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Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50<sup>th</sup> annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

**Proceeding:** 

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

#### Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com

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DNA barcoding of the tidal swamp rice ( <i>Oryza sativa</i> ) landraces from South Kalimantan, Indonesia DINDIN HIDAYATUL MURSYIDIN, YUDHI AHMAD NAZARI, BADRUZSAUFARI, MUHAMMAD RIDHO DINTA MASMITRA	1593-1599
Molecular identification of <i>blaCTX</i> -M and <i>blaTEM</i> genes encoding extended spectrum ß-lactamase (ESBL) producing <i>Escherichia coli</i> isolated from raw cow's milk in East Java, Indonesia RIBBY ANSHARIETA, SANCAKA CHASYER RAMANDINIANTO,	1600-1605
MUSTOFA HELMI EFFENDI, HANI PLUMERIASTUTI Short Communication:	1606-1611
Infanticide of Javan slow Ioris ( <i>Nycticebus javanicus</i> ) in captivity PANGDA SOPHA SUSHADI, WIRDATETI, NI LUH PUTU RISCHA PHADMACANTY, MOHAMAD WAHYUDIN	
Population dynamics of mistletoes species on <i>Cassia fistula</i> L. in Purwodadi Botanic Garden, Indonesia SOLIKIN	1612-1620
Characterization of Sardinella fimbriata and Clarias gariepinus bones WAN NOR ASIAH TUN MOHD ROSIDI, NURAINNI MOHD ARSHAD, NOR FAZLIYANA MOHTAR	1621-1626
Architectural and physical properties of fungus comb from subterranean termite Macrotermes gilvus (Isoptera: Termitidae) mound DINA TIARA KUSUMAWARDHANI, DODI NANDIKA, LINA KARLINASARI, ARINANA, IRMANIDA BATUBARA	1627-1634
DNA barcode of Enggano hill myna, <i>Gracula religiosa enganensis</i> (Aves: Sturnidae) based on mitochondrial DNA cytochrome oxidase subunit I JARULIS, CHOIRUL MUSLIM, SANTI NURUL KAMILAH, AHMAT FAKHRI UTAMA, DEBY PERMANA, MELISA MAYANG SARI, ALEX HADI PRAYITNO, IZUL MIFTAKHUL JANNAH	1635-1643
Genetic variation of longtail tuna <i>Thunnus tonggol</i> landed in four fish markets in Indonesia based on mitochondrial DNA	1644-1651
IDA AYU ASTARINI, ENEX YUNIARTI NINGSIH, DEVY SIMANUNGKALIT, SHELLA AYU ARDIANA, M DANIE AL MALIK, NI LUH ASTRIA YUSMALINDA, ANDRIANUS SEMBIRING, NI PUTU DIAN PERTIWI, NI KADEK DITA CAHYANI, ALLEN COLLINS	
The effect of biological agent and botanical fungicides on maize downy mildew JOKO PRASETYO, CIPTA GINTING, HASRIADI MAT AKIN, RADIX SUHARJO, AININ NISWATI, AULIANA AFANDI, REZA ADIWIJAYA, SUDIONO, MUHAMMAD NURDIN	1652-1657
Six new species and a new record of Curcuma L. (Zingiberaceae) from Thailand SURAPON SAENSOUK, THAWATPHONG BOONMA, PIYAPORN SAENSOUK	1658-1685
Combination of plant growth-promoting bacteria and botanical pesticide increases organic red rice yield and reduces the <i>Leptocorisa acuta</i> population MOHAMMAD HOESAIN, SIGIT PRASTOWO, SUHARTO, ANKARDIANSYAH PANDU PRADANA, IIS NUR ASYIAH, FARIZ KUSTIAWAN ALFARIZY, MUH ADIWENA	1686-1694
Agronomic characteristics of 30 promising lines of aromatic, red, and black rice and their antioxidant and cytotoxic effects in some cancer cells YUSUF LIMBONGAN, RICO RAMADHAN, KUNIYOSHI SHIMIZU, ENOS TANGKE ARUNG	1695-1700

