Biochemical and Molecular Characterization of Food Contaminating Bacteria Isolates from Food Stall Vegetables

**Ali Ramadan Ali ALatawi[1], Sutarno[1], Ari Susilowati[1]
and Hailu Weldekiros Hailu[1,2*]**

[1]*Bioscience Postgraduate Program, Department of Biology, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta, Central Java, Indonesia.*
[2]*Department of Biology, College of Science, Bahir Dar University, Bahir Dar, Amhara, Ethiopia.*

This research was carried out in collaboration between all authors. Authors ARAA and HWH managed literature search, analyzed the study and compiled the manuscript. Authors Sutarno and AS supervised the work. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Today the issue of food safety is global problem that gets main concern in setting public health policy. The eruption of diseases caused by food contamination occurs in places where sanitation and hygiene conditions are generally poor. Reliable identification of bacteria remains to be an important task in food microbiology. Molecular procedures are the most efficient tools of microbial characterization. The objectives of this research were: To identify food contaminating bacteria from vegetable sample taken from food stall using biochemical tests; And to conduct molecular characterization of food contaminated bacteria isolated from vegetable sample in food stall. Six different (pair of cooked and uncooked) vegetable samples were isolated from three food stalls at Solo, Indonesia. Biochemical tests for glucose, lactose, mannitol, maltose, sucrose, SIM (H_2S, Indole, and motility), Simmon's Citrate; Methyl Red and Voges Proskauer was conducted. 16S rRNA characterization and DNA sequence analysis was done. Both the biochemical and molecular characterization revealed that the dominant bacteria contaminants in the food stall vegetable samples were: 24 isolates of *Klebsiella* Spp, 3 isolates of *Pseudomonas aeroginosa,* 2 isolates of

_____

Corresponding author: E-mail: hawelbirtu@hotmail.com;

Aeromonas caviae and 6 isolates of *Enterobacter asburiae*. Only one out of 36 samples was uncontaminated with bacteria. In conclusion most of the bacteria studied were pathogenic bacteria. Therefore, to prevent food borne disease, attention should be given on hygiene and food handling in food stalls vegetables.

1. INTRODUCTION

Food serves as an important vehicle for pathogenic microorganisms such as bacteria, viruses and parasites [1]. Appropriate methods of detecting food contaminant pathogens are necessary to pave the way for effective food borne disease prevention [2,3].

Classical techniques of detecting coliforms include biochemical characterization, study of cell morphology, colony morphology and microbial culture techniques in lactose fermenting media [4]. However, due to the limited specificity and time consuming nature of these techniques, DNA based molecular techniques such as 16S rRNA gene sequencingis highly specific [5]. Molecular methods are necessary for the identification of environmental bacterial isolates and species with an incomplete biochemical/phenotypic description. Isolates well identified at the species level by conventional phenotypic methods served as a control for both conventional and molecular identification procedures [6].

Species of bacteria can be also identified by combining two or more techniques of phenotypic, biochemical and molecular techniques. It was suggested that phenotypic characters should be supported by molecular methods to obtain meaningful results for identification of *Pseudomonas*. For this reason, 16S rRNA characterization of Pseudomonas spp. was conducted. The protein profiles of *Pseudomonas aeruginosa* strains have high level of similarities when compared with each other [7]. *P. aeruginosa*, found to be distributed in soil, water, and marine environments and pathogenic to both humans and animals, is gaining much popularity in clinical scientific research where it accounts for increased mortality and morbidity rates in immuno-deficient patients who suffer from cystic fibrosis, burn wounds, and cancer [8].

The genus *Aeromonas* (particularly, family *Aeromonadaceae*) are human and animal pathogens which are widely spread in all environments. This genus has highly conserved 16S rRNA gene sequence which is therefore helpful for its identification [9].

It was reported that the gram negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped *Klebsiella pneumonia* bacteria had surpassed *E. coli* as the predominant isolate from patients with emerging disease pyogenic liver abscess (PLA) and it tended to spread globally [9]. Additionally, *Klebsiella pneumoniae* is frequent nosocomial pathogen causing urinary, respiratory and blood infections and the agent of specific human infections including Friedlander's pneumonia, rhinoscleroma [10].

