De Roever, 1998). In most of the reported outbreaks of gastrointestinal disease,

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fresh produce were found to be responsible for bacterial contamination (especially with members of *Enterobacteriaceae* family). In 1998, a case of *K. pneumoniae* infection was reported in Houston, Texas. The patient suffered from symptoms of gastroenteritis rapidly lead to multiorgan failure (Sabota *et al*., 1998). Mpuchane and Gashe, 1996, reported presence of *K. pneumoniae* in African spider herb (*Cleome gynandra*) and dried bush okra (*Corchorus olitorius*) in Botswana. Another report from Libya showed presence of *K. pneumoniae* in fruit juices (Ghenghesh *et al*., 2004).

Salad vegetables were found to be contaminated with some food-borne pathogens such as *Aeromonas hydrophila, Citrobacter freundii, Enterobacter cloacae* and *Klebsiella* (Adams *et al*., 1989; Albrecht *et al*., 1995; Brocklehurst *et al*., 1987; Ercolani, 1976; Francis *et al*., 1999; Garg *et al*., 1990; Gaurama *et al*., 1991; Odumeru *et al*., 1997; Van Gerwen *et al*., 1997). *K. pneumoniae* was also recognised as an important food-borne pathogen in fresh produce (Hamilton *et al*, 2006). Due to the high contamination rate of salad vegetables with food pathogens, it is essential to control the hygienic level associated with these products to reduce or minimize risk of food-borne disease (Francis *et al*., 1999; Nguyen and Carlin, 1994; Willocx *et al*., 1994).

The main objective of this study was to investigate the prevalence of *K. pneumoniae* in some salad vegetables marketed in hypermarkets and wet markets using MPN-PCR.

Materials and Methods

Sample collection

A total of 200 salad vegetable samples from five types (*Dacus carota* (Carrot), *Solanum lycopersicum* (Tomato), *Cucumis sativus* (Cucumber), *Lactuca sativa* (Lettuce) and *Cosmos caudatus* (Parsley) were purchased from wet markets and hypermarkets in Selangor, Malaysia. All fresh samples were transported on ice and analyzed immediately.

Detection and enumeration of Klebsiella pneumoniae by MPN-PCR

Briefly, 10 g of the each sample was placed in a sterile stomacher bag, added with 90 ml of Tryptic Soy Broth (TSB) (Merck, Germany) and homogenised for 60s. The suspension was incubated at 37°C for overnight. To perform a three tube MPN, serial dilution was carried out up to 10^{-11}. From each 10^{-9}, 10^{-10} and 10^{-11} fold dilutions, 1 ml was transferred into three sterile tubes and incubated for 24 h at 37°C. Boil cell method was used to extract DNA. A 500 µl of each broth was centrifuged for 5 min at 10,000 rpm. The pellet was then resuspended in 500 µl of TE (10:1) buffer and boiled for 10 min. The sample was cooled at -20°C for 10 min and centrifuged at 10,000 rpm for 10 min. The supernatant was used as DNA template in PCR analysis to detect *K. pneumoniae*. the following primers were used; Pf (5'-ATT TGA AGA GGT TGC AAA CGA T-3') and Pr1 (5'-TTC ACT CTG AAG TTT TCT TGT GTT C-3'). *K. pneumoniae* (ATCC 13773) was used as positive control.

PCR amplification was performed in 25 µl reaction mixture containing 5 µl of 5x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1 unit *Taq* DNA polymerase, 1 µM of each primer (Promega, Madison, USA) and 2 µl of the DNA template solution. The cycling conditions were as follows; 10 min at 94ºC followed by 35 cycles of 30s at 94ºC, 20s at 57ºC, and 20s at 72ºC. A final extension at 72°C for 10 min was performed. To visualize the PCR product, 10 µl of each product was run on agarose gel (1%) at 100V for 30 min. The gel was then stained with ethidium bromide and viewed under ultra violet light.

