

Identification of Gram-Negative Bacteria in the Oral Cavity of *Homalopsis buccata*

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Abstract

The *kadut belang* snake (*Homalopsis buccata*) is a semi-aquatic snake that can be aggressive when threatened. *H. buccata* is often used as a pet, a source of animal protein, and raw materials for leather crafts because of its relatively large size and unique pattern. The increasing trend of keeping reptiles as pets could increase the potential for transmission of bacteria to humans. The purpose of this study was to isolate and identify *Aeromonas hydrophila*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella* sp. from *H. buccata*. This type of research uses the accidental sampling method as the research design. The *H. buccata* criteria used in this study came from wild catches and were imported during the study period. Isolation was carried out to obtain separate bacterial colonies and identification of bacteria was carried out by Gram staining and biochemical reaction tests. Identification of Gram-Negative Bacteria in the Oral Cavity showed that *A. hydrophila*, *E. coli*, *K. pneumoniae*, and *Salmonella* sp. with a percentage of 33.3%, 11.1%, 55.5%, and 22.2% in the digestive tract of *H. buccata* snakes in the Mojokerto City area.

Keywords

Homalopsis buccata, infectious disease, oral cavity, tropical disease

Introduction

The *kadut belang* snake (*Homalopsis buccata*) is a semi-aquatic snake that is distributed in Southeast Asia, such as Peninsular Malaysia and Indonesia, especially on the islands of Sumatra, Kalimantan and Java (Murphy *et al.*, 2012). *H. buccata* has a natural habitat in swamp areas, rivers, and is often found in human settlements. *H. buccata* is classified as an exotic animal because it has a unique pattern of the dorsal head. When threatened, this snake may become aggressive and bite humans, potentially causing infected wounds (Setiawan and Marisa, 2015).

Bacteria from reptiles can be transmitted to humans through direct contact, such as snake bites, or through contamination by saliva or feces (Marin *et al.*, 2021; Dec *et al.*, 2022). Cold-blooded animals can harbor various bacteria harmful to humans, but the bacteria found in snakes may not always cause symptoms in humans (Ebani, 2017). Research by Marin *et al.* (2021) in Valencia, Spain found 72.0% of *Salmonella* sp. isolated from several types of reptiles (turtles, lizards and snakes). Dec *et al.* (2022) in Lubelskie, Poland, also found that 71.87% of the total *E. coli* isolated were resistant. Global findings of resistance bacteria isolated from snakes led to bacteriological reptile studies in Indonesia becoming important.

In the oral cavity of snakes, there are many bacteria that can cause secondary infections due to snake bites. This is because the oral cavity is a reservoir for various types of bacteria. In the Southeast Asia region, snake bite cases often occur, according to global records it can reach 1.2-5.5 million cases per year, of which 125,000 cases result in death or disability (Artavia-Leon *et al.*, 2017; Chuang *et al.*, 2022). *Aeromonas hydrophila*,

Enterobacteriaceae (*Escherichia coli*, *Salmonella* sp., *Klebsiella pneumoniae*), *Streptococcus* spp., *Staphylococcus aureus*, and *Clostridium* spp. are bacteria that can be transmitted through snake bites. These bacteria have the potential to cause illnesses like gastroenteritis, human salmonellosis, urinary tract infections, soft tissue infections, and pneumonia (Resiere *et al.*, 2018). Studies in Taiwan and South Africa revealed that the incidence of infected wounds after snake bites was 28.1% and 34.8%, respectively, and required a debridement procedure (Huang *et al.*, 2012; Wagener and Aldous, 2017).

E. coli, *A. hydrophila*, *Salmonella* sp., and *K. pneumoniae* are Gram negative bacteria that are zoonotic and can be found in the oral cavity of snakes (Abba *et al.*, 2017; Artavia-Leon *et al.*, 2017; Chuang *et al.*, 2022). The growing popularity of reptiles as pets has contributed to a rising risk of bacterial infections being transmitted from reptiles to humans (Puspita, 2015). Researchers found no bacteriological studies on *H. buccata*, although this species is commonly found in residential areas in Indonesia, where it is kept as an exotic pet and even used as an unconventional food source (Setiawan and Marisa, 2015). The snake *H. buccata* has the potential to transmit zoonotic bacteria to humans. Therefore, information about the Gram-negative bacteria present in *H. buccata* is important to research.

