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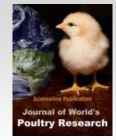

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Antibiotic Resistance Pattern of *Escherichia coli* Isolated from Layer Chicken in Bali-Indonesia

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ABSTRACT

Antibiotics have been used as growth promoters in the poultry industry worldwide, which might lead to the emergence of anti-microbial resistant bacterial strains. Theoretically, older animals should have been exposed to antibiotics and anti-microbial resistant (AMR) strains for longer periods, which may result in the discovery of more resistant strains. The present study aimed to evaluate the antibiotic resistance of *Escherichia coli* isolated from fecal samples of layer chicken that showed signs of watery diarrhea. In the current study, 134 fecal samples were taken from the layer chicken farms in Penebel village, Tabanan District, Bali, Indonesia. The chickens were classified into three groups including Group 1 under 7 days of age, group 2 aged 7-30 days, and Group 3 chickens older than one month. The samples were cultured in Eosin Methylene Blue agar. The suspected colonies were stained, and subjected to biochemical tests. *Escherichia coli*-positive colonies were subjected to a bacteria sensitivity test using multiple antibiotic discs. The result demonstrated multi-drug resistance (MDR) of *Escherichia coli,* while the isolated *Escherichia coli* was resistant to the most common antibiotics in layer farms in the study area including kanamycin, enrofloxacin, sulfamethoxazole-trimethoprim, streptomycin, and enrofloxacin. In addition, the present study confirmed that although all sample groups were sensitive to bacitracin, oxytetracycline, and clindamycin, they were resistant to sulfamethoxazole-trimethoprim, kanamycin, and ampicillin.

Keywords: Antibiotic resistance, Bali, Colibacillosis, *Escherichia coli*, Layer chicken

INTRODUCTION

Implementing strict biosecurity measures is essential to protect the health of chickens and ensure the sustainability of poultry farming. The risk of disease can be reduced by keeping the coop clean, avoiding high chicken population density, and limiting the number of people entering the coop. These efforts will help maintain the health of chickens and support the productivity and profitability of poultry farms (Islam et al., 2023).

One species of bacteria that often causes economic loss and is resistant to antibiotics is *Escherichia coli*, the causal agent of Colibacillosis. This disease is a serious threat to the poultry industry worldwide (Watts and Wigley, 2024). The overall total loss in Indonesia reached more than 10% of total national poultry assets (Wibisono et al., 2018). This bacterium is also zoonotic (Babazadeh and Ranjbar, 2021; Hu et al., 2022).

Colibacillosis is caused by *Escherichia coli* bacteria, which are rod-shaped and Gram-negative (Giubelan et al., 2024). The prevalence of colibacillosis infection in broiler chicken was 5%, with a mortality rate of 1.25% and the case fatality rate was 33.3% (Santoso et al., 2020). Up to 40% of colibacillosis occurs under three weeks of age, while 60% of colibacillosis infections happen in older poultry (Santoso et al., 2020). Colibacillosis occurs more often in young chickens, which is caused by the Avian Pathogenic *Escherichia coli* (APEC) type. The APEC bacteria are dominated by three serotypes, which are O1, O2, and O3. Approximately 10-15% of all *Escherichia coli* found in the intestines of healthy chickens are classified as the APEC type. The APEC type causes various clinical manifestations, such as respiratory, systemic, and reproductive tract infections in layer and broiler chickens (Peighambari et al., 2000; Ribeiro et al., 2023).

Antibiotics have been used as growth promoters in the poultry industry worldwide, which might lead to the emergence of anti-microbial resistant (AMR) bacterial strains. Many cases have been reported where bacteria were initially sensitive to antibiotics but gradually became resistant. Previously, we found that *Escherichia coli* isolated from layer chicken was sensitive to kanamycin, while to oxytetracycline, ampicillin, streptomycin, and sulfamethoxazole was resistant, and to chloramphenicol was intermediate resistant (Besung et al., 2019). Another study found that *Escherichia coli* isolated from broiler chickens in Subang, West Java, was 90% resistant to at least three or more types of antibiotics (Niasono et al., 2019). The most common resistance was against tetracycline 97.3%, sulfamethoxazole 87.8%, trimethoprim 74.3%, ampicillin 68.9%, nalidixic Acid 64.8%, ciprofloxacin 45.9%, enrofloxacin 40.5%, gentamicin 28.4%, and chloramphenicol 10.8%. Almost the same incident also occurred in China (Wang et al., 2021), in Iran (Jahantigh et al., 2020), and in Central Nigeria (Akanbi et al., 2022).