It was reported that *Enterobacter* species have become increasingly important nosocomial pathogens [11]. However, resistance to cephalosporin often complicates the treatment of *Enterobacter* infection. Various infections including bacteremia, gastroenteritis, cholangitis, and wound infections can be caused by *Aeromonas* species [12].

The aim of this research is to identify food contaminating bacteria from vegetable sample taken from food stall using biochemical tests and molecular characterization.

2. MATERIALS AND METHODS

2.1 Biochemical Test for Food Contaminant Bacteria

The research of biochemical and molecular characterization of food contaminating bacteria isolated from vegetables sample in food stall was conducted at the Micro and Molecular biology Laboratory of the Faculty of Math and Natural Sciences, Sebelas Maret University in June 2014.

Six different pair of cooked and uncooked vegetable samples (known by their local names as Tumis Kangkung, Lalapan Kubis, Terancam, Tumis Sawi Putih, Lalapan Kemang and Tumis Sawi Hijau) was randomly collected using sterile

plastic bags from three different food stalls in Solo, Indonesia.

25 g of each vegetable sample were mixed with 225 ml distill water in Erlenmeyer flasks separately. About 50 µl of the sample mix was taken and spread onto Macconkey agar aseptically and incubated at 37°C for 18-24 hours. Single colonies were streaked with a loop into other plates to get pure colonies and then incubated the plates at 37°C overnight. Finally, test microorganisms were inoculated into test tubes containing glucose, lactose, mannitol, maltose, sucrose, SIM (H2S, Indole, and motility), Simmon's Citrate, methyl Red and Voges Proskauer biochemical test and incubated at 37°C for 24 hours [13]. Longer incubation period was required to confirm a negative result.

2.2 16S rRNA Molecular Characterization

There are numbers of nucleic acid based methods for the detection and characterization of bacteria. Most methods are based on the use of the polymerase chainreaction (PCR) to amplify a specific gene target in the bacteria. Bacterial genomic DNA was extracted from the selected isolates by using (Presto™ Mini gDNA Bacteria Kit, Geneaid, USA).

2.3 PCR Amplification 16s rRNA Characterization

For amplification of 16S rRNA fragments, a pair of eubacteria universal primers having following sequence was used [10]:

Forward primer 63f: 5'-CAGGCCTAACACAT-GCAAGTC-3' and Reverse primer 1387r: 5'-GGGCGGAWGTGTACAAGGC-3'

A 25µl PCR reaction volume consisted 2.5 µl of IX PCR buffer, 1 µl of MgCl$_2$, 1µl of dNTPs, 1 µl of each primer, 0.5 µl of Taq DNA polymerase,2 µl of template DNA and 16 µl of ddH$_2$O. PCR was performed in 200ml thin walled tubes with a thermal cycler under the following cycling conditions: Initial denaturation at 95°C for 3 min, denaturation at 94°C for 15 sec, annealing at 62°C for 15 sec, extension at 72°C for 5 sec followed by final extension at 72°C for 10 min. Total number of cycles were 30. PCR products were electrophoresed in 0.8% agarose gel along with 100bp DNA ladder and stained with loading dye. Amplicons were visualized under UV light GelDoc.

3. RESULTS AND DISCUSSION

3.1 Biochemical Characterization

Here in Table 1, only nine representatives out of the 36 sample are presented due to similarity in biochemical test results. This is to avoid duplication of the similar results. The biochemical characterization (Table 1) supported by and molecular analysis (see Table 3 below) reveal that the dominant bacteria contaminants in the food stall vegetable samples were 24 isolates of *Klebsiella Sp.,* 3 isolates of *Pseudomonas aeroginosa,* 2 isolates of *Aeromonas caviae* and 6 isolates of *Enterobacter asburiae.* Only one out of 36 samples was uncontaminated with bacteria.

