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(RESEARCH ARTICLE)

Detection *Salmonella* sp. from swab clooca of broiler chicken at Keputran market, Surabaya Indonesia

Nissa Maulia Bestari [1], Adiana Mutamsari Witaningrum [1], Dadik Rahadrjo [1], Viski Fitri Hendrawan [2], Widjiati Widjiati [1], Epy Muhammad Luqman [1] and Wiwiek Tyasningsih [1,*]

[1] *Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.*
[2] *Department of Reproduction Faculty of Veterinary Medicine Universitas Brawijaya Malang, Indonesia.*

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Abstract

This study aims to determine the presence of *Salmonella* sp. from broiler cloacal swabs at Keputran Market, Surabaya Indonesia. The research methods used include sampling, isolation and identification using Tetrathionate Broth Base (TTB) Media, *Salmonella* Shigella Agar (SSA) Media and followed by Biochemical Test. Inoculation of samples on Tetrathionate Broth Base (TTB) Media followed by inoculation on *Salmonella* Shigella Agar (SSA) Media produces a transparent colony and there is a black spot in the middle. Microscopic examination was done by Gram staining. Suspected colony of *Salmonella* sp. were then purified and identify by biochemical tests. Bacterial biochemical test media used, such as Triple Sugar Iron Agar (TSIA), Simon Citrate Agar (SCA), Sulfide Indole Motility (SIM), Urea Test and Carbohydrate fermentation test. The results of the study from 30 samples of broiler cloacal swabs at Keputran Market, Surabaya found 2 positive samples detected of *Salmonella* sp.

Keywords: *Salmonella* sp.; Broiler; Cloacal swabs; Keputran Market; Surabaya Indonesia

1. Introduction

Salmonella sp. is a gram-negative bacterium that is pathogenic and is the agent that most often causes food borne disease in this world. Infection *Salmonella* sp. In animals and humans can cause salmonellosis which disrupts the digestive tract and in many cases, it can result in death. Salmonellosis in humans can be transmitted through food of animal origin contaminated by *Salmonella* sp. [1]. In animals, salmonellosis is the infectious disease that most affects commercial poultry production. Poultry products, especially broiler chicken meat, are contaminated by *Salmonella*. Occurrence in Switzerland (13.7%), Ireland (26.4%), Thailand (66.0%) and Korea (36.0%). Pathogenic agents that often cause salmonellosis in commercial poultry are *Salmonella pullorum*, *Salmonella gallinarum*, *Salmonella typhimurium*, *Salmonella enteritidis*. Based on the results of the Rapid Serum Agglutination (RSA) test in the laboratory on Day Old Chick (DOC) serum samples from broiler chickens sold by several companies in Lamongan Regency, the percentage of positive antibodies of *Salmonella pullorum* was 20% [2]. The incidence of salmonellosis in humans can be seen from the results of research which recorded that the incidences of Salmonellosis in the world in 2000, reported 21.6 million cases with 216 thousand deaths, and more than 90% occurred in Asia [3]. In 2001, Switzerland reported 2,677 salmonellosis attacks in humans (incidence rate 32 cases/100,000 population/year), this incidence increased by 8% from 2000.

Broiler chickens are the most economical livestock when compared to other livestock, their advantage is the speed of increase or production of meat in a relatively fast and short time or around 4 - 5 weeks of production, the meat can be marketed or consumed [4]. There is nutritional content in chopped chicken. 100 grams of chicken meat contain 74% water, 22% protein, 13 mg calcium, 190 mg phosphorus, 1.5 mg iron and around 30 IU of vitamins. Chicken meat is also rich in vitamin A, vitamin C and vitamin E. Besides being low in fat, chicken meat also includes unsaturated fatty acids [5]. Based on the Indonesian National Standard (SNI) about the maximum limit for microbial contamination in meat.

* Corresponding author: Wiwiek Tyasningsih, Email: witya_kh@yahoo.com

The food category of meat and meat products, including poultry meat and game meat, in the types of microbial contamination in *Salmonella* sp. the maximum limit is negative. The source of salmonellosis infection is contamination of broiler carcasses. The contamination process can occur during processing and can also come from recontamination of meat and other food ingredients. Processing thermal temperature at 66°C for 12 minutes or 60°C for 30 minutes can kill most *Salmonella* sp. [6]. There are several factors that influence contamination such as environmental factors, hygiene and equipment factors.

