October 31, 2021

Isolation and Biochemical Characterization of Enterobacter Cloacae Isolates from Ready-To-Eat Foods Using API 20E

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Received Date: 13-Sep-2021 Accepted Date: 30-Sep-2021 Published Date: 31-Oct-2021

Abstract.

Ready-to-eat foods have become a global concern because they are a source of income for low and middle-income people. Also, they constitute the main food for students and lower to middle-level workers. However, a lack of knowledge about good food processing practices results in microbial contamination that interferes with human health. Enterobacter cloacae is one of the most common bacterial contaminants found in ready-to-eat foods in many countries. Therefore, the purpose of this research is to identify E. cloacae using the API 20E kit on ready-to-eat foods in Yogyakarta city and its surroundings. Subsequently, 115 samples of 10 types of food were collected from various locations for isolation and identification of the contaminant. The results showed that 7 foods were found to be contaminated, with processed egg products having the highest contamination level at 40%. This was followed by various snacks and packaged dairy products at 30% each, then skewered meatballs, and other processed food products, at 20% and 10%, respectively. Meanwhile, dumplings, potato products, and assorted iced drinks were not contaminated by the bacteria.

Keywords: *Isolation, Biochemical Characterization*

INTRODUCTION

Congenital diseases caused by consuming food contaminated with pathogenic bacteria occur in many countries. Hence, reports of each case are important to trace the causative contaminating agent and ensure prevention can be performed as early as possible. The Foodborne Disease Outbreak Surveillance System (FDOSS), in 2009-2015, received reports of 5,760 extraordinary cases consisting of 100,939 illnesses, 5,699 hospitalizations, and 145 deaths [1]. Meanwhile, contamination can occur at any time, from agricultural land to dining tables, including the hands of restaurant workers, which are also a source of foodborne illness. In the period 1998-2013 found 17,455 outbreaks and 9788 (56%) cases related to food prepared in restaurants [2]. Also, studies in 2009-2018 showed that people with low to middle-income levels had the highest health problems due to the consumption of contaminated ready-to-eat (RTE) foods.[3] Some of the most common bacterial contaminants found in these foods were *Staphylococcus aureus, Bacillus, Listeria monocytogenes, Pseudomonas spp., Klebsiella pneumonia, Klebsiella oxytoca, Citrobacter freundii, Kluyvera ascorbate, Escherichia coli,* and *Enterobacter cloacae*. Most of the samples studied showed that *E. cloacae* were the highest contaminating bacteria at a level of 32-50%, especially on RTE vegetables [3, 4, 5]. The research also found that the pathogenic bacteria in processed meat and chicken snacks in Windhoek, Namibia, was also dominated by *E.*

cloacae [6]. Furthermore, this bacteria was the multidrug-resistant nosocomial pathogen that triggered outbreaks in susceptible patients in neonatal intensive care units (NICU) in France (2012-2018) and Nepal (2012-2013), causing gastrointestinal infections and meningitis [7, 8].

Ready-to-eat foods are taken directly without further processing and are widely consumed by school-age children and low-income workers. High contamination is caused by the lack of knowledge in RTE vendors about hygiene and good food-handling practices, from material preparation and processing to serving. Consumption of contaminated RTE foods causes foodborne illness and health problems depending on the amount and type of contaminants [3]. Subsequently, other sources of contaminants are linked to the food supply chain, such as animal feed. Research by Gana (2019) showed coliform contamination exceeding the $\geq$ log10 4.0 threshold, with the dominating contaminant being *Enterobacter cloacae* at 54.5%, followed by *Bacillus cereus* and *Klebsiella pneumoniae* at 27.3% and 18.2%, respectively. These isolates had multidrug resistance (MDR), which was dominated by *B. cereus* to 9 out of 11 antibiotics [9]. Meanwhile, Ready-to-eat foods, which are also menu choices for students in Yogyakarta, Indonesia, are often found around schools, offices, and public places. Yogyakarta has many popular snack menus, such as *cilok*, skewered meatballs, and others, and these snacks are sold by street vendors who lack knowledge and good food-processing practices and allow contamination with pathogenic bacteria that interfere with health. Therefore, this research aims to detect the level of *E. cloacae* contamination in food and snacks in schools and public places in Yogyakarta.