With the increasing development of resistance in poultry, the bacteria sensitivity situation becomes unclear, and bacteria sensitivity testing is needed. This testing is one of the ways to determine the bacteria's resistance to antibiotics. Theoretically, the older animal should have been exposed to antibiotics and AMR strains for a longer period, so more resistant strains might be discovered. The objective of this study was to find out the pattern of the antibiotic-resistant *Escherichia coli* isolated from fecal samples of layer chicken showing clinical signs of watery diarrhea in Bali, Indonesia.

MATERIALS AND METHODS

Ethical approval

Ethical clearance for this study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine Udayana University, Denpasar, Indonesia No. B/168/UN/14.2.9/PT.01.04/2023.

Sampling

All layer farms in Tabanan District Bali, Indonesia, were visited in August and September 2023. The swab samples were collected from the cloaca of chicken that had watery diarrhea using a bacterial Stuart transport medium (Oxoid). In one farm, three groups of chickens namely chickens under 7 days, 8-30 days, and over 30 days, if available, were observed separately. The clinical signs were observed and two cloacal swabs were collected from chickens in the same group. The total numbers of samples were 44, 41, and 49 for groups 1, 2, and 3, respectively. The number of samples was dependent on the availability of chicken in the respective group and the presence of suspect colibacillosis.

Bacteria isolation and identification

Bacteria in feces were cultured in Eosin Methylene Blue Agar (EMBA). A metallic green colony was classified using Gram staining and a series of biochemical tests, such as the Sulphide Indole Motility (SIM) test, Methyl Red (MR), Voges Proskauer (VP), and Simmons Citrate tests (SC) as previously described (Abu-Sini et al., 2023). The colonies were also tested in Triple Sugar Iron Agar (Koutsianos et al., 2020) Glucose, and Lactose Test.

Antibiotic sensitivity test

The colonies that were identified as *Escherichia coli* were tested by a sensitivity test using the agar diffusion method from Kirby-Bauer. Three colonies were taken from the EMBA media and grown in Mueller Hinton Broth (MHB) and incubated for 2 hours at 37°C. The culture turbidity should reach 0.5 MacFarland standard. The cultures were then spread in the Mueller Hinton Agar (MHA). Multiple antibiotic discs that contain bacitracin, oxytetracycline, clindamycin, sulfamethoxazole-trimethoprim, streptomycin, kanamycin, ampicillin, and enrofloxacin (Oxoid), which are commonly used for human and animal in Bali, were then attached to the MHA. The agar media was incubated for 18-24 hours, and the inhibitory diameter was measured and compared with the Clinical and Laboratory Standards Institute (CLSI) sensitivity test standard (Magiorakos et al., 2012; Weinstein and Lewis, 2020). The diameter of the zone was measured manually using a caliper.

Data analysis

The number of colonies that were resistant, intermediate, and sensitive against respective antibiotics were tabulated and analyzed descriptively. The comparison of the *Escherichia coli* inhibition zones against each antibiotic in layer chickens Group 1, Group 2, and Group 3 layers were analyzed using IBM SPSS Statistics software version 27, 2020. The level of significance measured was 5% ($p < 0.05$).