In biochemical tests of glucose in most samples positive results are obtained as the test solutions turn from red to yellow. Formation of gas bubbles that occur in the Durham tube fermentation reactions is caused by the glycolysis gaseous byproduct of glucose.

From the test results the majority of samples were positive for maltose, lactose, Manitol and sucrose which were confirmed by change of the medium from red to yellow color. This is because these bacteria have enzymes that can break down these carbohydrates.

Microbial fermentation is a biochemical activity that is carried out by microbes which convert organic macromolecular compounds into simpler compounds in anaerobic conditions. Fermentation can produce a variety of end compounds, such as carbohydrate fermentation to produce various acidic compounds such as lactic acid and propionic, esters, ketones and gas. Most of the microorganisms obtain energy from carbohydrates in the form of further substrate in fermentation produces organic acids (such as lactic acid, formic, acetic), accompanied or not accompanied by the formation of gas.

Indole test is used to see the ability of bacteria to degrade amino acid tryptophan enzymatically. All the samples were negative for indole test. This test is performed to help differentiate species of the family *Enterobacteriaceae.* It tests for the bacteria species' ability to produce indole. Bacteria use an enzyme, tryptophanase to break down the amino acid, tryptophan, which makes by-products, of which, indole is one.

Table 1. The results of biochemical tests for the identification a Bacteria isolated from vegetable samples from food stall

No	Biochemical test parameter	C3a	C5a1	A5a	A2b	B1a	A1a2	B4b1	B6a	A6a2
1	Glucose	+*	+*	+*	+*	+*	+*	+*	+*	+*
2	Lactose	+	+	_	_	+	+	_	_	_
3	Mannitol	+	+	+	+	+	+	+	+	+
4	Maltose	+	+	+	+	+	+	+	+	+
5	Sucrose	+	+	+	+	+	+	+	+	+
6	SIM H2S	_	_	_	_	_	_	_	_	_
	Indole	_	_	_	_	_	_	_	_	_
	Motility	_	_	+	_	_	_	_	_	_
7	Simmon's Citrate	+	+	+	+	+	+	+	+	+
8	Methyl RED	_	_	_	_	_	_	_	_	_
9	Voges Proskauer	_	_	_	_	_	_	_	_	_

+= Positive plus gas*

Positive results were obtained for most bacterial colonies grown test tubes containing citrate medium. This indicates that the bacteria have citritase enzyme which breaks down citrate into oxaloacetate and acetate. Simmon's Citrate test is used to look at the ability of enteric organisms based on the ability to ferment citrate as a carbon source. Citrate is selective test used to help differentiate species of the family *Enterobacteriaceae* media and is utilized as a single carbon and nitrogen source. To test this ability bacteria are incubated in medium that contains only citrate as a source of carbon and Ammonium phosphate as a nitrogen source. Simmon's Citrate test contains bromtimol blue indicator that will turn blue at the positive reaction and remain green if negative reactions [14].

Methyl Red, a test used to identify mixed acid fermenting bacteria that yield a stable acid end product causing the pH to drop below 4.4. Positive results are characterized by the color changes to red after adding Methyl Red. Methyl Red test result in most samples was negative.

All samples are negative, no change in color, for Voges Proskauer Test (a test used to identify bacteria capable of 2, 3 butanediol fermentation following mixed-acid fermentation).

3.2 Molecular Characterization

3.2.1 Isolation DNA

The Presto™ Mini gDNA Bacteria Kit was used for bacterial DNA isolation. DNA isolation results showed that the bacteria DNA were isolated with good quality when checked by Eppendrof biophotometer (Table 2). This is because the value of the isolated DNA at purity level of A260/280 ratio ranged from 1.90 to 1.99. DNA isolates are said to be pure and eligible to proceed to the molecular analysis if the A260/280 ratio values ranged from 1.8 to 2.0.

