High concentrations of pathogenic *Salmonella* spp. during the wet season on bathing beaches in Makassar City, Indonesia

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Abstract. Pathogenic bacteria in coastal areas have been found to contribute to the outbreak of acute infections among tourists, and can be transmitted through contact with polluted recreational water. *Salmonella* spp. are bacteria frequently associated with waterborne diseases, they can cause various symptoms from mild gastroenteritis to death. This research aimed to determine the concentration of *Salmonella* in bathing beaches and to evaluate whether the concentrations could be harmful to human health. Samples were collected for the analysis of bacteria and water quality at six different bathing beach sites in Makassar, Indonesia. *Salmonella* analysis was conducted using a membrane filtration method, where the filter was then placed on Bismuth Sulphite Agar (BSA), a selective medium for *Salmonella* growth. *Salmonella* identification was based on a suite of biochemical reaction tests. *Salmonella* concentration per membrane was determined using a colony counter. *Salmonella* concentrations were compared between sites using one-way ANOVA with post hoc test, while the correlation between *Salmonella* concentrations and water quality was evaluated using Principal Component Analysis (PCA). Three colony types grew on the BSA. *Salmonella* were detected at all six bathing beaches in Makassar. *Salmonella* concentrations were highest in Tanjung Bayang Beach, followed by Akkarena Beach, Kayangan Island, Lae-lae, Samalona Island, and Bulo Gading Beach. Salmonella concentration correlated with high total organic matter levels, and exceeded the EEC standard for recreational swimming and bathing water at all six bathing beaches in Makassar.

1. Introduction
Beaches are popular all around the world as places to visit for recreation, especially during the holiday season. Beach activities can include diving, snorkelling, swimming and bathing, fishing, surfing, sun bathing and watching the sunset. For example, in the USA beaches have been reported as the most popular summer vacation destination with 72% of Americans expressing a favourable opinion of going to the beach for the summer, and spending on average 44% of their summer vacation time on the beach (ABC/Washington Post 2012). In Australia, reported international and domestic visitors to the Gold coast in Queensland Australia increased 1% from July 2014 to December 2014 [1]. The tourism research company Longwoods International reported that in 2016 the Virginia Beach hosted 15.2 million visitors. Because beaches are a favourite tourist destination for many people in the world, sanitation in the beach environment is very important.

Pathogenic microorganisms present in coastal areas have contributed to the outbreak of acute infections among tourists. Preliminary studies on the outbreak of these infections focused attention on a variety of microorganisms such as *Salmonella*, *Staphylococcus*, and *Vibrio* [2]. A number of
pathogenic microorganisms are known to be transmitted through contact with polluted recreational water, however the most commonly used microbiological indicator for environmental sanitation is Salmonella spp., ubiquitous bacteria frequently associated with waterborne diseases. Pathogenic for both humans and animals, Salmonella spp. can cause various symptoms from mild gastroenteritis to death. More than 2600 Salmonella spp. serovars exist and all may be pathogenic [3]. Non-typhoidal Salmonella is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths worldwide each year [4]. It was also reported by [5] that a total of 42 typhoid cases required hospitalization: 6 (40% of all cases) in China, 2 (2%) in India, 26 (20%) in Indonesia, 3 (2%) in Pakistan, and 5 (28%) in Viet Nam. Salmonella invasion and intracellular replication within host cells can result in a range of diseases, including gastroenteritis, bacteremia, enteric fever and faecal infections; meanwhile particular Salmonella enterica subspecies or enterica serovars typhi and paratyphi are human-restricted pathogens that cause the systemic disease enteric (typhoid) fever [6]. Typhoid fever is recognized as a devastating disease in several regions in Asia, Africa and South America, while the disease is rare in developed countries [7]. Five Asian countries, which are considered to be endemic for typhoid are China, India, Indonesia, Pakistan, and Viet Nam [5].

Makassar, one of Indonesia’s larger cities, is situated on the coast. As in many other coastal areas around the world, the beaches in and around the city have become recreational areas and tourist destinations, especially during weekends, school holidays, and before and after festive holidays like Idul Fitri, Christmas and New Year. The presence of Salmonella in these waters is a potential threat to human health, because these bacteria can transmit disease through water [8]. The European Community Water Direction (EEC, 1976) established standards bathing water standards of no detectable presence of Salmonella in one litre of water. Salmonella concentrations exceeding the EEC standard have been detected in the coastal waters of many countries and regions, including Portugal and California. Of the seventeen serotypes of Salmonella derived from estuarine and coastal waters of Portugal [9], four can cause human disease, i.e. Salmonella enteritidis, Salmonella infantis, Salmonella typhimurium and Salmonella virchow, while total Salmonella concentrations in sand from California beaches ranged from 2.9 to 103.3 CE/g [10]. However, there are no data available on environmental sanitation based on pathogenic bacteria for the bathing beaches of Makassar City. This study aimed to determine the concentration of Salmonella on selected bathing beaches in Makassar and to evaluate if the concentrations present are harmful to human health.