RESULTS AND DISCUSSION

An example of the result of the antibiotics sensitivity tests using the Kirby-Bauer agar diffusion method is presented in Figure 1. The size of the inhibition diameter is standardized to determine the sensitive, intermediate, and resistant categories (Magiorakos et al., 2012; Weinstein and Lewis, 2020). In the picture, an isolate from G-1 resistant to discs 1, 3, 5, and 7 (left); an isolate from G-2 to 1, 2, 3, 4, and 6 (center); as well as an isolate from G-3 to 1, 2, 3, 4, and 5 (right) are presented. The discs 1-8 contained antibiotics (1) bacitracin, (2) oxytetracycline, (3) clindamycin, (4) sulfamethoxazole-trimethoprim, (5) streptomycin, (6) kanamycin, (7) ampicillin, and (8) enrofloxacin. The size of the inhibition shown in Figure 1 indicates isolated *Escherichia coli* from the samples is resistant to ≥ 3 antibiotics from different classes and was referred to as multi-drug resistant (MDR).

Improper and excessive use of antibiotics can cause bacteria to become resistant. Resistant bacteria will survive and reproduce, while sensitive bacteria will die (Van Boeckel et al., 2015). Resistant bacteria often avoid detection or attack the immune system through mechanisms. Some bacteria can produce enzymes that destroy antibodies or change their surface structure to avoid recognition by immune cells (Thomson et al., 2021).

The average diameters of antibiotic inhibition zones to isolates of *Escherichia coli* originating from different chicken age groups are presented in Table 1. The two lowest inhibition zones (tend to be resistant) were demonstrated in bacitracin and clindamycin, while the highest (sensitive) was in sulfamethoxazole-trimethoprim. The proportion (%) of *Escherichia coli* isolates from three different groups of chicken showing sensitive (S), intermediate resistant (I), and resistant (R) to various antibiotics are presented in Table 2. The result showed variation in the three most antibiotics that isolate resistant against, which listed from the highest were in group 1 K (kanamycin), E (enrofloxacin) and ST (sulfamethoxazole-trimethoprim), in group 2 K (kanamycin), S (streptomycin), ST (sulfamethoxazole-trimethoprim), in group 3 were ST (sulfamethoxazole-trimethoprim), K (kanamycin), and E (enrofloxacin).

The prevalence of antibiotic resistance among *Escherichia coli* isolates from layer chickens of different age groups in the Tabanan District, Bali, Indonesia, is presented in Table 2. Notably, *Escherichia coli* isolates from chickens less than 7 days old exhibited resistance to sulfamethoxazole-trimethoprim, kanamycin, and enrofloxacin with resistance rates exceeding 10%. However, resistance to other antibiotics, such as oxytetracycline, bacitracin, and ampicillin, was below 10%. As the chickens aged, the resistance changed. *Escherichia coli* isolates from chickens aged 8-30 days and over 30 days showed increased resistance to streptomycin, kanamycin, and ampicillin, while resistance to bacitracin, clindamycin, sulfamethoxazole-trimethoprim, and enrofloxacin decreased. Notably, none of the *Escherichia coli* isolates from any aged group exhibited resistance to certain antibiotics. Changes in antibiotic resistance on this farm were related to the unwise use of antibiotics. The antibiotic selection was determined by their availability in the market. Antibiotics that are often given to laying hens as treatments or growth stimulants can lead to the development of resistance. Conversely, antibiotics that are never used may result in increased bacterial sensitivity (Mann et al., 2021).

The prevalence of antibiotic resistance in layer chickens within breeding facilities poses a significant challenge to the livestock industry. The indiscriminate use of antibiotics as a preventive measure, rather than solely for treatment, exacerbates the emergence of resistant bacterial strains (Rahman and Hollis, 2023). Previous studies indicated that *Escherichia coli* and *Salmonella* isolated from layer chickens frequently exhibit resistance to commonly used antibiotics, such as ampicillin, tetracycline, and sulfonamides (Zhao et al., 2021). Comprehensive surveys conducted in breeding centers reveal a substantial prevalence of resistance, with some studies reporting resistance rates exceeding 50% for specific antibiotics (Ahmed et al., 2024).

