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TETRASIKLIN RESISTANCE IN *Escherichia coli* ISOLATED FROM PIG FARM, PIG SLAUGTERHOUSE, AND THE ENVIRONMENT IN BANTEN PROVINCE

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ABSTRACT

This study aims to determine the prevalence of tetracycline antibiotic resistance to *Escherichia coli* **(***E. coli*) isolated from farms, pig slaughterhouses, and the environment in Banten Province. This study used 44 samples of solid waste/feces and effluent from pig farms, 10 samples of floor swab and effluent from pig slaughterhouses and 4 samples of the environment (river water). Isolation and identification of *E. coli* using Tryptone Bile X-Glucuronide (TBX), MacConcey Agar (MCA) media and confirmed by indole test. Sensitivity test against oxytetracycline and tetracycline antibiotics was carried out using Kirby-Bauer method (disc diffusion). The results showed that oxytetracycline was the most resistant antibiotic. The prevalence of oxytetracycline resistance was highest in farms in solid waste/feces samples (56.82%), pig slaughterhouses in effluent samples (80%) and environment in river samples (upstream, downstream) (100%). Tetracycline showed the highest prevalence in solid waste/feces samples of farm (40.91%), in floor swab samples of pig slaughterhouses (70%), in upstream river samples of environment (100%). This study indicates that waste from farms and pig slaughterhouses could be a source of transmission of *E. coli* resistant to tetracycline antibiotics to the environment.

Key words: antibiotics, *E. coli,* sewage, swine, tetracycline resistance

ABSTRAK

Penelitian ini bertujuan mengetahui prevalensi resistansi antibiotik golongan tetrasiklin terhadap to Escherichia coli **(***E. coli*) *yang diisolasi dari peternakan, tempat pemotongan babi, dan lingkungan di Provinsi Banten. Penelitian ini menggunakan sampel limbah padat/feses dan limbah cair (effluent) dari peternakan babi masing-masing 44 sampel, sampel swab lantai dan effluent dari tempat pemotongan babi masing-masing 10 sampel dan 4 sampel lingkungan (air sungai). Isolasi dan identifikasi E. coli menggunakan media Tryptone Bile X-Glucuronide (TBX), MacConcey Agar (MCA) dan dikonfirmasi dengan uji indol. Uji kepekaan terhadap antibiotik oksitetrasiklin dan tetrasiklin dengan metode Kirby-Bauer (difusi cakram). Hasil penelitian menunjukkan bahwa, oksitetrasiklin merupakan antibiotik yang paling banyak mengalami resistansi. Prevalensi oksitetrasiklin yang resistan paling tinggi pada peternakan yaitu sampel limbah padat/feses (56.82%), tempat pemotongan babi pada sampel effluent (80%) dan lingkungan pada sampel sungai (upstream, downstream) (100%). Tetrasiklin menunjukkan prevalensi paling tinggi pada peternakan yaitu sampel limbah padat/feses (40.91%), tempat pemotongan babi pada sampel swab lantai (70%), lingkungan pada sampel sungai bagian upstream (100%). Hasil penelitian ini mengindikasikan bahwa, limbah dari peternakan dan tempat pemotongan babi dapat menjadi sumber penyebaran E. coli resistan terhadap antibiotik golongan tetrasiklin ke lingkungan.*

Kata kunci: antibiotik, , E. coli, limbah, babi, resistansi tetrasiklin

INTRODUCTION

Tetracyclines are antibiotics that are widely used in pig farming (Kallau *et al*. 2018a; Detha *et al*. 2021) and humans because they have a broad spectrum, are readily available and reasonably priced (Jurnalis *et al*. 2009). Several studies have shown that tetracycline have developed resistance (Wang *et al.* 2016; Zhang *et al.* 2017; Kallau *et al.* 2018b). Antibiotic resistance is a serious problem not only nationally but also globally (CIVAS 2021; O'neill 2014). Antibiotic resistance has far-reaching impacts on public, animal and environmental health. Several reports mentioned that resistant pathogenic and commensal bacteria can be transmitted through the consumption of food of animal origin and environmental contamination (WHO 2010; O'neill 2014). Antibiotic resistance causes the use of antibiotics to be ineffective in inhibiting or killing bacteria and leads to treatment failure, prolong hospital stays, increased risk of complications, and increased morbidity and mortality (Castro-Sánchez *et al*. 2016; Coyne *et al*. 2016).