2. Methods

2.1. Study site
Samples were collected from six bathing beach sites in Makassar City (Figure 1). Three replicate samples were taken at each site, and each replicate was divided into six sampling points. Site I, Bulo Gading Beach, is located in the city centre, and is characterized by shipping docks, restaurants, hotels and urban residential areas. Site II, Akkarena Beach, is close to a shopping mall with no residential area. Site III, Tanjung Bayang Beach, is surrounded by residential development. Site IV, Lae-lae Island, is a small but densely populated island, very close to the mainland of Sulawesi Island. Site V, Kayangan Island, is close to the major sea port of Makassar. Site VI is Samalona Island, a small island with a very small number of permanent inhabitants. A total of 90 marine water samples were taken for Salmonella analysis and another 90 samples for water quality. Sampling was conducted during the rainy season.
2.2. Data Collection

At each sampling point, a 100 mL water sample for *Salmonella* analysis was taken from the subsurface water layer (30 cm depth) following [11], using a sterile high-density polyethylene (HDPE) plastic bottle. The samples were transported to the laboratory in a cool box and maintained at 1–4°C. The physical and chemical parameters monitored in this study were water temperature, salinity, pH, current, dissolved oxygen and total organic matter. Temperature and dissolved oxygen were measured on site using a DO meter, salinity was recorded with a refractometer, pH with a pH meter. Total organic matter was measured from sub-samples prepared according to the Winkler method [12], then analysed using a spectrophotometer (HAC-USA type LPG 422.99.00012) at 660 nm wavelength.

2.3. Isolation and identification of *Salmonella*

*Salmonella* bacteria were isolated using membrane filtration [12]. A 100 mL volume of water was filtered through a 47 mm diameter 0.45 pore-size filter. The filter was then placed on Bismuth sulphite Agar (BSA) medium (Merck). The bacterial culture was incubated at 37°C for 24 hours. BSA medium is a modification of the original [13] selective diagnostic medium used for the isolation of *Salmonella typhi* and other salmonellae from food and other materials, e.g. sewage, water, etc. The BSA content formula per litre of water is in the Difco & BBL Manual [14]. Each typical colony was selected and streaked onto nutrient agar/bismuth sulphite agar.

To identify the presence of *Salmonella* we conducted morphological and biochemical tests. Morphology of the bacteria (shape, size, elevation, colour and surface texture) was observed and determined [15]. The biochemical tests used were Gram Stain, oxidase, phenol red, triple sugar iron (TSI), citrate, sulphide indole motility (SIM), methyl red (MR), voges proskauer (VP), glucose, lactose, sucrose, and mannitol [15]. Bacterial isolates were identified according to Bergey’s Manual of Determinative Bacteriology (9th Edition) and Probabilistic online bacterial identification which can be accessed from http://www.microrao.com/identify.htm.

2.4. *Salmonella* concentration and statistical analysis

After 24 hours of incubation, the BSA agar plates were analysed to determine the presence of *Salmonella* bacteria. Bacterial colonies were counted as colony forming units (cfu) on modified agar plates. The colonies that appeared on each plate were counted. The density of the bacteria recovered...
from the water and sediment were expressed as cfu/100mL of water. Concentration of *Salmonella* per membrane using colony counter, was calculated by means the equation in [12]:

\[
\text{Salmonella colonies counted X 100} = \frac{\text{Total Salmonella/100 mL}}{\text{Volume of sample filtered (mL)}}
\]

*Salmonella* concentrations between locations were compared using one-way analysis of variance (ANOVA), followed by post hoc Tukey honestly significant difference (HSD) test. Principal Component Analysis (PCA) was performed to evaluate the correlation between *Salmonella* concentration and water quality.

3. Results

3.1. *Salmonella* Colony typology and biochemical tests

Colonies on the membrane filter of the Bismuth sulphite Agar (BSA) were brown and black. There were three colony types observed: medium sized black coloured colony with a surrounding dark zone (isolate 1); small black coloured colony (isolate 2), and small brownish coloured colony (isolate 3) (Figure 2). The biochemical tests confirmed the presence of *Salmonella* (Table 2).