RESEARCH METHODOLOGY

Sample Collection

A total of 115 samples of snacks and beverages were collected from street vendors around schools and public places in Yogyakarta. They were placed in sterile containers and taken to the Microbiology Laboratory of Duta Wacana Christian University for microbiological testing with less than two hours for sampling.

Enumeration and Isolation of *Enterobacter cloacae*

A sample quantity of 10 grams was placed into 90 ml (1:10) Buffered Peptone Water (BPW) to allow the bacterial cells injured during the food processing to return to health and grow well in the isolation medium. BPW was incubated for 24 hours at 37°C [10, 11], and a series of dilutions were performed using 1ml of the healthy cell cultures and 9 ml of 0.1% to 10^{-7} peptone water. The cell cultures were homogenized using a vortex, and 0.1 ml was taken from the 10^{-4}, 10^{-5}, $10^{-6,}$ and 10^{-7} dilutions to be grown on a Chromocult Coliforms Agar (CCA) medium. Subsequently, the suspected *E. cloacae* colony expressed a typical salmon-red color [12, 13]. Then, it was separated by a streak plate using CCA to obtain a single isolate, which was grown on Brain Heart Infusion Agar (BHIA) medium as a collection of isolates. Subsequently, the isolates were tested biochemically at the genus level using Indole production, Methyl red, Voges–Proskauer, Citrate, Hydrogen sulfide, Fermentation of Lactose, and D-sorbitol assays [14, 15]. The isolates that were suspected at the genus level were confirmed biochemically using API 20E.

Confirmation of *Enterobacter cloacae* using API 20E

The confirmation stage from the level of the genus *Enterobacter spp* to the species *E. cloacae* was performed biochemically using the API 20E kit, Biomereoux. Here, the suspected isolate was regenerated on BHI Agar medium for 18-24 hours at 37^{o}C on a streak plate. The growing colonies were taken using an aseptic tube and aseptically dissolved in a 5ml physiological salt diluent medium containing 0.85% NaCl. Cells were suspended at a turbidity level equivalent to a 0.5 McFarland solution, followed by the inoculation of the suspension into 20 biochemical test wells on the API 20 E test kit (Biomeriux) using a sterile Pasteur pipette. Test wells with bottom lines, namely ADH, LDC, ODC, H2S, and URE, were inoculated with only half of the cell suspension. Then, mineral oil was added to the cupule until it was full to create anaerobic conditions. The cell suspension that was fully inoculated to the top (cupule) was only in the CIT, VP, and GEL test wells, while the suspension was half the well height or up to the tube boundary for other test wells. After the inoculation, the API 20E kit

was incubated at 37°C for 18-24 hours to observe each test for a positive or negative reaction. Some test wells, namely VP, IND, TDA, and GLU, required an additional drop of reagent to determine whether the results were positive or negative. Subsequently, VP1 and VP2 reagents were added to the VP test well, James reagent was added to IND, and TDA reagent to TDA. The addition of Nit 1 and Nit 2 reagents was necessary for the GLU test well to identify the presence of NO_2. A positive reaction was marked by a change to red, while a consistent yellow color meant the result was negative, which was followed with Zn powder to test for the presence of N_2 gas. The results from each test well were confirmed using web API software to obtain the identity of the tested isolates. Furthermore, the species name and %ID showed the profile indexes or the magnitude of the similarity between the tested biochemical characters and the database of the *Enterobacteriaceae* family in the software of the API 20E system, Biomereoux [16, 17, 18].