Table 2. DNA concentration results of bacteria DNA isolates

Bacterial isolates	DNA concentration (µg/ml)	The ratio A260/A280
C3a	1.8	1.85
C5a1	5.2	1.95
A2b	1.2	1.96
B1a	1.5	1.99
A1a2	4.1	1.95
B4b 1	1.6	1.90
B6a	1.6	2.0
A6a2	1.5	1.94

3.2.2 Amplification 16S rRNA genes

Amplifying the 16S rRNA gene can be well compared with other types of primary and consistently amplify 16S rRNA genes from different organisms. The results of 16S rRNA gene amplification by PCR were analyzed by agarose gel electrophoresis 0.8% (w / v) for 45 minutes at a voltage of 90 volts and a current of 400 mA. The results of 16S rRNA gene PCR amplification followed by electrophoresis and Gel Doccan be seen in Fig. 1.

Fig. 1 shows that bacterial DNA was successfully amplified. This is indicated by the presence of bright bold bands. This success indicates that the primers used attaches to specific sites on the DNA template with the optimum temperature used for primer annealing. The optimum temperature for DNA template annealing byprimer can be known by looking at the information contained in the primary packaging.
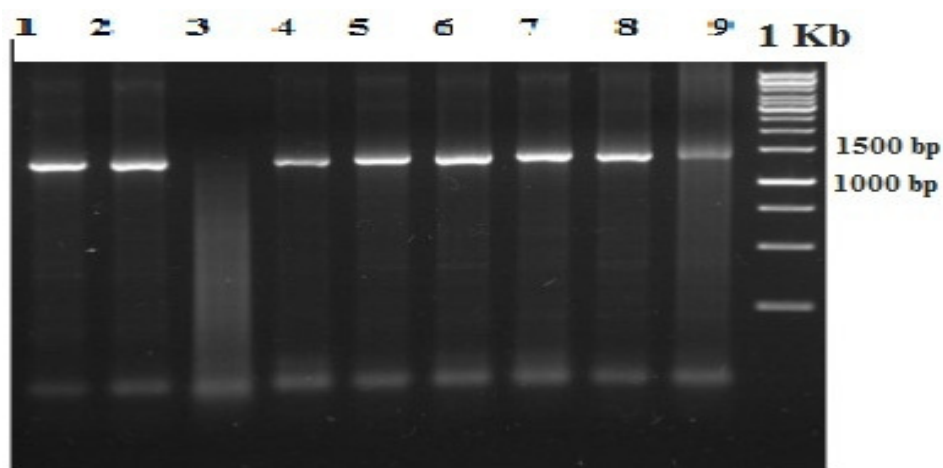
Fig. 1. Electrophoresiso16S rRNA gene by using the primers 63F and 1387r: 1 Kb DNA Marker M, 1). C3a, 2). C5a. 3). A5a, 4). A2b, 5). B1a, 6). A1a2, 7). B4b2, 8). B6a, 9). A6a2. Note: No band was observed for sample number A5a (may be due to DNA transportation conditions)

PCR product size can be determined by comparing the length of the DNA bands migrating with DNA markers of known size and concentration. The results of 16S rRNA gene amplification with primers 63F and 1387r are approximately 1,300 bp as seen in a DNA marker size 1 Kb.

3.2.3 Bioinformatics (sequence analysis of 16S rRNA genes)

The DNA sequencing data for the amplified 16S rRNA gene in this study was obtained through the route of PT. Genetics Science Jakarta-Singapore 1stBase, cycle sequencing performed on ABIprism™ 310 Automated DNA Sequencer (PE Applied Bio systems).

The nucleotide sequences of genes encoding 16S rRNA were analyzed with the Gen Bank database using the BLAST program to confirm the identity of the isolates with previously known

bacteria species (Table 3). BLAST is a software algorithm to compare the primary sequences of biological information, such as the amino acid sequences of different proteins or DNA sequences of nucleotide bases seen.

Based on the existing database in Gen Bank, all of the isolates have similarity with pathogenic bacteria. These nine isolates include: C3a with 94% similarity to *Klebsiella pneumoniae* strain CCFM8369;C5a1 98%with similarity *Klebsiella pneumoniae*PMK1;A2b with 99% similarity to Pseudomonas aeruginosa strain SMVIT;B1a with 97% similarity to *Klebsiella sp.* CCFM8373;A1a2 with 96% similarity to *Klebsiella sp.* T-3-1; B4B1 with 99% similarity to *Aeromonas caviae* strain VIT-SHRJ;B6a with 98% similarity *Enterobacter asburiae* L1 complete genome; And A6a2 with 97% similarity *Enterobacter asburiae* strain M-T-MRS_50. However, no sequence data was obtained for A5a.

