Isolation and Detection of *Escherichia Coli* Trimethoprim Resistance Gene from Layer Chickens in East Java Province, Indonesia by Polymerase Chain Reaction

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Abstract: Aim: The aim of this research was to detect the presence of a gene which is responsible for *Escherichia coli* trimethoprim resistance (TEM gene) using Polymerase Chain Reaction from layer chickens in East Java Province, Indonesia. Methods: Purposive sampling of 60 infundibulums has been done in laying chicken farms in those indicating Colibacillosis from 6 districts in East Java Province, Indonesia including Sidoarjo, Mojokerto, Jombang, Kediri, Bojonegoro and Blitar. Ten samples were collected from each district. Samples were isolated using Eosin Methylene Blue Agar and five biochemical tests of Sulfide Indol Motility (SIM), Simons Citrate Agar (SCA), Triple Sugar Iron Agar (TSIA), Urea Agar (UA) and Sugar Test were used for bacteria identification. The DNA was then isolated from *Escherichia coli*-positive samples prior to Polymerase Chain Reaction (PCR) amplification to investigate TEM gene that indicated trimethoprim antibiotic resistance. Result: Bacteriological tests showed that 30 of 60 samples (50%) were *E. coli* positive. Ten of those were from Blitar, 10 from Bojonegoro, 5 from Sidoarjo, and 5 from Jombang. Two samples from Kediri and Mojokerto districts were negative. From PCR amplification showed that 28 of 30 samples were negative due to TEM gene, and there were 2 positive samples shown from Bojonegoro and Blitar. This indicated that 6.66% of total samples were positive containing TEM gene. Conclusion: Based on bacteriological examination 50% of samples from six districts in East Java, Indonesia were *E. coli* positive, and 6.66% were trimethoprim resistant based on occurrence of TEM gen in PCR amplification.

1 INTRODUCTION

Avian colibacillosis is an infectious avian disease of caused by *Escherichia coli* infection and is responsible for significant economic losses in the poultry industry (Matsuda et al., 2010). Avian Pathogens *Escherichia coli* (APEC) are increasingly encountered in the field and its relationship to various conditions of the disease, either as a primary pathogen or as a secondary pathogen and may be infectious to humans or zoonotic. Chicken colibacillosis is characterized in acute form by septicemia resulting in death and subacute form by pericarditis, air sacculitis and perihepatitis. Avian colibacillosis has been recognized as a major contagious disease of all ages. Avian Pathogenic *Escherichia coli* (APEC), spreads to various internal organs and causes colibacillosis characterized by systemic fatal disease. The diseases that are infected by *E. coli* enter the host through ingestion or inhalation, after which it trans-locates across mucosal layers, then colonizes in other tissue via bloodstream, it can cause air sacculitis, enteritis, arthritis, panoptalmitis, reproduction genital infections, bursitis stenalis (Krisnaningsih, 2005), and in layers can cause salphingitis, omphalitis, misshapen, pedunculated eggs and egg peritonitis (Anyanwu, 2014).

Treatment with many antibiotics causes resistance. In the use of antibiotics in APEC it is

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important to note the different sensitivity of *E. coli* serotype because some serotypes were resistant to some antibiotics. The development of resistance properties of *E. coli* bacteria is a serious problem today especially related to the treatment and handling of some diseases caused by *E. coli*, so it is necessary to do a safe antibiotic replacement. The use of inappropriate antibiotics with no benefit to therapy is a feed stimulator or growth promoter for livestock and poultry is one cause of loss of antibiotic effectiveness. It can cause disruption of normal microbial ecology balance and eliminate a group of sensitive bacteria while a group of resistant bacteria will grow and well developed becomes a pathogenic population (Dibner & Richards, 2005).