RESULTS AND DISCUSSION

The Results of the Isolation and Enumeration of *E. cloacae* Colonies

Field observations of street food and beverages sold by street vendors in schools and public places were performed before sampling. Yogyakarta has many popular snacks of interest to students and the public, including skewered meatballs, cilok, dumplings, various snacks, various iced drinks, packaged milk, and bottled drinking water. Subsequently, the samples were first grown on BPW medium for 24 hours to detect *E. cloacae* in the ready-to-eat foods. The BPW medium, used as a pre-enrichment medium, allowed the bacterial cells damaged by processing treatments to become healthy and be grown on selective media [10, 11]. Then, the Chromocult Coliform Agar (CCA) medium was used for the enumeration of bacteria suspected to be *E. cloacae*. Figure 1 shows the colonies that appeared on the CCA medium from the *Enterobacteriaceae* family members and the suspected *E. cloacae* colony, which gave a typical red color. The total colonies that grew on the CCA medium were from the *Enterobacteriaceae* family.

FIGURE 1. The Appearance of Typical Bacterial Colonies From the *Enterobacteriaceae* Family Members on CCA Medium

This is a selective differential medium capable of giving the appearance of different bacterial colonies from each genus in the Enterobacteriaceae family, including *E. cloacae*. The CCA medium is composed of a chromogenic substrate for the expression of the synthesis of the enzyme β-glucuronidase (5-Bromo-4-Chloro-3-Indoxyl-β-D-glucuronide), otherwise known as X-GLUC or BCIG and β-galactosidase substrate (6-Chloro -3-Indoxyl-β-D-galactoside), known as Salmon-GAL. Enterobacter, Klebsiella, and *Citrobacter* are members of the *Enterobacteriaceae* family that can express the enzyme β-glucuronidase. Hence, they use the X-GLUC substrate to produce salmon to red or mauve colonies on CCA appearance.[12,19] Other members, such as *Salmonella*, *Shigella*, and *Yersinia*, are incapable of using 6-chloro-3-indoxyl-β-D-galactoside as a substrate but can use X-GLUC substrate to give white, bright blue, or transparent colonies. Meanwhile, *Escherichia coli* uses these two chromogenic substrates to produce typical dark blue colonies [19, 20].

Table 1 shows the enumeration of total bacterial colonies on the CCA medium. On counting, they showed a varying range in each type of snack food tested, and the difference in the number of bacteria indicates that the contamination level varies greatly. The reason was the differences in the sample originating from differences in the raw material preparation and treatment, processing, personal hygiene, and different knowledge levels of food preparation practices. Another factor was the difference in the selling locations, containers, and the cleanliness of the environment. Furthermore, over 90% of the total snack samples exceeded the threshold based on the National Agency for Drug and Food Control (BPOM) regulation Number 13 of 2019 concerning the maximum limit of microbial contamination in processed food, at above 10^2 CFU/ml or grams. Meanwhile, the high microbial contamination level

in processed food products also occurs in South Africa. Here, foodborne pathogen test results from 252 ready-to-eat foods, including vegetables, potatoes, rice, pies, beef, and chicken stews from roadside cafeterias and retail outlets, showed contamination levels between 2.3 - 6.8 (log 10 CFU g^{-1}). The analysis of bacterial contaminants from the *Enterobacteriaceae* family on ethnic street foods in Gangtok, India, showed a range that also exceeded the threshold of 2.8 - 5.2 Log CFU/g sample [21, 22]. Furthermore, the total bacteria (aerobic plate count) in ready-to-eat foods from Middle Thailand showed that 56 of 135 samples (42%) had a contamination level of more than 6 logs CFU/g [23].