Table 1 Biochemical test results for three bacterial isolates growing on Bismuth Sulphite Agar selective medium for *Salmonella*

| Characteristic         | Isolate A         | Isolate B | Isolate C |
|------------------------|-------------------|-----------|-----------|
| Colony colour          | metallic black    | black     | brownish  |
| Cell morphology        | Rod               | Rod       | Rod       |
| Gram stain reaction    | Negative          | Negative  | Negative  |
| Motility               | +                 | +         | +         |
| Indole Production      | -                 | -         | -         |
| VP test                | -                 | -         | -         |
| MR test                | +                 | +         | +         |
| Urease activity        | -                 | -         | -         |
### Characteristic

| Utilization of citrate | Isolate A | Isolate B | Isolate C |
|------------------------|-----------|-----------|-----------|
|                        | +         | +         | +         |

**Triple Sugar Iron (TSI)**

- **Butt**
  - Isolate A: Yellow
  - Isolate B: Yellow
  - Isolate C: Yellow
- **Slope**
  - Isolate A: Yellow
  - Isolate B: Yellow
  - Isolate C: Yellow
- **H2S**
  - Isolate A: +
  - Isolate B: +
  - Isolate C: -
- **Gas**
  - Isolate A: -
  - Isolate B: +
  - Isolate C: +
- **Oxidase activity**
  - Isolate A: -
  - Isolate B: -
  - Isolate C: -

**Utilization of**

- **Glucose**
  - Isolate A: +
  - Isolate B: +
  - Isolate C: +
- **Lactose**
  - Isolate A: -
  - Isolate B: -
  - Isolate C: -
- **Sucrose**
  - Isolate A: -
  - Isolate B: -
  - Isolate C: -
- **Mannitol**
  - Isolate A: +
  - Isolate B: +
  - Isolate C: +

**Tentatively identified genus**

- Isolate A: *Salmonella*
- Isolate B: *Salmonella*
- Isolate C: *Proteus*

### 3.2. *Salmonella* concentration

*Salmonella* concentrations were highest in Tanjung Bayang Beach, followed Akkarena Beach, Kayangan Island, Lae-lae, Samalona Island, and Bulo Gading Beach (Figure 3). The differences among sites were significant (F₅,₈₄ = 6.005 P < 0.05). Tukey post-hoc tests indicated significant between sites differences in *Salmonella* concentration between Bulo Gading Beach and Akkarena Beach (p = 0.012); Tanjung Bayang Beach and Bulo Gading Beach (p= 0.000); and Tanjung Bayang Beach with Lae-lae Island (p = 0.021) and Samalona Island (p = 0.004).

![Figure 3. Average concentration of *Salmonella* at each of the six sites (n=15). Small case letters indicate statistically significant between site differences (p < 0.05)](image_url)

Bulo Gading Beach is the site closest to the city centre. Visitors to this site are mostly older people or people recovering from certain illnesses such as stroke, bronchitis, etc. The next closest sampling location is Akkarena Beach, is in a non-residential area with a few snack food and drinks retail stalls. At Akkarena, the management has built breakwaters particularly aimed to protect children when they
are swimming. Site number three, Tanjung Bayang Beach, is the most crowded with more visitors compared to the others due to its low entrance fee and easy access. Lae-lae Island, the fourth sampling location, has relatively small numbers of visitors due to limited space for bathing and swimming. Seaweed is cultivated in some of the waters around Lae-lae. The fifth site, Kayangan Island, has the second largest number of visitors after Tanjung Bayang, most of which are young people. The last site is Samalona Island, a beautiful small island with a white-sand beach popular with visitors mostly from outside Makassar; visitors must rent a boat to reach this island, as there is no regular transportation.

3.3. Correlation between Salmonella concentrations and water quality

The Principal component analysis (PCA) biplot (Figure 4) showed a clear separation between sites based on Salmonella concentration in axis 1. The highest Salmonella concentrations were at Tanjung Bayang Beach and Akkarena Beach. The high concentrations were strongly related to the concentration of total organic matter (TOM) and salinity, with correlation values of 0.62 and 0.65 (Table 2), respectively. Whereas, lower Salmonella concentrations identified in Bulo Gading Beach and Samalona Island were associated with high temperature.

![Fig 4. Principal component analysis (PCA) biplot of Salmonella concentration with water quality](image)

| Concentration of Salmonella | Water Quality | Site | Correlation |
|-----------------------------|---------------|-----|-------------|
| TOM (mg/L)                  | TB            | AK  | 0.62        |
| Salinity (ppt)              | 43.19, 43.19  |     | 0.62        |
| Temperature (°C)            | 31.53, 31.6   |     | 0.65        |
|                             | 30.32, 30.51  |     | -0.79       |
4. Discussion

Bacteria grew well on the selective Bismuth Sulphite Agar (BSA) medium with black and brown colonies. The BSA technical sheet indicates that *Salmonella* spp. colonies appear black on the BSA medium after 18 hours incubation, and usually after a few hours a metallic sheen appears on certain species. (http://www.liofilchem.net). The characteristics of the *Salmonella* spp. colonies on this medium have also been reported by previous studies [16,17,18]. Sulphite is mixed into bismuth glucose sulphite to isolate *Salmonella typhi* and *S. Paratyphi* [19]. Ferrous sulphate found in the BSA medium is an indicator for hydrogen sulphide (H2S) production. When H2S is present, *Salmonella* spp. reacts with iron salt to form iron sulphate; it is this reaction which produces black or green colonies and brown deposits [20].