Table 3. Similarity of 16S rRNA gene sequences of isolates with bacteria on the NCBI/GenBank database based on BLAST analysis

Isolate code	Similarity from BLAST	Accession	Identity
C3a	*Klebsiella pneumoniae* strain CCFM8369	KJ803926.1	94%
C5a1	*Klebsiella pneumoniae* strain PMK1	CP008929.1	98%
A5a			
A2b	*Pseudomonas aeruginosa* strain SMVIT	KJ671465.1	99%
B1a	*Klebsiella sp.* CCFM8373	KJ803930.1	97%
A1a2	*Klebsiella sp.* T-3-1	FJ577968.1	96%
B4b1	*Aeromonas caviae* strain VIT-SHRJ	KJ716457.1	99%
B6a	*Enterobacter asburiae* L1	CP007546.1	98%
A6a2	*Enterobacter asburiae* strain M-T-MRS_50	JQ795798.1	97%

In this study, 94-99% 16S rDNA sequence similarity appeared reasonable cut-off values to delineate different genera. The phylogenetic relationships of the genus Klebsiella to other genera of the family *Enterobacteriaceae* have been previously studied by genomic DNA relatedness. The genus Klebsiella consists of three phyletic lines shared with other members of the *Enterobacteriaceae*, including *Enterobacter aerogenes*, Erwinia and Tatumella. The genus Klebsiella is found to be heterogeneous based on the BLAST analysis. The medical importance of the genus Klebsiella led to its subdivision into three species corresponding to the diseases they caused: *Klebsiella pneumoniae*, *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis*. *Klebsiella pneumoniae* is a member of the *Enterobacteriaceae*. *Klebsiella* spp., particularly *Klebsiella pneumoniae*, is important causes of nosocomial infections [15].

The rRNA gene is the most conserved DNA in all cells. The 16S rRNA gene is used as the standard for classification and identification of microbes, because it is present in most microbes and shows proper changes [16]. Due to the reason that portions of the rDNA sequence from distantly related organisms are remarkably similar, sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria species. For example, *Klebsiella pneumoniae* strain CCFM8369, *Klebsiella pneumoniae* str. Kp52.145, *Pseudomonas aeruginosa* strain SMVIT, *Klebsiella* sp. CCFM8373, *Klebsiella sp.* T-3-1, *Aeromonas caviae* strain VIT-SHRJ, *Enterobacter asburiae* L1, complete genome, and *Enterobacter asburiae* strain M-T-MRS_50. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms. Therefore, the use of 16S rRNA gene sequences to study bacterial taxonomy has been by far the most common and helpful housekeeping genetic marker used.

4. CONCLUSION

The result from biochemical test indicated that *Pseudomonas aeruginosa, Klebsiella sp.* (eg. *Klebsiella pneumonia)* isolates are able to ferment glucose, lactose, mannitol, maltose and sucrose. However, *Aeromonas caviae* and *Enterobacter asburiae* are lactose non-fermenting. Most the bacteria samples were negative for methyl red, vogesproskauer, and SIM (H_2S, Indole, and Motility). Most of the samples are positive for Simmon's Citrate test which means the organisms are able to utilize citrate as a carbon source.

The molecular characterization of pathogenic bacteria isolated from salad vegetable taken from food stall shows that the samples were contaminated with bacteria such as *Klebsiella* Spp*, Pseudomonas aeroginosa, Aeromonas caviae and Enterobacter asburiae* which were compared for their similarity to some pathogenic bacteria, according to the results obtained from NCBI.