Considering the potential for bacterial transmission through snake bites and equipment contamination by snake saliva, the oral cavity was chosen as a place to isolate and identify Gram-negative bacteria. Bacteria from the species *A. hydrophila*, *E. coli*, *K. pneumoniae*, and *Salmonella* sp. were the focus of detection in the oral cavity of *H. buccata* snakes from wild catches. The limitations of

research on the *H. buccata* snake are by considering the closeness of the animal and its close distribution to human life. Meanwhile, the research limitations concerning the chosen types of Gram-negative bacteria are influenced by the snake's natural habitat, its hunting behavior, and results from prior bacteriological studies.

Materials and Methods

Sample Collection and Procedures

The research was conducted from August to December 2022. The samples used were swabs from the snake's oral cavity. *H. buccata* were obtained from collectors in Mojokerto City, East Java, Indonesia. The snake *H. buccata* was then transported to the research site at the Instrument Laboratory, Faculty of Health, Medicine, and Life Sciences, Airlangga University by expedition service. The number of samples used the calculating proportion formula with a prevalence of 95% and a standard error of 15% (Abba *et al.*, 2017; Adhikari, 2021). The calculation results showed that the minimum number of samples used was nine samples with rounding. Ethical approval was required for this study and continued with an oral cavity swab for *H. buccata*. The oral cavity swab begins with the anesthesia using ketamine by intramuscular injection with dose of 10-20 mg/kg. Swabbing of the oral cavity is carried out thoroughly.

Isolation and Identification

The oral cavity swabs from *H. buccata* were then placed in enrichment media, specifically Tetrathionate Broth, and general growth media, such as Nutrient Broth. The samples were incubated at 37°C for 18 to 24 hours.

Aeromonas hydrophila

Bacterial isolation of *Aeromonas* sp. was accomplished by inoculating the swab sample onto solid agar media, specifically Tryptic Soy Agar (TSA) and incubating at 27°C for 24 hours. Identification tests for the bacteria *A. hydrophila* are conducted microscopically through Gram staining. Subsequent identification involves tests for motility, indole production, and H₂S production using SIM media. Additional tests include assessments on methyl red-Voges Proskauer media, catalase tests, and oxidase tests (Niamah, 2012).

Escherichia coli

The isolation stage starts from making scratches on the Eosin Methylene Blue Agar (EMBA) media and then incubating at 37°C for 18 hours to 24 hours. Suspected colony of *E. coli* will be metallic green on EMBA media. After acquiring a pure isolate, an identification test was conducted using the Indole, MR-VP, Citrate (IMViC) biochemical testing method. Pure isolates in EMBA media were inoculated in TSIA media and then incubated at 37°C for 24 hours. Cultures on TSIA media were then inoculated on Sulfide Indole Motility (SIM), Simmons Citrate Agar (SCA), and Methyl Red-Voges Proskauer (MR-VP) media. Kovac's reagent was added to SIM media after incubation at 37°C for 24 hours for the indole test. Methyl red reagent was given to MR media, alpha-naphthol and Potassium Hydroxide (KOH) reagents were given to VP media after incubation at 37°C for 24 hours.

Klebsiella pneumoniae

Bacterial isolation of *K. pneumoniae* was performed by inoculating an oral cavity swab sample onto MacConkey Agar (MCA) media and incubating it at 37°C for 18 to 24 hours.

Suspected colony of bacteria *K. pneumoniae* was further identified using Gram staining and IMViC test. *K. pneumoniae* showed negative results for the indole and motility tests on SIM media, positive results for the citrate test, negative reaction results for the MR reagent but positive for the VP reagent in the test with MR-VP media (Ramaditya *et al.*, 2018).

Salmonella sp.

Bacterial isolation of *Salmonella* sp. began by placing the swab sample into selective enrichment media, specifically Tetrathionate Broth. The mixture was then homogenized using a vortex and incubated at 37°C for 18 to 24 hours, then followed by inoculating the bacterial isolates in enrichment media onto selective solid media *Salmonella* sp., namely Salmonella Shigella Agar (SSA). and then separated according to colony morphology (Artavia-Leon *et al.*, 2017). Identification of

suspected colony of *Salmonella* sp. was done by Gram staining and biochemical test namely IMViC and Triple Sugar Iron Agar (TSIA) (Nesa *et al.*, 2011; Amirudin *et al.*, 2017; Samad *et al.*, 2018).