Efforts to control salmonellosis can be started at the production level on the farm by including day old chick (DOC) which comes from *Salmonella*-free parents stock, providing feed and drinking water free from *Salmonella*. Handling with the hygienic way for post-harvest livestock products (meat and eggs) and store it in a clean condition. Production equipment before and after use must be cleaned. Personnel involved in production must wash their hands before and after work. It is best to cook meat or eggs thoroughly before consuming them [7]. There are several diseases caused by the *Salmonella* sp., namely in humans such as diarrhea, vomiting, stomach cramps, abdominal pain and typhoid fever. Typhoid fever is a serious infectious disease and is an endemic disease which has become a global health problem, including in Indonesia and Southeast Asian countries such as Malaysia and Thailand. The incidence rate is among the highest in the world, namely between 358-810/100,000 population every year. This disease has a fairly high mortality rate, namely 1-5% of sufferers (Punjabi, 2004) [8].

According to data from the World Health Organization (WHO) in 2014, there were 21 million cases of typhoid fever worldwide with a death rate of 200,000 cases. In developing countries, it is reported that 95% of typhoid fever cases are outpatients. In Indonesia there are 900,000 cases with a death rate of around 20,000 cases. In Indonesia, typhoid must receive serious attention from various parties, because this disease is endemic and threatens public health. The problem is increasingly complex with the increase in career cases or relapse and resistance to the drugs used, making treatment and prevention efforts difficult. The typhoid morbidity rate in Indonesia is reported to be 81.7 per 100,000 population, with a distribution according to age group of 0.0/100,000 population (0–1 year), 148.7/100,000 population (2–4 years), 180.3/100,000 (5-15 years), and 51.2/100,000 (≥16 years). This figure shows that the majority of sufferers are in the 2–15-year age group [9]. According to Presidential Regulation no. 112 of 2007, traditional markets are markets built and managed by the central government, regional governments, BUMN, BUMD and private parties whose business premises are kiosks, shops, tents and stalls owned or managed by small, medium, cooperative, independent traders people whose buying and selling process is carried out through a bargaining process [10].

Keputran Market is the largest traditional vegetable market in Surabaya. Its distribution reaches almost the entire Surabaya area, especially in parts of North Surabaya and East Surabaya Indonesia. This market is located on Jalan Keputran, where the area is included in the central Surabaya area. This market is opposite the Kalimas River which is also the largest river in Surabaya. Keputran Market starts operating optimally at 18.00 WIB - 05.00 WIB. Keputran Market is the center of the agribusiness market in the heart of the city. The capital market is referred to as “the biggest traditional market in hero city” [11]. According to Setiowati et al. [12], the percentage of chicken meat samples from traditional markets in Indonesia that were positively contaminated with *Salmonella* is 10.06%. Contamination *Salmonella* sp. in chickens originating from infected farms [13].

2. Material and methods

The samples used for this research were cloacal swabs from broiler chickens at Keputran Market, Surabaya Indonesia. The cloacal swab sample size used was 30 samples. Broiler chicken cloacal swab samples were obtained at Keputran Market, Surabaya. Each sample was putted into the sterile flocked swab which already contains Buffer Peptone Water (BPW) and closed tightly, wrapped in plastic and stored in cool box filled with ice, then taken to the laboratory. The research was carried out from December 2021 to February 2022 at the Bacteriology and Mycology Laboratory, Veterinary Microbiology Division, Faculty of Veterinary Medicine, Universitas Airlangga.

2.1. Materials

The research materials used were Broiler Cloaca Swab Samples, Tetrathionate Broth Base (TTB) Media, *Salmonella* Shigella Agar (SSA) Media, 70% Alcohol, Identification Media namely Triple Sugar Iron Agar (TSIA), Simon Citrate Agar (SCA), Sulfide Indole Motility(SIM), Urea Agar, Carbohydrate (glucose, mannitol, maltose, sucrose, lactose), Gram Staining Materials (crystal violet, Lugol, 95% alcohol, safranin) and Buffer Peptone Water (BPW).

2.2. Tools

The equipment used in this research is cool box, flocked swab, gloves, masks, labels, markers, lab coats, tissue, scissors, isolation, Erlenmeyer flasks, test tubes, autoclaves, incubators, Petri dishes, stoves, object glass and cover glass, Bunsen flame, hose, tweezers.