The comparative analysis presented in Table 3 demonstrates an absence of statistically significant differences ($p > 0.05$) between the inhibition zones of bacitracin and clindamycin antibiotics against *Escherichia coli* strains isolated from layer chicken farms, specifically from chickens aged 1-7 days, 8-30 days, and over 30 days. This observation can be attributed to the presence of a lipopolysaccharide layer in the outer membrane of *Escherichia coli*, which acts as a barrier, limiting the penetration of various antibiotics, including clindamycin and bacitracin. Clindamycin is typically effective against Gram-positive bacteria, while bacitracin works well on bacteria with a thick peptidoglycan cell wall. However, since *Escherichia coli* has a thin peptidoglycan layer protected by an outer membrane, these antibiotics are less effective (Puvaca and de Llanos Frutos, 2021). The inhibition zones for the antibiotics oxytetracycline and sulfamethoxazole-trimethoprim against *Escherichia coli* from farms with chickens older than 30 days were significantly lower compared to those with chickens aged 1-7 days and 8-30 days ($p < 0.05$).

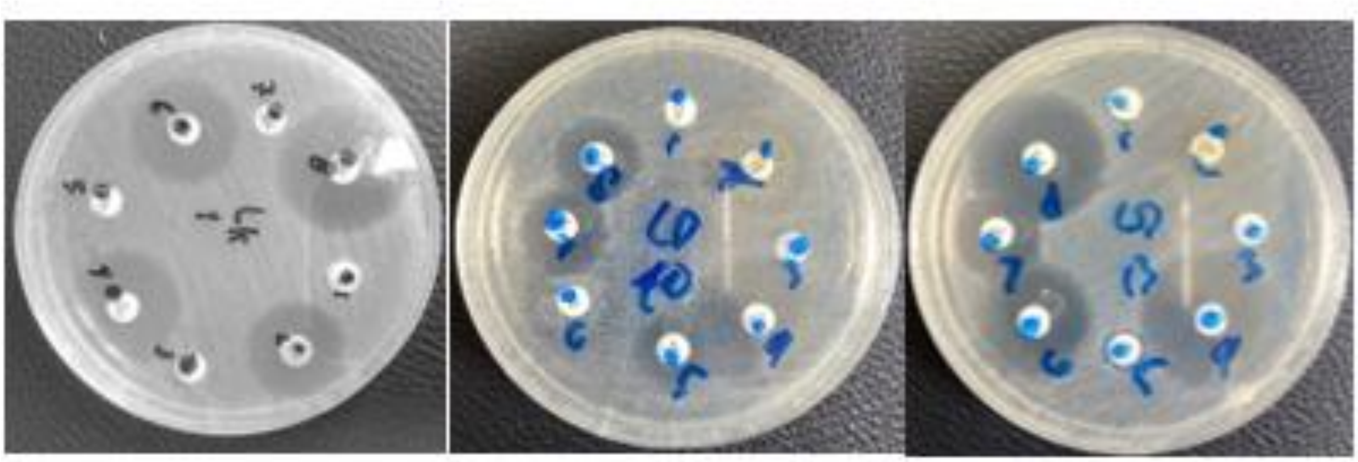

Figure 1. The antibiotics sensitivity tests using the Kirby-Bauer agar diffusion method. Left was a colony from a sample of chicken group 1, middle from group 2, and right from group 3, respectively. Handwritten numbers 1-8 were discs 1 to 8 that contained antibiotics bacitracin, oxytetracycline, clindamycin, sulfamethoxazole-trimethoprim, streptomycin, kanamycin, ampicillin, and enrofloxacin.

Table 1. Average diameters of antibiotic inhibition zone to isolates of *Escherichia coli* originated from different layer chickens in Tabanan District, Bali, Indonesia

	B	O	C	ST	S	K	A	E
Group 1	8.57 ± 6.11	17.07 ± 3.46	7.83 ± 2.91	27.36 ± 5.32	18.93 ± 4.76	19.12 ± 2.03	20.10 ± 3.49	23.40 ± 3.11
Group 2	7.13 ± 0.51	18.10 ± 4.02	7.48 ± 2.53	20.33 ± 6.59	21.28 ± 1.92	22.75 ± 2.38	23.05 ± 2.50	24.78 ± 4.07
Group 3	7.10 ± 0.47	15.44 ± 3.99	7.40 ± 2.32	26.04 ± 4.14	20.08 ± 2.27	22.10 ± 1.97	19.29 ± 3.58	22.04 ± 4.86
Average of respective antibiotics	7.60	16.87	7.57	24.58	20.10	21.32	20.81	23.41