The pig farming industry plays a major role in causing antibiotic resistance, especially due to the overuse of antibiotics. Based on a study conducted by Kallau *et al*. (2018a) showed that 55.21% of antibiotics were used for medication, 42.71% for disease prevention and 2.08% for increasing production, in pig farms in Kupang City, NTT,

The use of sub-lethal doses of antibiotics has a negative impact on the incidence of bacterial resistance. Sub-lethal doses of antibiotics can exert selective pressure on bacteria in the animal's digestive tract to acquire and maintain antibiotic resistance genes and lead to an increase in the relative abundance of resistant bacterial populations (Gullberg *et al.* 2011). In addition, more than 85% of the administered antibiotics or their metabolites can be excreted through the urine or feces of livestock and then discharged into the environment, resulting in antibiotic residues, which can also increase the abundance of resistant bacterial populations in the environment (Tao *et al*. 2014).

Escherichia coli (*E. coli*) is a commensal, Gram-negative enteric bacterium that is common in humans and animals. In addition, some strains of *E. coli* are pathogenic to animals and humans which can cause *foodborne diseases* that are harmful to health. *E. coli* is one of the most widely used indicator organisms in antimicrobial resistance monitoring because it is susceptible to high selective pressure from antimicrobial agents and carries Mobile Genetic Elements (MGE) that play an important role in transferring antibiotic-resistant genes to other bacteria (Gekenidis *et al*. 2018; Brisola *et al.* 2019). In addition, *E. coli* is one of the most widely used indicator organisms for monitoring the sanitation and microbial quality of water (He *et al*. 2016).

Banten Province has a number of pig farms and pig slaughterhouses to fulfil the need for pork in the community. According to the Central Bureau of Statistics (BPS) of Banten Province in 2021, the pig population in Banten Province totalled 7819 pigs (BPS 2023). Currently, there is limited data related to antibiotic-resistant *E. coli*, especially tetracycline, from pig farms, pig slaughterhouses and the environment in Banten Province, so it is necessary to conduct research related to this issue. In addition, waste from pig farms and slaughterhouses that are not handled properly certainly have great potential to pollute the environment, ecosystems and pose a serious threat to public health.

This study aims to determine the incidence (prevalence) of antibiotic resistance of tetracycline group to *E. coli* isolated from farms, pig slaughterhouses and the environment in Banten Province. This data is needed as a basis for strategies in developing prevention and control measures against antimicrobial resistance.

MATERIALS AND METHODS

Sampling Size and Technique

All pig farms and slaughterhouses in Banten Province were sampled. The total number of farms in Banten Province was 44 with each farm sampled for solid waste (feces) and liquid waste. The total number of pig slaughterhouses in Banten Province was 10 pig slaughterhouses with each sampled consisting of swabbed pig slaughterhouse floors and effluent, and environmental samples in the form of streams drained by waste from pig slaughterhouses comprising 2 rivers with each river sampled at 2 points (upstream and downstream).

Sampling of effluent referred to SNI 6989.59-2008 on Wastewater Sampling Methods (BSN 2008) and ISO 19458:2006 on sampling for Microbiological Analysis in Water Quality Tests (ISO 2006) which are carried out aseptically and transported to the laboratory at 4° C. Sampling at the pig slaughterhouse was carried out at two different times, during the slaughtering process and after slaughtering with 250 mL samples each (total sample 500 mL) for effluent samples. River water sampling consisted of 2 spots, upstream with a distance of 10 metres from the waste source and downsream with a distance of 5 metres from the waste source. The number of river water sampled was 500 mL.

Isolation and Identification of *Escherichia coli*

Isolation and identification of *E. coli* referred to the Global Tricycle Surveillance ESBL *E. coli* from WHO, 2021 (WHO 2021). All samples were serially diluted to 10-5 in duplicate using sterile PBS solution in a ratio of 1:9. Samples at each dilution were collected as a dose of 0.1 mL and transferred to a petri dish containing Tryptone Bile X-Glucuronide agar (TBX; Merck, Germany) and flattened on the surface of the agar (spread plate method). Colonies suspected to be *E. coli* on TBX media were greenish-blue in color. Dishes with colonies ≤100 CFU/mL were proceeded to the next stage. A minimum of 5 colonies of each colony selected from TBX agar were inoculated onto MacConcey's Agar Medium (MCA; Oxoid, UK). Colonies of suspected *E. coli* on MCA media were round, smooth, dark pink in color and surrounded by a turbid zone. After that, colonies suspected of *E. coli* were planted on Tryptic Soy Agar (TSA; Oxoid, UK) media, then planted on Sulfide Indol Motility (SIM; Oxoid, UK) media for indole biochemical testing as confirmation of *E. coli*. The result was positive for *E. coli* if a cherry red ring is formed. This study used a positive control of *E. coli* ATCC 25922.