Based on Bergey's Manual of Determinative Bacteria (9th Edition) and Probabilistic Online Bacterial Identification, the bacteria that grew on the BSA medium were identified as three different isolates: *Salmonella* spp. isolate (1), *Salmonella* spp. isolate (2) and the third isolate appeared to be *Proteus* spp. Although the BSA medium is selective for the growth of *Salmonella* bacteria, *Proteus* spp. is sometimes found, characterised by small green-brown colonies (http://www.liofilchem.net). *Proteus* spp. Bacteria can be found in soil and water but most live in the digestive tract of humans and animals, so that the presence of these bacteria in the aquatic environment is often used as an indicator of faecal pollution. An opportunistic pathogen, *Proteus* spp. poses a threat of infection when bathing in contaminated sea water. Pathogenesis of *Proteus* spp. has been reported in human respiratory tract infections, diarrhoea, infection of burns, and meningitis [21]; furthermore, the species *Proteus mirabilis* can cause urinary tract infections, particularly when catheters are used [22].

The presence of *Salmonella* spp. and *Proteus* spp. was detected at high concentrations in the waters of all six bathing beaches in Makassar City. The average concentrations obtained in colony forming units (cfu) per 100 mL were: Bulo Gading Beach, 648.67; Kayangan Island, 717.60; Tanjung Bayang, 1081.20; Akkarena Beach, 979.60; Samalona Island, 716.60; and Loe-Lae Island, 768.80. It is likely that the high concentration of *Salmonella* spp. was related to the sampling period being during the rainy season. Runoff can carry trash contaminated with *Salmonella* into sea waters. Significant correlations between rainfall and the presence of *Salmonella* in rivers and coastal waters have been found [23,24], and higher diversity of strains has been found during floods due to heavy rain [25].

In this study the highest concentration of *Salmonella* was found in Tanjung Bayang, the most densely populated area, which also has very few waste storage facilities, so that both the local community and visitors generally still dump garbage directly into the sea. Similarly, the disposal of human waste is most often directly into the sea, as toilets owned by each house are only used for visitors. Previous research has found *Salmonella* at locations near human habitation [26], with a tendency to increase with higher human population density [27]. The prevalence of *Salmonella* resistant to antimicrobials is also more common in areas near to human settlements compared to areas with little anthropogenic influence [28]. Although the majority of salmonellosis outbreaks have been linked to the consumption of contaminated food, *Salmonella* is also considered a major cause of waterborne disease epidemics, with sources of exposure including contaminated coastal recreational areas. The lowest *Salmonella* concentration was found in Bulo Gading Beach. Hotels and restaurants that dominate the area around Bulo Gading Beach already have garbage disposal containers so that less waste is disposed of or washed into the sea. Visitors who come to this beach generally only come to treat a range of disease or health disorders with sea water, not for recreation.

The principal component analysis (PCA) biplot graphical analysis of the correlation between *Salmonella* concentration and water quality showed that the high *Salmonella* concentrations in Tanjung Bayang and Akkarena Beach are characterized by high total organic matter concentrations. *Salmonella* is included in the chemoorganotrophic group, absorbing energy from the oxidation of organic compounds, and is considered heterotrophic [29]. Heterotrophic bacteria require organic sources of carbon such as sugar, fat and amino acids, fermenting sugars with gas, ethanol, acetate or lactate production [30]. Therefore higher levels of organic matter can provide nutrients to support higher concentrations of *Salmonella*. The low concentration of *Salmonella* at Bulo Gading Beach and
Samalona Island was associated with comparatively high seawater temperatures (30.32°C–30.51°C). This might be related to the statement in [11] that marine bacteria achieve maximum cell growth and multiplication at a temperature of 18°C, and if it they experience a temperature of 30°C for more than 10 minutes, cell propagation can be inhibited by 20%-30%. At 37°C, 42% of marine bacteria died, and at 45°C only 15% remained alive [31].

The concentration of Salmonella found in all six coastal areas of Makassar City was very high, well beyond the threshold considered safe for bathing and swimming. Based on this study, bathing in these waters is not recommended, as it could be dangerous to health.

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