Results and Discussion

Results showed that *K. pneumonia*e was detected in 65% of raw vegetables from hypermarkets (Table 1). Improper food handling practices, poor hygienic condition of places where vegetables were displayed use of contaminated equipments and containers during transportation can contribute to contamination with *K. pneumoniae*. According to some researchers (Ponniah *et al*., 2010; Tunung *et al*., 2010; Usha *et al*., 2010; Yang *et al*., 2008), poor hygienic practices and improper handling are considered as major factors for contamination of food at hypermarkets level.

In contrast, contamination of *K. pneumonia*e in vegetables from wet market is low comparing to the hypermarket vegetables. The minimum temperature for the growth of the mentioned pathogens is between 8 to 10°C. As vegetables in wet market are immediately sold, the percentage of the contamination can be minimized while vegetables are not stored for long period in humid conditions.

High contamination level of *K. pneumoniae* in salad vegetables is a great concern since there is always high demand for salad vegetables. *K. pneumoniae* contamination was found to be very high in lettuce (100%) and cucumber samples (82.5%) and lowest in carrot samples (30%). This is because leafy vegetables (such as lettuce, celery, spinach, basil, leek, Chinese cabbage and parsley) provide large surface areas and topographical features which can foster

Table 1. Prevalence of *K. pneumoniae* in raw vegetables

Vegetable	Wet market			Hypermarket			Total		
	n	PCR positive	%	n	PCR positive	%	n	PCR positive	%
Carrot	20	5	25	20	7	35	40	12	30
Tomato	20	13	50	20	7	65	40	20	50
Cucumber	20	15	75	20	18	90	40	33	82.5
Salad	20	20	100	20	20	100	40	40	100
Parsley	20	10	65	20	13	35	40	23	57.5
Total	100	63	63	100	65	65	200	128	64

n = Number of samples , (%) =Percentage

Table 2. Microbial load (MPN/g) of *K. pneumoniae* in salad vegetables

Vegetable	Wet market			Hypermarket		
	Min.	Med.	Max	Min.	Med.	Max
Tomato	<3	<3	1100	<3	<3	1100
Cucumber	<3	<3	93	<3	6.5	120
Salad	<3	<3	43	<3	<3	460
Parsley	<3	<3	24	<3	<3	1100
Carrot	<3	3.7	1100	<3	<3	93

Min= Minimum (MPN/g) value; Med= Median (MPN/g) value; Max= Maximum (MPN/g) value

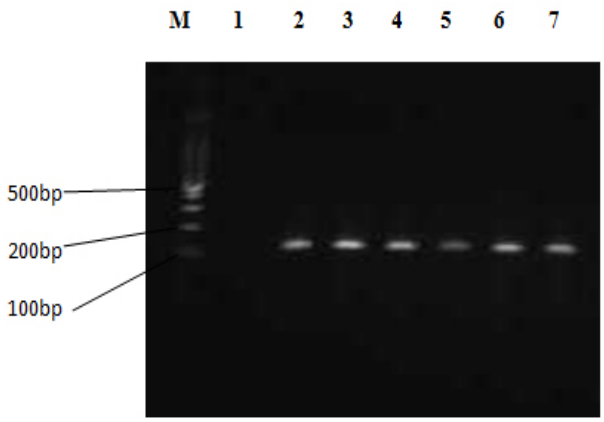

Figure 3. Agarose gel electrophoresis of the *Klebsiella pneumoniae* (130bp). Lane 1= blank; Lane 2-4= positive samples from wet market; Lane 4-7= positive samples from hypermarket; M= 100bp DNA marker

the entrapment or attachment of microorganisms. Besides these vegetables have high relative humidity which favours the spread and survival of bacteria on plant surfaces (Adams *et al*., 1989). Isolation of *Enterobacteriaceae* and other bacterial species from vegetables has been reported by several researchers (Bennik *et al*., 1998; Brocklehurst *et al*., 1987; Ercolani, 1976; Garg *et al*., 1990; King *et al*., 1991; Sahilah *et al*., 2010; Tunung *et al*., 2011).