Antibiotic Sensitivity Test

Antibiotic resistance phenotypes were determined through *E. coli* sensitivity testing against antibiotics by the *Kirby-Bauer disc diffusion* method on *Mueller Hinton Agar* (MHA; Oxoid, UK) media according to the Clinical and Laboratory Standards Institute (CLSI 2018). The assay was performed in duplo. Bacterial isolates were determined for their sensitivity to antibiotics by measuring the inhibition zone formed on MHA media in the form of sensitive (S), intermediate (I) and resistant (R) referring to CLSI 2018. The antibiotics used in this study were tetracycline 30 µg and oxytetracycline 30 µg.

Data Analysis

The test data was displayed in the form of tables and figures, then analysed descriptively.

RESULTS AND DISCUSSION

Isolation and Identification of *E. coli*

All samples tested on farms, pig slaughterhouses and the environment showed positive results for *E. coli* (100%). The complete isolation and identification results of *E. coli* can be seen in Table 1.

The results of isolation and identification of *E. coli* in livestock samples (solid waste/feces and effluent) in Banten Province showed a high prevalence, reaching

100%. This finfing was in line with research conducted by Kallau *et al.* (2018b) which showed a high prevalence of *E. coli* on pig farms in Kupang City, which was 85.40%. *E. coli* is a commensal bacterium in the digestive tract of animals and some are pathogenic, so it is commonly found in pig farms and the surrounding environment (Park *et al.* 2017). Based on observations in the field, most pig farms in Banten Province are located not far from community settlements, lack of cleanliness and sanitation and their waste is not handled properly, potentially polluting the surrounding environment.

High prevalence of *E. coli* was also shown in pig slaughterhouse samples (floor swab and effluent) and the environment (river) at 100%. Some previous studies also reported that, wastewater treatment plants from pig slaughterhouses in Germany showed a high *E. coli* prevalence of 85.1% (Savin *et al*. 2020). In addition, high prevalence was also shown in slaughterhouse effluent (pigs and cattle) in Portugal (Amador *et al*. 2019), and slaughterhouse wastewater in Addis Ababa, Ethiopia (Tesfaye *et al*. 2019). According to Blaak *et al*. (2015), the percentage of *E. coli* present in different slaughterhouses can vary depending on various factors such as the condition of the sampling area, the level of faecal contamination, the time of sampling, the speed of effluent flow and the condition of the effluent drain.

Poor hygiene and sanitation practices, high faecal contamination of animal intestines and poor health conditions of slaughtered animals are responsible for the high prevalence of *E. coli* detected in floor and effluent samples from pig slaughterhouses (Onuoha *et al*. 2016). Blood is a protein-rich medium and can be an ideal source of nutrients for the growth and proliferation of *E. coli* (Dankaka *et al*. 2018), so this can lead to high numbers of *E. coli* on the floor and effluent of pig slaughterhouses. Poor hygiene and sanitation conditions in pig slaughterhouses lead to the spread of *E. coli* from animals, humans and the environment and even ecosystems through contaminated effluents. Most of the effluent from pig slaughterhouses in Banten Province is discharged into the Cisadane river without any treatment, posing a risk to the health of the people who use the river water for their daily needs. Previous research reported that there was high *E. coli* contamination in the Cisadane river ranging from 2 x 10^2 to 3 x 10^5 cfu/100 mL (Purwati *et al*. 2019). The presence of *E. coli* may also indicate the possible presence of other pathogenic organisms of public health importance (Atuanya *et al*. 2018).

Tetracycline Sensitivity Test

The results of the *E. coli* sensitivity test against tetracycline antibiotics showed that oxytetracycline was the most resistant antibiotic with the highest prevalence reaching 100% in river samples (upstream and downstream), followed by pig slaughterhouse effluent (80%) and pig slaughterhouse floor *swabs* (70%). Meanwhile, pig farm samples showed the highest prevalence in solid waste (56.82%), then in pig farm liquid waste (47.73%). Tetracycline also showed a high prevalence of 100% in upstream river samples and then pig slaughterhouse floor swabs at 70% (Figure 1). The sensitivity test results to tetracycline antibiotics can be seen in Table 2.