Results

The results of isolation and identification of Gram-negative bacteria in the oral cavity of the Kadut Belang snake (*Homalopsis buccata*) obtained bacteria from the species *A. hydrophila*, *E. coli*, *K. pneumoniae*, and *Salmonella* sp. The percentage of bacterial findings in all samples included *A. hydrophila* of 33.33% (3/9), *E. coli* of 55.55% (5/9), *K. pneumoniae* of 11.11% (1/9), and *Salmonella* sp. of 22.22% (2/9). The isolation and identification of Gram-negative bacteria in the oral cavity of the Kadut Belang snakehead (*Homalopsis buccata*) can identify 11 bacterial samples.

Table 1. Results of Isolation and Identification of Gram-Negative Bacteria in the Oral cavity of the Kadut Belang Snake (*Homalopsis buccata*)

Sample	<i>A. hydrophila</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>Salmonella</i> sp.
Hb-1	-	+	-	-
Hb-2	-	-	-	-
Hb-3	+	-	+	+
Hb-4	-	+	-	+
Hb-5	-	+	-	-
Hb-6	-	-	-	-
Hb-7	+	+	-	-
Hb-8	-	+	-	-
Hb-9	+	-	-	-

Isolation and Identification of *Aeromonas hydrophila*

The bacteria *A. hydrophila* was successfully isolated and identified from the Kadut Belang snakehead (*Homalopsis buccata*) in samples Hb-3, Hb-7, and Hb-9 with a percentage of 33.33%. Oral swab samples were taken from nine Kadut Belang snakes (*Homalopsis buccata*). The results of the isolation of *Aeromonas hydrophila* bacteria from *H. buccata* oral swab samples showed colonies growing on Tryptic Soy Agar (TSA) media. The characteristics of colonies growing on TSA media show yellowish cream-colored colonies.

Colonies were identified on Gram staining and showed red-stained bacteria. The motility test and indole test showed positive results as indicated by the formation of an inverted cypress on all samples and the formation of a red ring when dripped with Kovac's reagent for samples Hb-1, Hb-3, Hb-4,

to Hb-9 on Sulfide Indole Motility (SIM) media. The color change of the SIM media to black also indicates a positive reaction in testing the ability of bacteria to produce hydrogen sulfide (H₂S) and was found in samples Hb-1, Hb-2, Hb-3, Hb-4, Hb-7, and Hb-9. The Methyl red (MR) test indicated positive on all samples by showing a red color change when dripped with reagent and Voges-Proskauer (VP) indicated positive on Hb-2, Hb-3, Hb-7, and Hb-9 samples by showing changes to red color when dropped with alpha-naphthol and KOH reagent. The catalase test results indicated positive in all samples with the formation of gas bubbles when the bacterial isolate was dropped with 3% H₂O₂. The oxidase test was also declared positive on all samples by showing a blue color change on the oxidase strip. The results of bacterial identification can be seen in Table 2.

Table 2. Results of *A. hydrophila*

Biochemical Test	Hb-1	Hb-2	Hb-3	Hb-4	Hb-5	Hb-6	Hb-7	Hb-8	Hb-9
Indole	+	-	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+
VP	-	+	+	-	-	-	+	-	+
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
H ₂ S	+	+	+	+	-	-	+	+	+

Isolation and Identification of *Escherichia coli*

Based on research, *E. coli* bacteria were obtained from Kadut Belang snakeheads (*Homalopsis buccata*) in samples Hb-1, Hb-4, Hb-5, Hb-7, and Hb-8 with a percentage of 55.5%.

The results of the isolation of *Escherichia coli* bacteria from oral swab samples from *H. buccata* on Eosin Methylene Blue Agar (EMBA) media has characteristic blackish colored colonies with a metallic green glow.

Identification of *E. coli* bacteria begins with a Gram staining test and shows that the bacteria are colored red with a short rod-shaped bacterial morphology. Identification of *E. coli* was continued with the biochemical reaction test using the IMViC method. On SIM media, the sample isolate produced an inverted spruce shape, a red ring shape when reacted with Kovac's reagent on samples Hb-1, Hb-2, Hb-4 to Hb-9. In the MR test, positive results in the form of a change in the color of the medium

to reddish when dripped with reagent were shown in samples Hb-1, Hb-4, Hb-5, Hb-6, Hb-7, and Hb-8. The VP test did not show a color change in the medium after being reacted with the reagent and indicated negative results in all samples. The citrate test carried out on Simmons Citrate Agar (SCA) media showed the color change of the media from green to blue in the Hb-6 sample, which indicated a positive result. The results of bacterial identification can be seen in Table 3.