Trimethoprim is a potent broad-spectrum antibacterial agent with a slow bactericidal action against susceptible bacterial infections (gram-negative and gram-positive). It acts by inhibiting the enzyme dihydrofolate reductase, which is responsible for the conversion of folate to folinate and reduces their pools of folinic acid that is required as a co-factor (Papich, 2016). Nowadays, there is widespread antibiotic resistance in the poultry industry and can lead to reconsideration of antibiotic use, especially trimethoprim which has been widely used primarily by combining with the sulfonamide group. Bacteria may become resistant to trimethoprim by the following: reduced bacterial uptake, alterations or mutations in dihydrofolate reductase, overproduction of dihydrofolate reductase (Kester, et al., 2012).

The aim of this research was to detect the presence of trimethoprim antibiotic resistance gene in isolated samples taken from layer chicken farms from East Java Province, Indonesia using the latest method of matching genes using PCR.

2 MATERIALS AND METHODS

The survey areas were the layer chicken farms owned by breeder farmers in East Java Province, Indonesia, at Sidoarjo, Mojokerto, Jombang, Kediri, Bojonegoro and Blitar districts.

2.1 Sampling Areas

Ten samples of infundibulum were taken from each district indicating colibacillosis and the total number of samples was 60 samples.

2.2 Isolation of Samples

Samples were taken from layer chicken's infundibulum and then isolated using Eosin Methylene Blue Agar medium and five biochemical tests of Sulfide Indol Motility (SIM), Simons Citrate Agar (SCA), Triple Sugar Iron Agar (TSIA), Urea Agar (UA) and Sugar Test were used for bacteria identification. Positive results of *E. coli* gained from biochemical tests showed that SIM had a cloudy streak area and a red ring on top of the medium, TSIA medium showed A/A, H_2S (-), and gas (+), Urea Agar changed colour from pink to orange. On Sugar Test almost all of the sugar was fermented except mannitol, and for SCA Medium did not change into any colour.

2.3 DNA Isolation

After biochemical test showed positive results, *E. coli* was inoculated in Nutrient Agar to avoid any disturbing colours as PCR processes take place from EMBA medium. These were the following steps for the extraction of DNA:

Took 20 µl QIAGEN Protease (or proteinase K) using pipet into 1,5 ml micro-centrifuge tube. Add 180 µl ATL Buffer, then add 2-3 bacteria colony from Nutrient Agar and 5 µl Lysozim. Incubation at 60°C for 30 minutes. Add 200 µl AL buffer into the sample, then vortex for 15 seconds. Add 200 µl ethanol 96% and mix up with vortex for 15 seconds, then spin down. Put into QIAamp Mini spin column (2 ml collection tube) compound from step before. Centrifuge at 8,000 rpm for 1 minute. Throw 2 ml filtrate from collection tube and then replaced tube with the new one (new 2 ml collection tube). Add 500 µl AW1 Buffer, then centrifuge at 8,000 rpm for 1 minute. Throw 2 ml filtrate from collection tube and then replace tube with the new one (new 2 ml collection tube). Add 500 µl AW2 Buffer, then centrifuge at 13,000 rpm for 3 minutes. Throw 2 ml filtrate from collection tube and then replace tube with the new 2 ml collection tube. Centrifuge again at 13,000 rpm for 1 minute. Move QIAamp Mini spin column to 1.5 ml micro-centrifuge tube. Add 50 µl AE Buffer or distilled water. Incubation at room temperature (15-25°C) for 1 minute, then centrifuge at 8,000 rpm for 1 minute. Then obtain 50 µl DNA template.

2.4 Polymerase Chain Reaction (PCR) and Electrophoresis

Materials for Polymerase Chain Reaction running were as follows: 20 µl QIAGEN Protease (proteinase K), 180 µl Buffer ATL, 5 µl Lysozyme, 200 µl buffer AL, 200 µl ethanol 96%, 500 µl Buffer AW1, 500 µl Buffer AW2, 50 µl Buffer AE, 2x PCR Master mix (Intron), 1µl (50Pmol/µl) of TEM F3 (the sequence of primer pair is GTA TCC GCT CAT GGA GAC AAT AAC CCT G) and TEM R3 (the sequence of primer pair is CCA ATG CTT AAT CAG TGG AGG CAC C) primer, and 5µl of DNA template, agarose gel 1.5%.