B: Bacitracin, O: Oxytetracycline, C: Clindamycin, ST: Sulfamethoxazole-Trimethoprim, S: Streptomycin, K: Kanamycin, A: Ampicillin, E: Enrofloxacin. Group 1: Layer chickens under 7 days old, Group 2: 8-30 days old, Group 3: Over 30 days old

Table 2. Proportion (%) of sensitive *Escherichia coli* isolated from layer chickens of Tabanan District, Bali, Indonesia to various antibiotics

Antibiotics	Proportion (%) of isolates that were sensitive (S), intermediate resistant (I) and resistant (R)								
	Group 1			**Group 2**			**Group 3**		
	S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Bacitracin	93.02	2.33	4.65	97.56	2.44	0.00	100	0.00	0.00
Oxytetracycline	90.70	9.30	0.00	95.12	4.88	0.00	100	0.00	0.00
Clindamycin	95.35	0.00	4.65	97.56	0.00	2.44	100	0.00	0.00
Sulfamethoxazole-Trimethoprim	69.77	0.00	30.23	70.73	0.00	29.27	14.29	0.00	85.71
Streptomycin	88.37	0.00	11.63	29.27	26.83	43.90	48.98	10.20	40.82
Kanamycin	34.88	2.33	62.79	14.63	0.00	85.37	18.37	0	81.63
Ampicillin	90.70	0.00	9.30	75.61	0.00	24.39	18.37	20.41	61.22
Enrofloxacin	46.51	16.28	37.21	85.37	0.00	14.63	12.24	30.61	57.15

S: Sensitive; I: Intermediate; R: Resistant to various antibiotics.

Table 3. The analysis of *Escherichia coli* sensitivity to various antibiotics based on the age of layer chickens in Tabanan District, Bali, Indonesia

Antibiotics \ Inhibition zone (mm)	Group 1	Group 2	Group 3	Average	p-value
Bacitracin	8.50 ± 0.92[a]	7.12 ± 0.80[a]	7.10 ± 0.67[a]	7.57 ± 0.307	0.105
Oxytetracycline	18.12 ± 1.628[a]	17.00 ± 1.522[a]	15.45 ± 1.570[b]	16.78 ± 1.342	0.005
Clindamycin	7.80 ± 0.438[a]	7.46 ± 0.395[a]	7.39 ± 0.331[a]	7.54 ± 0.222	0.729
Sulfamethoxazole- Trimethoprim	27.14 ± 1.464[a]	25.92 ± 1.990[a]	20.34 ± 1.020[b]	24.61 ± 3.963	0.000
Streptomycin	18.86 ± 1.762[b]	20.06 ± 1.268[a]	21.22 ± 1.917[a]	20.02 ± 1.344	0.004
Kanamycin	19.14 ± 2.030[c]	22.61 ± 2.376[b]	22.96 ±1.972[a]	21.31 ± 2.605	0.000
Ampicillin	19.20 ± 1.576[b]	20.11 ± 1.486[b]	22.95 ± 1.500[a]	20.65 ± 1.595	0.000
Enrofloxacin	21.92 ± 0.864[b]	23.41 ± 1.113[a]	24.16 ± 1.067[a]	23.28 ± 1.243	0.006

[a, b, c] Means with different superscript letters in the column represent significant differences at $p < 0.05$. Group 1: Layer chickens under 7 days old, Group 2: 8-30 days old, Group 3: Over 30 days old.

Conversely, the inhibition zones for the antibiotics streptomycin, kanamycin, ampicillin, and enrofloxacin against *Escherichia coli* from chickens older than 30 days were significantly greater ($p < 0.05$) compared to those from chickens aged 1-7 days and 8-30 days. Accordingly, resistance to oxytetracycline and sulfamethoxazole-trimethoprim has increased, whereas resistance to streptomycin, kanamycin, ampicillin, and enrofloxacin has decreased. The changes in resistance on these farms are suspected to be due to the use of antibiotics in disease treatment or prevention.