High prevalence of tetracycline-resistant *E. coli* was also reported in a previous study in pig farm effluent (98%) and pig slaughterhouse effluent (80%) samples in Portugal (Amador *et al.* 2019). Healthy pig feces from a pig slaughterhouse in Spain (90%) (Marchant *et al*. 2013) and river water fed by pig farm effluent also showed high prevalence of tetracycline-resistant *E. coli* (Jia *et al.* 2014).

It is noteworthy, that there is a high potential to become resistant to tetracycline antibiotics, namely in samples that have an intermediate level of resistance. *E. coli* that experienced resistance at the intermediate level to the antibiotic oxytetracycline showed the highest prevalence in pig farm liquid waste samples at 50%, then in pig farm solid waste (feces) samples at

Table 1. Results of isolation and identification of *E. coli*

Samples	Sample type	Number of culture isolates	Percentage of test results on various media (%)			Number of positives *E. coli* (%)
			TBX media culture	MCA media culture	Indole Test	
Pig farming	Solid waste (feces)	44	100	100	100	100
	Liquid waste	44	100	100	100	100
Pig slaughterhouse	Floor swab	10	100	100	100	100
	Liquid waste (effluent)	10	100	100	100	100
Environment	River	4	100	100	100	100

Table 2. Sensitivity test results of tetracycline antibiotics against *E.* coli

Samples	Type Sample	Number of samples	% resistance					
			Tetracycline			Oxytetracycline		
			S	I	R	S	I	R
Pig farming	Solid waste (feces)	44	45.45	13.67	40.91	2.27	40.91	56.82
	Liquid waste	44	38.64	22.73	38.64	2.27	50	47.73
Pig slaughterhouse	Floor swab	10	0	30	70	0	30	70
	Liquid waste (effluent)	10	10	30	60	10	10	80
Environment	Upstream river	2	0	0	100	0	0	100
	Downstream river	2	0	50	50	0	0	100

S (sensitive); I (intermediate); R (resistant)

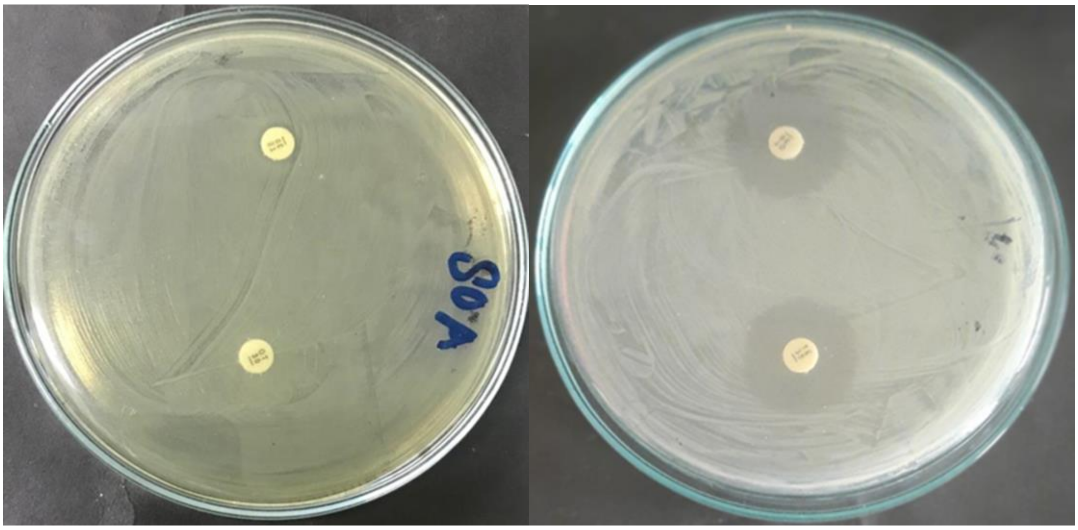

Figure 1. Sensitivity test of *E. coli* to tetracycline

40.91%, while intermediate resistant *E. coli* to tetracycline antibiotics showed the highest prevalence in downstream river samples at 50% (Table 2). Intermediate resistance levels have a high potential to become resistant, posing a serious threat if no control is exerted on the use of antibiotics in the future.