Amplification of bacterial DNA was performed with a volume of 20 µl as follows: 12.5 µl of 2X master mix (Intron), 0.5µl of distilled water, 1 µl of TEM F3 primer, 1 µl of TEM R3 primer, and 5 µl of DNA template. Polymerase chain reaction steps are Pre-Denaturation at 94^0C for 5 minutes, then Denaturation at 94^0C for 1 minute; Annealing at 53^0C for 30 seconds for 30 times; Extension at 72^0C for 1 minute, these 3 steps are repeated for 35 cycles and last Final Extension at 72^0C for 5 minutes.

PCR products are electrophoresed on a 1.5% agarose gel then running for 30 minutes. A digital image of the gel is captured in a computer, and the amplification patterns were evaluated by visual examination of inverted gel pictures.

3 RESULTS

The survey showed that layer chickens in Sidoarjo, Mojokerto, Jombang, Kediri, Bojonegoro and Blitar district indicating colibacillosis showed by number of cases found in the field and from the checking of post-mortem showed symptoms of colibacillosis. There were 10 layer chickens obtained from each district. The bacteriological test showed that 10 samples from Blitar, 10 samples from Bojonegoro, 5 samples from Sidoarjo, and 5 samples from Jombang were *E. coli* positive. Samples from Kediri and Mojokerto were negative.

Table 1 : Result of biochemical test for *E. coli*

No.	District	Samples Amount	Result	
			Positive	Negative
1.	Sidoarjo	10	5	5
2.	Mojokerto	10	0	10
3.	Jombang	10	5	5
4.	Kediri	10	0	10
5.	Bojonegoro	10	10	0
6.	Blitar	10	10	0

Table 2. Detection of TEM gene in samples from East Java Province, Indonesia

No.	District	Samples Amount	Result	
			Positive	Negative
1.	Sidoarjo	5	0	5
2.	Mojokerto	0	0	0
3.	Jombang	5	0	5
4.	Kediri	0	0	0
5.	Bojonegoro	10	1	9
6.	Blitar	10	1	9

Table 3 : Results of isolation and identification for *E. coli* using bacteriological tests

Area	Number of Sample	EMBA	SIM	SCA	TSIA	G	L	S	Malt	Man
Sidoarjo	1	-	-	-	-	-	-	-	-	-
	2	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	3	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-
	5	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-

	6	-	-	-	-	-	-	-	-	-
	7	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	8	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	9	-	-	-	-	-	-	-	-	-
	10	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
Mojokerto	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-
Jombang	1	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	5	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	6	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-
	9	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	10	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-

Kediri	1	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-
Bojonegoro	1	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	2	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	3	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	4	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	5	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	6	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	7	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	8	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	9	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	10	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
Blitar	1	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	2	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-

	3	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	4	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	5	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	6	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	7	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	8	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	9	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-
	10	+	+/motile	-	A/A, H_2S (-) gas (+)	+	+	+	+	-

Figure 1 : Colony of *E. coli* on EMBA medium

4 DISCUSSION

Colony of *E. coli* on EMBA medium showed metallic green colour with uniform colony, with circular colony shape, rough surface, low convex elevation, and erose edges. EMBA medium is a selective and differential medium for gram-negative bacteria coliform containing eosin and methylene blue which is an indicator of pH and positive gram bacterial inhibitors. In addition, EMBA media can distinguish coliform bacteria as normal flora and *E. coli* in the presence of lactose and sucrose indicator.

All sugar tests resulted in yellow colour of the medium due to the fermentation of sugar which caused the pH of the media to turn into acid. In this research, the result shows that no changing colour of SCA medium indicated that *E. coli* bacteria did not use citrate as carbon sources.