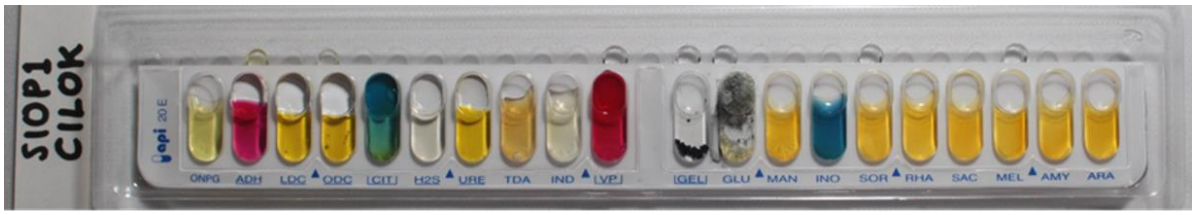
TABLE 1. Results of Total Enumeration of Bacteria Growing on CCA Medium

No.	Sample Origin	Number of Samples	The Range of Bacteria Growing on CCA Medium
1	**Processed Egg Products**	10	7.0×10^1 - 9.1×10^6 CFU/gr
2	**Snack**	10	4.6×10^5 - 6.1×10^8 CFU/gr
3	**Packaged Dairy Products**	20	1.4×10^5 - 7.2×10^9 CFU/ml
4	**Skewered Meatball**	10	1.7×10^9 - 3.5×10^9 CFU/gr
5	**Cilok**	10	1.0×10^3 - 2.0×10^8 CFU/gr
6	**Dumpling**	10	1.0×10^3 - 3.0×10^6 CFU/gr
7	**Potato Products**	10	1.8×10^4 - 2.0×10^8 CFU/gr
8	**Bottled Water**	10	6.6×10^5 - 2.3×10^8 CFU/ml
9	**Packaged Tea Products**	10	6.0×10^8 - 1.5×10^{10} CFU/ml
10	**Assorted Iced Drinks**	15	5.3×10^2 - 6.6×10^6 CFU/ml

Characterization of Suspected Isolates of *Enterobacter cloacae*

A series of biochemical tests are required for screening at the genus level, alongside an API 20E confirmatory tests at the species level to ensure the isolated colony is *E. cloacae*. The biochemical test used to select the genus *Enterobacter spp* was the IMViC test, where most of the members gave negative reaction for indole and methyl red test but positive reaction for voges proskauer and citrate or (- - + +) reactions, as opposed to the *Escherichia spp* genus, which gave a + + - - reaction. Further tests that led to the species estimation were its ability to hydrolyze urea alongside ferment lactose and sorbitol [14]. As shown in Figure 2, the isolates were confirmed biochemically using API 20E, which was selected as a bacterial identification system for the *Enterobacteriaceae* family because of the high accuracy level. There are twenty biochemical characters tested in the 20E kit to be able to distinguish the identity of species in the Enterobacteriaceae family. Confirmation results of the tested isolates (Figure 2) were isolates from the cilok sample which gave confirmation results as Enterobacter cloacae with a %ID of 95.3%. These results indicate that the character of the isolates tested has a 95.3% match between the identities of E. cloacae and the biomereoux database. These results are included in the category of very good identification (Biomeroux). Meanwhile, the results of the evaluation conducted by Devenish and Barnum (1980) on 235 isolates from 240 bacterial strains from the *Enterobacteriaceae* family showed a 97.5% validity. In 2014, Maina *et al*. also assessed the accuracy of the API 20E test on 1425 out of 1658 bacterial isolates with an 87.6% conformity or validity [24. 25]. The confirmation

results of the suspected isolates of *E. cloacae* in this research showed a %ID or Analytical Profile Index within a range of 91.3% - 99.3%. These results indicated high similarity between the biochemical characters of the isolates with the database in the Biomereoux software. According to Table 1, the RTE foods that were uncontaminated with *E. cloacae* among the 10 food types were dumplings, potato products, and assorted iced drinks. Of the 7 types, processed egg products had the highest level of contamination at 40%, followed by various snacks and packaged dairy products at 30% each, alongside skewered meatballs and other processed food products at 20% and 10%, respectively. The high level of *E. cloacae* contamination in RTE foods is presumably due to many factors, such as food vendors without formal education in clean food-processing techniques. They also lack good knowledge about the causes of foodborne illness, dirty equipment, non-standard processing, and the occurrence of cross-contamination after heating. Consequently, the risk of contamination was supported by changing sale locations, food being sold in the open on roadsides, public places, markets, or around schools and campuses [22, 23]. Based on the field observations, the food vendors did not maintain hygiene when serving food by s not washing their hands after counting money, holding body parts, such as their mouth, nose, and wiping sweat. Contamination also came from the food supply chain and raw materials used to make snacks like meat, chicken, fish, or dough [3, 6, 9].