Usage of antibiotics, whether to treat or prevent disease, can lead to increased antibiotic resistance. Over time, bacteria exposed to these antibiotics may develop mechanisms to survive their effects, reducing the antibiotics' effectiveness. This resistance can spread, posing significant challenges for managing infections and ensuring effective treatment in animal and human health contexts. The emergence of antibiotic resistance is a complex issue that requires a holistic approach, including the regulation of antibiotic use, improved management practices, and education for farmers. Integrated efforts are needed to mitigate the risks and impacts of antibiotic resistance in livestock farming (Ahmed et al., 2024).

Antimicrobial resistance (AMR) is a growing global health crisis that occurs when microorganisms evolve to resist the effects of medications used to treat infections (Tang et al., 2023). Antimicrobial resistance due to veterinary use, including in layer chicken, is a significant contributor to the global AMR crisis (Caneschi et al., 2023; Hedman et al., 2020). Poor biosecurity in layer chicken farms in Bali and uncontrolled antibiotic administration have led to the emergence of resistant bacteria. It is also believed that antibiotic resistance in these farms may have originated from surrounding farms.

The results of the present study showed that all three isolates were resistant to three or more antibiotics, which is referred to as MDR (Magiorakos et al., 2012). The MDR cases have been reported from various environments including chicken farms in Dakahlia and Sharkia Governorates, Egypt (Awad et al., 2023), in Malang Regency, Indonesia (Dameanti et al., 2022), and in Karatu, Northern Tanzania (Sonola et al., 2021). Furthermore, the current study revealed that resistance to certain antibiotics, including bacitracin and clindamycin, was observed across all age groups. Notably, resistance to bacitracin has been previously reported in *Escherichia coli* isolates obtained from chicken in Malang Regency, Indonesia (Dameanti et al., 2022), Farnham, Quebec, Canada (Thibodeau et al., 2008), and Zambia (Mudenda et al., 2023). Additionally, multidrug-resistant *Escherichia coli* O157 has been identified in poultry farms located in eastern Ethiopia (Shecho et al., 2017).

Antibiotic resistance in veterinary medicine significantly impacts animal health and public safety. Since antibiotics are commonly used to promote growth and prevent disease in livestock, their use can lead to the development of resistant bacteria in animals, which can then be transmitted to humans through the food chain. It results in serious risks, as resistant infections are more difficult to treat, leading to increased morbidity and healthcare costs (Van Boeckel et al., 2015). Additionally, the presence of antibiotic-resistant bacteria complicates treatment protocols and may result in higher treatment failure rates. There is a need for more responsible use of antibiotics, including enhanced monitoring and regulation. Addressing this issue is critical not only for maintaining animal welfare but also for protecting public health from the impacts of antibiotic resistance (Ajayi et al., 2024).

CONCLUSION

It can be concluded that the *Escherichia coli* isolated from layer chicken in the study area were sensitive to bacitracin, oxytetracycline, and clindamycin, but resistant to sulfamethoxazole-trimethoprim, streptomycin, kanamycin, and ampicillin. As the age of the chicken increases, the resistance to some antibiotics increases. Further research is needed to cover a wider study area. Biosecurity should be strictly implemented in farms that have a history of infection.

DECLARATIONS

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Authors' contributions

I Nengah Kerta Besung and Ni Ketut Suwiti designed the research, collected the samples, and provided the media for the research. Putu Henrywaesa Sudipa and I Gusti Ketut Suarjana conducted the research process in the laboratory. All authors reviewed the analyzed data and approved the final draft of the manuscript.

Competing interests

The authors have disclosed no conflicts of interest.

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time. Additionally, all authors checked the originality of data and sentences via plagiarism checkers.

Availability of data and materials

The original data presented in the study are included in the article. For inquiries, please contact the corresponding author.

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