2.3. Sample

The technique used is to insert a sterile swab into the cloaca quite deep in the mucosal wall with little feces, place the swab on the flocked swab with Buffer Peptone Water (BPW) transport media. Sample Inoculation on Tetrathionate Broth Base (TTB) Media: Samples in the form of homogeneous cloacal swabs were dipped in TTB media and incubate at 37^0C for 24 hours. Streaking Samples on *Salmonella* Shigella Agar (SSA) Media: The samples used for streaking on *Salmonella* Shigella Agar (SSA) media are culture that already grow on Tetrathionate Broth Base (TTB) media. After streaking, followed by incubation at 37^0C for 24 hours [14]. The colonies resulting from inoculation on SSA media are transparent colonies and black spot in the middle [15]. Microbes reduce thiosulfate to sulfate so that it appears as black colonies. Several *Salmonella* sp. produces black spots in the center of the colony (black center) because of H_2S production.

Bacterial Identification *Salmonella* sp.: The research has found several results indicating the presence of bacteria *Salmonella* sp. characterized by a black spot in the middle of the colony (black center) and to find out the results of bacterial growth, identification is carried out such as Gram staining, purification, identification of bacteria using biochemical tests. Gram staining: In Gram staining, a smear preparation was done and fixed it over a Bunsen flame until dry, then dripped it with a crystal violet solution (Gram A), and left for one minute, then washed using distilled water. Next, dripped with Lugol's solution (Gram B) and left for 2 minutes, then washed using distilled water and dried. Acetone alcohol 95% solution (Gram C) is dripped for 30 seconds, and washed using distilled water and dried. After that, dripped it with a solution of safranin (Gram D) and left it for 30 seconds, then washed using distilled water and dry it. Next, it was observed under the microscope with 1000x magnification [16]. The results of Gram staining are characterized by bacilli-shaped, red in color and showed Gram negative bacteria. Next, the bacterial colonies which showed the form of bacilli and red in color from the gram staining results, were multiplied on *Salmonella* Shigella Agar (SSA) media. Incubation is carried out at 37^0C for 24 hours.

2.4. Identification of Bacteria with Biochemical Tests

Suspected colonies of *Salmonella* on *Salmonella* Shigella Agar (SSA) media are identified by biochemical test. The bacterial biochemical test media used include Triple Sugar Iron Agar (TSIA), Simon Citrate Agar (SCA), Sulfide Indole Motility (SIM), Carbohydrate Media, Urea. In the Triple Sugar Iron Agar test, it is method which is used to see abilities microorganisms in fermenting sugar. TSIA medium contains 3 types of sugar, namely glucose, lactose, and sucrose, also phenol red as indicator as well as $FeSO_4$ to show H_2S formation which is indicated by its existence black precipitate. TSIA medium was inoculated by pricking until the bottom of media then streak it on the slant part of media (Lay, 1994). Positive research results show a yellow color (A = acid) on the slant or bottom of the media, and there are black precipitates. Meanwhile, negative results will show a red color (Alk= alkali) on the slant or bottom of the media.

In the Simon Citrate Agar Test is a selective medium based on the use of citrate. The media tests the ability of organisms to utilize citrate as a sole carbon source. The SCA medium is inoculated with a loop and then spread on the media. *Salmonella* gives positive results on the citrate test [17]. Positive results will be colored blue, while negative results will remain green. In the test, the Indole test is a component of the test series of IMViC, which is used to differentiate Enterobacteriaceae. Motility tests are useful for testing a variety of organisms. Overall, the SIM test is primarily useful for differentiating *Salmonella* and *Shigella*. SIM medium is inoculated by pricking on the media. SIM test can be distinguished based upon their ability to release sulfide gas (H_2S), produce indole from tryptophan, and move into the medium via the use of flagella. Specific test results for *Salmonella* is negative for the indole test, motile, and result in black precipitation for sulfide. In the Urea test, urea is a product of amino acid decarboxylation. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia makes the medium change to alkaline detected by changing the red color of phenol from light orange at pH 6.8 to magenta (pink) at pH 8.1. Urea medium is inoculated by piercing on the media. Specific test result of *Salmonella* is a negative urease test. Positive result is red, while negative results remain yellow.

In the Carbohydrate test, Hanes [18] stated that in the carbohydrate test *Salmonella* sp. is unable to ferment lactose but only glucose. It is characterized by a change in the color of the media from red to yellow, meaning that these bacteria form acid from glucose fermentation. The catalase test of *Salmonella* sp. is positive, while the oxidase test is negative. Carbohydrate media is inoculated by inserting a loop into a liquid medium, then homogenizing it. According to SNI

(2008), *Salmonella* sp. able to ferment mannitol and glucose. Positive fermentation results are characterized by a change in the red color to yellow due to the production of acid from bacterial metabolism. According to Latif et al. [19] *Salmonella* sp. has the characteristic of not fermenting lactose and the inability to ferment lactose is one of the important things in the diagnostic examination of bacterial criteria to distinguish bacteria from other members of bacteria.