In conclusion most of the bacteria identified based on molecular and biochemical characterization are pathogenic. It can be said that salad provided in food stall may become the source of food borne disease.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Newell DG, Koopmans M, Verhoef, L, Duizer E, Aidara-Kane A, Sprong H, Opstegh M, Langelaar M, Threfall J, Scheutz F, Giessen VJ, Kruse H. Food-borne disease the challenges of 20 years ago still persist while new ones continue to immerge. Int J Food Microbiol. 2010;139:S3-S15.
2. Hanashiro A, Morita M, Matté GR, Matté MH, Torres, EAFS. Microbiological quality of selected street foods from a restricted area of Säo Paulo city, Brazil. Food Control. 2005;16:439-444. Available: http://dx.doi:10.1016/j.foodcont.2004.05.004
3. Schlundt J. New directions in food borne disease prevention. Int J Food Microbiol. 2002;78:3-7. Available: http://dx.doi:10.1016/S0168-1605(02)00234-9
4. Kumar RS, Roymon MG, Ipe JA. Isolation, biochemical and molecular characterization of strains of coliforms from the water sample collected from Shivnath River. Recent Research in Science and Technology. 2013;5(2):57-63.

5. Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. J. Mol. Diagn. 2001;6:313-321. [PubMed].

6. Karunasagar I, Kumar HS. Molecular methods for rapid and specific detection of pathogens in seafood. Aquacult Asia. 2002;3:34-37.

7. Uğur A, Özgür Ceylan, Belma Aslım. Characterization of *Pseudomonas spp.* from seawater of the southwest coast of Turkey. J. Biol. Environ. Sci. 2012;6(16):15-23.

8. Osayande J. Easy Identification of Difficult-to-Type *Pseudomonas aeruginosa* clinical and environmental isolates. The Internet Journal of Microbiology. 2008;7:2.

9. Kupfer M, Kuhnert P, Korczak BM, Peduzzi R, Demarta A. Genetic relationships of *Aeromonas* strains inferred from 16S rRNA, gyrB and rpoB gene sequences. International Journal of Systematic and Evolutionary Microbiology. 2006;56:2743–2751.

10. Brisse S, Fevre C, Passet V, Jeanjean SI. Tournebize R, Diancourt L, Grimont P. Virulent clones of *Klebsiella pneumoniae*: Identification and Evolutionary Scenario Based on Genomic and Phenotypic Characterization. 2009;4(3):1-13. DOI: 10.1371/journal.pone.0004982

11. Drancourt M, Bollet C, Carta A, Rousselier P. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. Nov., with description of *Raoultellao rnithinolytica* comb. Nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. International Journal of Systematic and Evolutionary Microbiology. 2001;51:925–932.

12. Kimura M, Araoka H, Yoneyama A. *Aeromonas caviae* is the most frequent pathogen amongst cases of *Aeromonas* bacteremia in Japan. Scandinavian Journal of Infectious Diseases. 2013;45(4):304-309.

13. Hussain T, Roohi A, Munir S, Ahmed I, Khan J, Veronique, Edel-Hermann, Kim KY, Anees M. Biochemical characterization and identification of bacterial strains isolated from drinking water sources of Kohat, Pakistan. African Journal of Microbiology Research. 2013;7(16):1579-1590. DOI: 10.5897/AJMR12.2204.

14. Vincent CC, Cheng, Yam W, Tsang L, Miranda CY, Yau, Gilman KH, Siu, Sally CY, Wong, Hung, Josepha, Tai WM, Ho P.and Yuen K, Jasper FW, Chan, Kelvin KWT, Tse H, Ivan, FN. Epidemiology of *Klebsiella oxytoca*-Associated Diarrhea with detected by simmons citrate Agar supplemented with Inositol, tryptophan, and bile salts. J. Clin. Microbiol. 2012;50(5):1571.
DOI:10.1128/JCM.00163-12.

15. Liu Wang J, Jiang W. An increasing prominent disease of *Klebsiella pneumonia.* Gastroenterology Research and Practice. 2013;1-12.

16. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. Genes coding for bacterial 16S rRNA bacterium-specific PCR primers that amplify design and evaluation of useful. Appl. Environ. Microbiol. 1998;64(2):795.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=825&id=8&aid=6875