DOUBTFUL PROFILE	
Strip	API 20 E V5.0
Profile	3 2 0 5 5 7 3 6 7
Note	POSSIBILITY OF Erwinia spp

Significant taxa	% ID	T	Tests against			
Enterobacter cloacae	95.3	0.17	ODC 92%	NO2 100%	N2 1%	
Pantoea spp 2	4.5	0.04	ADH 1%	NO2 85%	N2 0%	

Next taxon	% ID	T	Tests against			
Cronobacter spp	0.1	0.0	ODC 91%	INO 75%	SOR 1%	NO2 100%
			N2 0%			

Complementary test(s)	GLUCOSEg	YELLOW	MDG	
Enterobacter cloacae	100%	0%	85%	
Pantoea spp	20%	76%	7%	
Erwinia spp	-(+)	-(+)	-(+)	

FIGURE 2. The Results of The Identification of Suspected Isolates of *E. cloacae* from Cilok Snacks using API 20 E with a Conformity Index of 95.3%

Table 2. Identification of *E. cloacae* Isolates from RTE Foods

No.	Sample Origin	Number of Samples	The Results of the Identification using API 20E			Percentage of Contaminated Samples
			Isolate Code	Identified	% ID	
1	**Processed Egg Products**	10	S1.1M	*Enterobacter cloacae*	96,7%	4/10 (40%)
			S3.2M	*Enterobacter cloacae*	98,3%	
			S4.1M2	*Enterobacter cloacae*	98,4%	
			S6.2M	*Enterobacter cloacae*	97,6%	
2	**Snack**	10	S_1KSkBMU	*Enterobacter cloacae*	98,9%	3/10 (30%)
			S_3TtPdMU	*Enterobacter cloacae*	94,7%	

			S_{11}KKtKjMU	*Enterobacter cloacae*	98,9%	
3	**Packaged Dairy Products**	20	S_3MM_2 S_7MM_1 S_2P_1 S_9MU	*Enterobacter cloacae* *Enterobacter cloacae* *Enterobacter cloacae* *Enterobacter cloacae*	91,3% 97,3% 99,2% 95,7%	4/20 (20%)
4	**Skewered Meatball**	10	S9M1	*Enterobacter cloacae*	98,6 %	1/10 (10%)
5	**Cilok**	10	S10PM1	*Enterobacter cloacae*	95,3%	1/10 (10%)
6	**Dumpling**	10	S_1H_{P1}	*Enterobacter cloacae*	98,9%	1/10 (10%)
7	**Potato Products**	10	S1BG1	*Enterobacter cloacae*	99,1%	1/10 (10%)
8	**Bottled Water**	10	-	-	-	-
9	**Packaged Tea Products**	10	-	-	-	-
10	**Assorted iced drinks**	15	-	-	-	-

CONCLUSION

Of the ten types of RTE with a total sample of 115 studied, 7 types of RTE were contaminated with *E. cloacae.* The highest level of contamination was found in processed egg products, namely 40%, followed by various snacks (30%), packaged milk products (20%) and other snack products only 10%. The results of confirmation of the suspected bacterium *E. cloacae* using API 20E showed that it was identified as *Enterobacter cloacae* with the Conformity Index of (%ID) of 91.3% - 99.3%.

ACKNOWLEDGMENT

The authors are grateful to the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for the financial support provided under research funding contract no. 3278.5/LL5/PG/2021,and no. 266/D.01/LPPM/2021. The authors are also grateful to the members of Duta Wacana Christian University's Microbiology Laboratory.

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