Table 1 Biochemical Characteristics of *Salmonella* sp. (Bell and Kyriakides, 2002) [20]

Characteristics	**Reaction**
Catalase test	+
Oxidation test	-
Use urease	-
TSIA (Triple Sugar Iron Agar) Test	Alk/A, gas (-), $H2S$ (+)
Citrate test	+
Production of acid in lactose	-
Gas production in glucose	+, gas (+)
Indole	-

Information: (+)= positive reaction, (-) = negative reaction

2.5. Data analysis

The growth of bacterial colonies of *Salmonella sp*. are analyzed by identifying bacteria microscopically and by biochemical testing of bacteria.

3. Result and Discussion

Salmonella sp detection results on the cloacal swab of broiler chickens at Keputran Market, Surabaya, of the 30 samples examined, 2 samples were found to be positive *Salmonella* sp. can be seen in the table (Table 2).

Table 2 Bacteria Detection Results *Salmonella* sp.

No	**Sample Code**	**Detection Results *Salmonella sp.***	**No**	**Sample Code**	**Detection Results *Salmonella sp.***
1	1	Negative (-)	16	16	Negative (-)
2	2	Negative (-)	17	17	Negative (-)
3	3	Negative (-)	18	18	Negative (-)
4	4	Negative (-)	19	19	Positive (+)
5	5	Negative (-)	20	20	Negative (-)
6	6	Negative (-)	21	21	Negative (-)
7	7	Negative (-)	22	22	Negative (-)
8	8	Negative (-)	23	23	Negative (-)
9	9	Negative (-)	24	24	Negative (-)
10	10	Positive (+)	25	25	Negative (-)
11	11	Negative (-)	26	26	Negative (-)
12	12	Negative (-)	27	27	Negative (-)
13	13	Negative (-)	28	28	Negative (-)
14	14	Negative (-)	29	29	Negative (-)
15	15	Negative (-)	30	30	Negative (-)

Information: (+) Positive of *Salmonella* sp., (-) Negative of *Salmonella* sp.

Bacteria detection *Salmonella* sp. by carrying out isolation and identification, isolation on selective media *Salmonella* Shigella Agar (SSA) previously the sample was inoculated on the media enrichment Tetrathionate Broth Base (TTB). Identify bacteria *Salmonella* sp. carried out by gram staining and followed by biochemical tests including the Triple Sugar Iron Agar (TSIA) test, the Simon Citrate Agar (SCA) test, the Sulfide Indole Motility (SIM) test, the Urea test and the Carbohydrate test. Results of isolation on SSA media, suspected colonies *Salmonella* sp. showing a colorless colony with a black color in the middle (Picture 1). Colonies that show no color with black in the middle are then subjected to Gram staining. Gram staining results, bacteria *Salmonella* sp. shows a rod shape, red color and Gram-negative bacteria (Figure 2).

Figure 1 Suspected colony of *Salmonella* sp. on SSA media

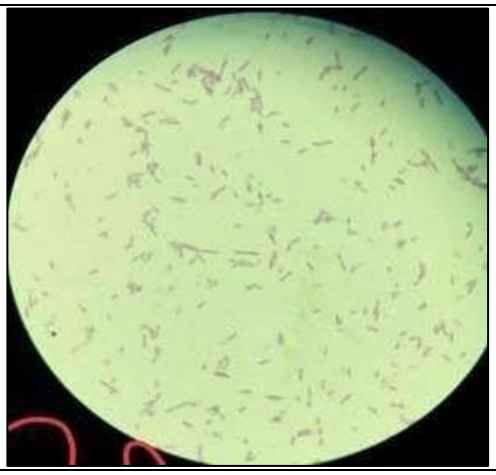

Figure 2 Gram staining for suspected colony of *Salmonella* sp.

A biochemical test is carried out to determine the bacteria based on biochemical reactions, which include Triple Sugar Iron Agar (TSIA) test for the suspected *Salmonella* sp. showed red color change on the slant and yellow color change on the bottom media. The results of this study showed negative results, the Sulfide Indole Motility (SIM) test for suspected bacteria *Salmonella* sp. indicated by the results of negative indole, positive motile and present H_2S. The results found in this test were negative.