Table 3. Results of Identification of Suspected *E. coli* Using the IMViC Method

Biochemical Test	Hb-1	Hb-2	Hb-3	Hb-4	Hb-5	Hb-6	Hb-7	Hb-8	Hb-9
Indole	+	+	-	+	+	+	+	+	+
MR	+	-	-	+	+	+	+	+	-
VP	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	+	-	-	-

Isolation and Identification of *Klebsiella pneumoniae*

As a result of the isolation and identification, the bacteria *K. pneumoniae* was found from the Kadut Belang snakehead (*Homalopsis buccata*) in the Hb-5 sample with an incidence of 11.1%. The results of the isolation of *K. pneumoniae* bacteria from *H. buccata* oral swab samples showed characteristic mucoid pink bacterial colonies with large diameters in samples Hb-2, Hb-3, Hb-4, Hb-5, Hb-6, Hb-7, and Hb-8.

The research continued with identification through the Gram staining test. Microscopic observation using Gram staining showed that the bacteria were colored red with

short rod-shaped bacterial morphology. Bacterial identification was then carried out using the IMViC biochemical reaction test method (Permatasari et al., 2020). The red ring on SIM media forms when reacted with Kovac's reagent and indicated positive results for indole in Hb-6, Hb-8, and Hb-9 samples. The MR test did not show color changes and indicated negative results in Hb-3 and Hb-5 samples. The VP test indicated positive results in Hb-4, Hb-5, and Hb-6 samples. SCA media was used for the citrate test with positive results indicated by a change in the color of the media to blue in the Hb-5 sample. The results of bacterial identification can be seen in Table 4.

Table 4. Results of Identification of Suspected *K. pneumoniae* Using the IMViC Method.

Biochemical Test	Hb-1	Hb-2	Hb-3	Hb-4	Hb-5	Hb-6	Hb-7	Hb-8	Hb-9
Indole	-	-	-	-	-	+	-	+	+
MR	+	+	-	+	-	+	+	+	+
VP	-	-	-	+	+	+	-	-	-
Citrate	-	-	-	-	+	-	-	-	-

Isolation and Identification of *Salmonella* sp.

Based on research that has been held, it was found that *Salmonella* sp. from the Kadut Belang snake (*Homalopsis buccata*) in samples Hb-3 and Hb-4 with an incidence of 22.2%. Isolation of *Salmonella* sp. started by inoculating an oral swab sample from *H. buccata* into enrichment media in the form of Tetrathionate Broth (TTB) and incubating at 37°C for 24 hours. The isolate was then inoculated in a specific growth medium in the form of Salmonella-Shigella Agar (SSA). On SSA media, bacterial isolates showed characteristic translucent colonies with black spots.

Identification of bacteria began with Gram staining. Microscopic observation after Gram staining showed that the bacteria were colored red with the morphological

characteristics of rod-shaped bacteria. Identification was then continued with the biochemical reaction test using the IMViC method and growth test on TSIA media. Isolates of Hb-2, Hb-3, Hb-4, Hb-6, and Hb-8 samples on SIM media did not form reddish rings when reacted with Kovac's reagent. In the MR test, only the Hb-8 sample did not show color changes and was indicated as negative. The VP test carried out on all samples showed negative results. All samples showed positive results on SCA media. The results of the bacterial growth test on TSIA media showed a red color on the slant, acid at the bottom of the tube, and a black color change on the Hb-1, Hb-3, Hb-4, Hb-5, and Hb-7 samples. Results of identification of *Salmonella* sp. in the oral cavity of *H. buccata* can be seen in the following table.

Table 5. Results of Identification of Suspected *Salmonella* sp. Using the IMViC Method.

Biochemical Test	Hb-1	Hb-2	Hb-3	Hb-4	Hb-5	Hb-6	Hb-7	Hb-8	Hb-9
H2S	+	-	+	+	+	-	+	+	+
Indole	+	-	-	-	+	-	+	-	+
MR	+	+	+	+	+	+	+	-	+
VP	-	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+	+

Conclusion

This study concluded that it was proven that there were bacteria such as *A. hydrophila*, *E. coli*, *K. pneumoniae* and *Salmonella* sp. in the digestive tract of *H. buccata* snakes in the Mojokerto City area. Additional research on the sensitivity of bacteria from the oral cavity of *H. buccata* is necessary to assess the level of bacterial resistance to antibiotics.

Approval of Ethical Commission

The research protocol was approved by the Animal Care and Use Committee of Airlangga University (Approval number, 995/HRECC.FODM/XII/2022).

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Author's Contribution

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data Availability Statement

The data that support the findings in this study are available to access and can be requested from the corresponding author on reasonable request.

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