Contamination of *K. pneumoniae* was 82.5% in cucumber (33/40). This is because of the presence of normally present microorganisms on the surface of cucumber the close contact of it with soil. Presence of soil bacteria or fungi can result in growth and colonization of microorganism on the vegetable and subsequently utilizing nutrients from plant tissues. Among these microorganisms, some commonly found bacteria are listed (Coliforms or faecal coliforms e.g. *Klebsiella* and *Enterobacter*) (Soriano *et al*., 2000; Splittstoesser *et al.*, 1980; Duncan and Razzell, 1972; Zhao *et al.*, 1997). The main sources for these contamination are as follows; animal waste fertilizers, contaminated irrigation water and post harvest washing using contaminated water. Usually the upper layer of the soil (30 cm^2 from the ground) contains 10^6 - 10^7 bacteria/g as some farmers use animal manure or faecal as a fertilizer to enrich soil. On the other hand, contamination may occur through the systemic contamination starting from cultivation site to storage and handling.

The maximum microbial load for *K. pneumoniae* in most samples was 1100 MPN/g (Table 2). Contamination of vegetables probably occurred in their display site which is not effectively cleaned and sanitized. Contamination in such places can lead to the development of biofilms (Blackman and Frank, 1996). Exopolysaccarides secreted by microorganism are able to form a bound capsule layer or create a matrix structure (Leigh and Coplin, 1992). Microbial aggregates that may harbour bacteria, yeast and molds within this matrix have been found on plant surfaces and are referred to as biofilms (Morris *et al*., 1997). The formation of biofilms on leaf surfaces of some vegetables (lettuce, celery, spinach, parsley, Chinese cabbage, basil, leek and endive) has been reported (Morris *et al*., 1997). High relative humidity in vegetables is a factor that favours the survival of bacteria on plant surfaces (Leben, 1988).

The vegetables in hypermarkets are usually washed using normal water without any treatments, packed and stored in at ambient temperature (refrigerator temperature) for a period of one week. This can increase bacterial replications rate and probability of cross-contamination.

Coliforms are associated with both soil and decaying vegetation. The survival of enteric bacteria in soil is particularly one of the main reasons in the contamination of raw vegetables (Geldreich *et al*., 1962). Microbial contamination of fresh vegetables with *K. pneumoniae* is an important food safety concern. Consumption of contaminated fresh vegetables can represent a potential risk to consumer's health, particularly in immunocompromised individuals. As mentioned before, *K. pneumoniae* is considered opportunistic pathogens, so healthy adults are not considered to be at high risk of developing infections and illness.

In order to avoid food-borne disease risk, special attention must be paid to improvement and control of

the hygienic quality of fresh vegetables. According to Rao and Rao (1983), *Klebsiella* cannot be easily dislodged from the surface of vegetables by gentle washing. Washing vegetables using disinfectants is one of the suggested ways to reduce risk. Sodium hypochloride or potassium permanganate solutions can be used against a wide number of microorganisms (Adams *et al*., 1989; Albrecht *et al*., 1995; Beuchat *et al*., 1998; Brackett, 1992; Garg *et al*., 1990; Lisle *et al*., 1998; Reynolds *et al*., 1989). Potassium permanganate is as antimicrobial compound that is used in washing process of vegetables and is used to indicate if the rinsing procedure is correctly accomplished.

Conclusion

In conclusion, it was found that *K. pneumoniae*, was present in the studied vegetables sold at wet markets and hypermarkets in Selangor, Malaysia. The study also showed that the MPN-PCR is an effective method for quantitative detection of *K. pneumoniae*. The data presented here will be useful for the microbiological risk assessment of *K. pneumoniae* associated with raw vegetables consumption in Malaysia.

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