Simon Citrate Agar (SCA) test for suspected bacteria *Salmonella* sp. indicated by a color change from green to blue. In the results of this test, two positive samples were found which changed color from green to blue, the suspected bacterial Urea test *Salmonella* sp. indicated by a color change from yellow to pink on the media. The results of this Urea test research are positive. The test results for all biochemicals can be seen in Figure 3.

Figure 3 Biochemical test results on bacteria *Salmonella* sp. Description: From left – right (TSIA, SCA, Urea, SIM), TSIA: alkali/acid, gas (-),H_2S (+), SCA: citrate test (+), Urea : Urea (+), SIM: Indole negative, motile positive, H2S (+)

Figure 4 Results from the Carbohydrate test of *Salmonella* sp. Description: From left – right (Glucose, Maltose, Mannitol, Lactose, Sucrose), Glucose (+), Maltose (+), Mannitol (+), Lactose (-), Sucrose (-)

Positive carbohydrate Test of the suspected *Salmonella* sp. indicated by color changes in glucose, mannitol and maltose. The results of this research are positive (can be seen in Figure 4). In this study, several types of biochemical tests were used, namely the TSIA Test (Triple Sugar Iron Agar), SIM Test, Citrate Test, Urea Test and Carbohydrate Test. Biochemical test results on bacterial colonies originating from 30 suspected samples *Salmonella* from the SSA media, 2 samples were found positive for bacteria *Salmonella* sp. Based on the results of isolation and identification of 30 samples originating from cloacal swabs of broiler chickens at Keputran Market, Surabaya, 2 samples were positive for bacteria. *Salmonella* sp. with prevalence (0.1%).

Salmonella is a rod-shaped bacteria (1- 2 μm), Gram negative, non-spore forming bacteria, usually motile bacteria with peritrichous flagella. *Salmonella* is a facultative anaerobe that is biochemically characterized by its ability to ferment glucose producing acid and gas, and its inability to use lactose and sucrose. Optimum growth temperature 380C. *Salmonella* can grow at low water activity (aw ≤ 0.93) whose response depends on the strain and type of food. *Salmonella* actively grows in the pH range of 3.6 – 9.5 and is optimal at pH values close to normal [21].

Salmonella in the digestive tract is difficult to eliminate because the bacteria are in the circulation of the bile system and intermittently the bacteria will enter the lumen of the digestive tract with the bile and are excreted through feces which can pollute the environment and can infect other animals or humans, not infrequently. *Salmonella* survives in lymphatic tissue [22]. The presence of contamination and infection *Salmonella* sp. on a farm with good sanitation and poor sanitation can be caused by the spread *Salmonella* sp. which often occurs through contaminated feces and contaminates feed, drinking water and hatching eggshells. There is contamination *Salmonella* sp. in the cloaca is also positively associated with contamination rates *Salmonella* sp. on eggs. The cloaca is formed by three systems, namely the digestive, urinary and reproductive systems. *Salmonella* sp. known to be bacteria (shedding) from chickens suffering from salmonellosis, the feces will pass through the cloaca as a result of which bacteria can be found in that area. To isolate and identify bacteria *Salmonella* sp several things are discussed to find these bacteria in broiler chickens at Keputran Market, Surabaya, totaling 30 samples. What is done is taking cloacal swabs from broiler chickens, inoculating samples on Tetrathionate Broth Base (TTB), and *Salmonella* Shigella Agar (SSA) media, Gram staining, purification, bacterial biochemical tests.

Cloacal swab samples from broiler chickens at Keputran Market, Surabaya were taken twice during the research. The first sampling was 15 samples, while the second sampling was 15 samples. Samples that have been taken will be moved inside flocked swab which already contains transport media in it. Cloacal swab samples from existing broiler chickens are then inoculated in the media Tetrathionate Broth Base (TTB). Tetrathionate Broth Base recommended for selective enrichment methods to isolate *Salmonella typhi* and other *Salmonella* from dirt, waste, etc. Organisms that reduce tetrathionate such as *Salmonella multiply* in the medium while many fecal organisms are inhibited [23]. From inoculating samples in the Tetrathionate Broth Base (TTB), Next, the sample is cultured in the *Salmonella* Shigella Agar (SSA). *Salmonella* Shigella Agar is a highly selective medium for isolation *Salmonella* sp. SSA is a selective medium for isolating *Salmonella* sp. and Shigella sp. from feces, urine and food samples [24]. Results of inoculating samples on SSA, there were 10 positive samples found and 7 negative samples. Shown by transparent characteristics, black spot center and yellow zone. It can be seen from the results that only a few samples were detected positive for bacteria *Salmonella* sp.