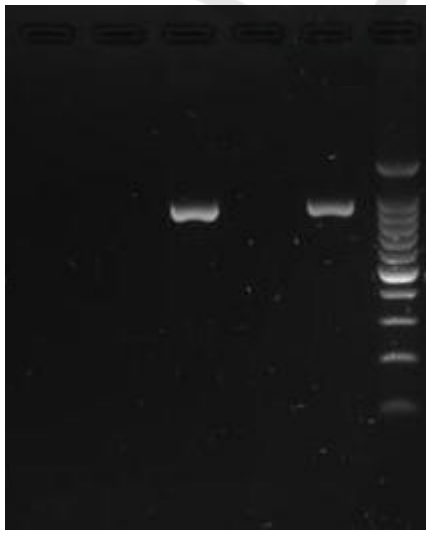
In the SIM media, indol test aims to identify the ability of bacteria to produce indole by using tryptophanase enzyme (Leboffe, 2011). In this research, observations on the SIM media found a cloud clump around the streak area. This prompts the movement of bacteria that grow around the streak area. The movement of the bacteria due to semisolid media (motility test) is designed by reducing the concentration of agar to the media that is about 0.4% on the medium which is only sufficient to maintain its shape while allowing the movement of bacteria (Leboffe, 2011).

Urease test is useful for identifying organisms capable of hydrolyzing urea which can produce ammonia and carbon dioxide, especially to know if the microorganisms have urease enzyme or not. Urease is a constitutive enzyme that hydrolyzes urea into carbon dioxide and ammonia. In this research, Urea Agar used was not pink, but a little orange in colour and from the observational result the agar media did not change in colour.

In this research, results from observations for the TSIA test showed A/A with negative H_2S and presence of gas. The yellow colour of the entire medium is due to the fermentation of carbohydrates and will bring up the gas as a gap in the media or will lift the agar from the bottom of the tube (Leboffe, 2011).

Using the PCR method can give faster, cheaper and more efficient results. If using another method such as dilution with MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bacteriosid concentration) that takes longer time to see the result. Based on the results of resistance test antibiotic trimethoprim using PCR it can be seen that the results of the Bojonegoro and Blitar districts show positive antibiotic resistance trimethoprim. This is indicated by the emergence of bands on electrophoresis results at 861 bp. The presence of bands that appear on the results indicates that there are bacteria that have gene matches with the existing primary pieces. This means that the bacteria have adapted from the previous environment so that it has a gene that can withstand the sensitivity of the trimethoprim antibiotic that matches the existing primer. With the emergence of this band, it can be concluded that bacteria found in Bojonegoro and Blitar districts have genetic compatibility with the primary TEM gene, which indicates antibiotic resistance to trimethoprim in *Escherichia coli*.

The resistance rate of trimethoprim among *Escherichia coli* from layer chickens in East Java, Indonesia was 6.66% based on the result of this research. The resistance occurs because bacteria produce enzyme decomposers antibiotics so that antibiotics become inactive. These bacteria encode genes that produce enzymes that break down antibiotic molecules before they kill bacteria. An example is the lactamase beta enzyme, this enzyme will describe the beta structure of lactam in antibiotics, so antibiotics become inactive again and cannot kill bacteria. It reduces the accumulation of



Explanation:
Target band 861 bp, 2 positive samples
M : Marker DNA 100bp
C- : Negative control
E4 : Sidoarjo
E5 : Bojonegoro
E6 : Jombang
E7 : Blitar

Figure 3 : Trimethoprim Resistance by Polymerase Chain Reaction Result

intracellular antibiotics by decreasing permeability and/or enhancing the active efflux of antibiotics. The efflux mechanism occurs when a resistant gene encodes a protein that actively pushes antibiotics out of a bacterial cell, resulting in low levels of antibiotics in the cell and inability to kill bacteria.

5 CONCLUSION

Based on biochemical examination, 50% of samples from six districts in East Java, Indonesia were *Escherichia coli* positive, and 6.66% were trimethoprim resistant.

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