The occurrence of antibiotic-resistant *E. coli* in pigs can contaminate the farm environment, pig slaughterhouse environment, pig meat and workers who handle pigs and has the potential to be spread to the extensive environment and ecosystem through the waste produced. The spread of antibiotic-resistant bacteria can also occur through direct or indirect contact such as through food, water and animal feeces to agricultural land (Marshall and Levy 2011; Sanders *et al*. 2013). Several studies have also reported tetracycline-resistant *E. coli* contamination in pork (Rizaldi *et al*. 2019) and processed meat of pork origin (Amalo *et al*. 2021), pig slaughterhouse wastewater (Amador *et al.* 2019), well water around pig farms (Huang *et al*. 2019), river water (Jia *et al*. 2014) and soil fertilised with pig feces (Kang *et al.* 2018).

Based on observations made in the field, most pig farms in Banten Province are located not far from community settlements and the waste generated is not handled properly. Likewise, pig slaughterhouses dispose of their waste into the river without any waste treatment, so this is very risky to pollute the environment and the broader ecosystem from antibiotic-resistant bacteria and Antibiotic Resistance Genes (ARGs) and can have a serious impact on public health through the contaminated food chain. This is evidenced by the river water samples tested in this study showing a high prevalence of tetracycline-resistant *E. coli* both upstream and downstream.

Excessive and inappropriate use of antibiotics in pig farms is one of the causes of resistance to oxytetracycline and tetracycline. The results of previous studies showed that in pig farms, antibiotics were used for medication by 55.21%, disease prevention by 42.71% and for increased production by 2.08%. Tetracycline antibiotics, especially oxytetracycline, are the most widely used antibiotics in pig farms (Kallau *et al*. 2018a; Rizaldi *et al*. 2019; Detha *et al*. 2021).

Tetracyclines are a class of broad-spectrum antibiotics against various pathogenic bacteria in animals and humans, so they are widely used for the treatment of bacterial infections and have also been used in the livestock industry for many years. Pig farms often use tetracycline for the treatment of gastrointestinal, respiratory, urogenital tract infections, atrophic rhinitis and others (Callens *et al*. 2012; Jensen *et al.* 2012). In addition to treatment, tetracycline is also widely used for prophylaxis and as a growth promoter mixed in animal feed at sub-therapeutic dose levels (Sabino *et al*. 2019).

Administration of sub-lethal doses of antibiotics can slow the growth of susceptible bacterial populations, consequently exerting selective pressure on bacteria in the animal's digestive tract to acquire and maintain antibiotic resistance genes and triggering an increase in the relative abundance of resistant bacterial populations (Gullberg *et al.* 2011). In addition, administered antibiotics or their metabolites can be excreted more than 85% through the urine or feces of livestock and then discharged into the environment, which can lead to antibiotic residues and can increase the abundance of resistant bacterial populations in the environment (Tao *et al*. 2014). Animals and humans can excrete tetracycline in its active form into the environment due to its highly hydrophobic nature and low volatility, so tetracycline is very stable in aquatic environments and is commonly detected in Wastewater Treatment Plants (WWTPs) (Daghrir and Drogui 2013). Tetracycline compounds form stable complexes with cations making them more likely to bind to suspended matter or sewage sludge during wastewater treatment (Collado *et al*. 2014). Several studies have reported that tetracyclines are the most common substances detected in WWTPs (Opriş *et al.* 2013; Vergeynst *et al.* 2015).

Tetracycline is also the most widely available antibiotic in health care units, especially Community

Health Centres (Puskesmas) (Jurnalis *et al.* 2009). The use of the same antibiotic in humans and production animals is one of the causes of the emergence and spread of antibiotic-resistant bacteria in humans which can have an impact on treatment failure, longer hospital stays, increased risk of complications, and result in increased morbidity and mortality (Castro-Sánchez *et al*. 2016; Coyne et *al.* 2016).

CONCLUSIONS

E. coli was resistant to oxytetracycline and tetracycline with the highest prevalence in pig farms in solid waste/feces samples, in pig slaughterhouses in effluent samples and in the environment in upstream and downstream river samples. The results of this study indicate that effluents from pig farms and slaughterhouses can be a source of spread of tetracycline-resistant *E. coli* to the environment.

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