Suspected colonies from SSA media is subjected to Gram staining. Gram Positive staining is the staining of bacteria that are resistant to alcohol and the bacteria will be blue purple, while Gram Negative staining is the staining of bacteria that are not resistant to alcohol and the bacteria will be red. The gram staining results showed that 2 samples were positively detected for bacteria *Salmonella* sp. with rod-shaped characteristics and red color which indicates Gram negative characteristics, including bacterial characteristics *Salmonella* sp. which had undergone Gram staining. Positive sample results from Gram staining, followed by purification. Purification is the process of separating two or more substances that are mixed with each other and to obtain a pure substance from a substance that has been contaminated or mixed. The results of the purification contained 2 samples that detected bacteria *Salmonella* sp. Shows the presence of characteristics of a positive sample of bacteria *Salmonella* sp. which exists black spot center.

The purification samples that showed positive were subjected to bacterial biochemical tests, namely Triple Sugar Iron Agar (TSIA), Simon Citrate Agar (SCA), Sulfide Indole Motility (SIM), UREA, Carbohydrate test. Triple Sugar Iron Agar (TSIA)) to see the ability of bacteria to ferment glucose and/or lactose and the ability to produce gas *H*2S. TSIA contains three sugar or carbohydrates namely glucose, sucrose and lactose. Positive results are indicated by the media changing to yellow due to acid production. *Salmonella* on TSIA will make the slanted part change to red, and the bottom becomes yellow. When produced *H*2S then it will react with ferrous sulfate (III) and black precipitation of ferric sulfide FeS is formed (II) at the bottom (Midorikawa, 2014). The results found from this research that there were no samples that had color changes on the slant and on bottom, some samples had *H*2S is thick. Simon Citrate Agar (SCA) used to differentiate

between families Enterobacteriaceae and groups Aerogenes based on citrate utilization as the only carbon source. Positive test results Simmons Citrate indicated by the media changing to blue due to the presence of the indicator bromthymol blue [25]. The results of this research found 2 positive samples that changed from green to blue.

Sulfide Indol Motility (SIM) aims to determine the movement of bacteria [26]. This media contains ferrous ammonium sulfate and sodium thiosulfate which is an indicator of formation hydrogen sulfide (H_2S). There are positive results H_2S is characterized by blackening of the inoculated medium, while a positive result of motility is the widening of the growth diffusion zone from the inoculation line [27]. The results of this study found negative indole, H_2S black, positive motile. Urea aims to determine the ability of bacteria to convert urea into ammonia. The results of this research were positive, because samples were found that changed color from yellow to pink to red. Sugar Solution - In general, sugar bacteria ferment certain carbohydrates and it is important to know the characteristics of the bacteria. When making confectionery media, a Durham tube is usually equipped into the media to detect gas production as a result of the fermentation process. The results of this research are there were several samples of glucose that experienced a color change from red to yellow.

The study shows that the final results of the bacterial biochemical test were that 2 samples were found to be positive for bacteria *Salmonella* sp. The increasing incidence of salmonellosis is also caused by long transportation distances and lack of attention to sanitary hygiene. The microorganisms that cause salmonellosis can be transmitted both horizontally and vertically. Chicken can be contaminated *Salmonella* sp. starting from livestock which is influenced by the cage and livestock environment [28]. The infectious disease that usually attacks chickens is Salmonellosis. The cause of this disease is *Salmonella* sp. [29]. Salmonellosis, apart from being economically detrimental, will also have a negative impact on public health. Although many other pathogens, transmission of *Salmonella* sp. through food will cause the emergence of a disease. Based on statistical data, more than 90% of the causes of disease in humans are related to food or what is known as foodborne disease [30].

4. Conclusion

From the results of this research, conclusions can be drawn regarding the cloacal swabs of broiler chickens at Keputran Market, Surabaya. It was found that 0.1% were positive (2 out of 30 samples) contaminated with bacteria *Salmonella* sp. From the results of this research, the author can suggest that we continue to pay attention to the cleanliness and hygiene of the production site. As well as communicating to the parties.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee of Universitas Airlangga. All variables were considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain was caused to the animals during sampling(certificate registration number: 2021/112-KE).

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