The potential of bird diversity in the urban landscape for birdwatching in Java, Indonesia INSAN KURNIA, HARNIOS ARIEF, ANI MARDIASTUTI, RACHMAD HERMAWAN	1701-1711
Comparison of the effectiveness of pregnancy diagnosis in Aceh cow through measurement of interferon-tau and progesterone concentrations BUDIANTO PANJAITAN, TONGKU NIZWAN SIREGAR, HAFIZUDDIN, ARMAN SAYUTI, MULYADI ADAM, TEUKU ARMANSYAH, SYAFRUDDIN	1712-1716
Potential of fungi isolated from a mangrove ecosystem in Northern Sulawesi, Indonesia: Protease, cellulase and anti-microbial capabilities AGUS TRIANTO, OCKY KARNA RADJASA, SUBAGIYO, HARTUTI PURNAWENI, MUHAMMAD SYAIFUDIEN BAHRY, RIGNOLDA DJAMALUDIN, AIYEN TJOA, IAN SINGLETON, KAREN DIELE, DARREN EVAN	1717- 1724
Isolation, characterization, activity test and molecular identification of thermophilic bacteria producing proteases from Dolok Tinggi Raja Natural Hot Springs, North Sumatra, Indonesia EDY FACHRIAL, VISENSIUS KRISDIANILO, HARMILENI, I NYOMAN EHRICH LISTER, TITANIA T. NUGROHO, SARYONO	1725-1732
Implementation of species protection act for the conservation of Tanimbar corella, <i>Cacatua goffiniana</i> (Finsch, 1863) TRI HARYOKO, MARK O'HARA, BERENIKA MIODUSZEWSKA, HARI SUTRISNO, LILIK BUDI PRASETYO, ANI MARDIASTUTI	1733-1740
Chemical composition, antibacterial and antioxidant activities of essential oils extracted from dry and fresh <i>Brocchia cinerea</i> NISRINE CHLIF, ABDELAZIZ ED-DRA, MOHAMMED DIOURI, NOUREDDINE EL MESSAOUDI, BADR ZEKKORI, FOUZIA RHAZI FILALI, AMAR BENTAYEB	1741-1749
Application of selected teak clone and organic fertilizer to accelerate rehabilitation of lowland forest in Java, Indonesia SURYO HARDIWINOTO, FIQRI ARDIANSYAH, WIDIYATNO	1750-1756
Morphometric analysis of Gorontalo (Indonesia) native chickens from six different regions ALFI SOPHIAN, ABINAWANTO, UPI CHAIRUN NISA, FADHILLAH	1757-1763
DNA barcoding of lamp shells (Brachiopoda: <i>Lingula anatina</i> ) from Probolinggo, East Java, Indonesia RENI AMBARWATI, DWI A. RAHAYU, FIDA RACHMADIARTI, FIRAS KHALEYLA	1764-1774
Growth rate and yield response of several sweet potato clones to reduced inorganic fertilizer and biofertilizer HANNY HIDAYATI NAFI'AH, REGINAWANTI HINDERSAH, SYARIFUL MUBAROK,	1775-1782
Characterization and delineation of two infraspecific taxa of <i>Dioscorea esculenta</i> (Lour.) Burkill: The leaf architecture approach MENISA A. ANTONIO, INOCENCIO E. BUOT JR.	1783-1789
Snake pet ownership in the city: A case study in Greater Jakarta, Indonesia MIRZA D. KUSRINI, SHARON PRATIWI PALESA, BURHANUDDIN MASY'UD	1790-1798
Azolla microphylla and Pseudomonas aeruginosa for bioremediation of bioethanol wastewater KHOIRUL ANNISA, SUTARNO, SLAMET SANTOSA	1799-1805
Ethnoecology of <i>Zanthoxylum acanthopodium</i> by local communities around Lake Toba, North Sumatra, Indonesia YATI NURLAENI, JOHAN ISKANDAR, DECKY INDRAWAN JUNAEDI	1806-1818
Screening of actinobacteria-producing amylolytic enzyme in sediment from Litopenaeus vannamei (Boone, 1931) ponds in Rembang District, Central Java, Indonesia DIAH AYUNINGRUM, ANINDITIA SABDANINGSIH, OKTAVIANTO EKO JATI	1819-1828

Identification of conserved peptide upstream open reading frames (CPuORFs) in oil palm (Elaeis guineensis) genome ANDREA PUTRI SUBROTO, REDI ADITAMA, ZULFIKAR ACHMAD TANJUNG, CONDRO UTOMO, TONY LIWANG	1829-1838
Antimicrobial activity and GC-MS analysis of bioactive constituents of Aspergillus fumigatus 269 isolated from Sungai Pinang Hot Spring, Riau, Indonesia ZONA OCTARYA, RIRYN NOVIANTY, NABELLA SURAYA, SARYONO	1839-1845
Species richness and conservation priority of dragonflies in the Suranadi Ecotourism Area, Lombok, Indonesia MOHAMMAD LIWA ILHAMDI, AGIL AL IDRUS, DIDIK SANTOSO, GITO HADIPRAYITNO, MUHAMMAD SYAZALI	1846-1852
The diversity and abundance of phytoplankton and benthic diatoms in varying environmental conditions in Kok River, Chiang Rai, Thailand as bio-indicators of water quality TIPPAWAN PRASERTSIN, KRITTAWIT SUK-UENG, KITTIYA PHINYO, EKKACHAI YANA	1853-1862
Evaluation of a promising tomato line ( <i>Solanum lycopersicum</i> ) derived from mutation breeding ENIK NURLAILI AFIFAH, RUDI HARI MURTI, ADITYA WAHYUDHI	1863-1868
Short Communication: Diversity of cellulolytic bacteria isolated from coastal mangrove sediment in Logending Beach, Kebumen, Indonesia HENDRO PRAMONO, AFIFAH MARIANA, DINI RYANDINI	1869-1878
Modified culture assay to obtain a diversity of hyphal structures of <i>Ceratobasidium</i> theobromae-VSD pathogen on cocoa MUHAMMAD JUNAID, DAVID GUEST	1879-1886
Fieldwork during pandemic: Backyard bird survey and making student's biological field practice works NURUL L. WINARNI, BHISMA G. ANUGRA, SHANIA ANISAFITRI, NABILLA N. KAUNAIN, DIMAS H. PRADANA	1887-1894
Epiphytic yeasts from Piperaceae as biocontrol agents for foot rot of black pepper caused by <i>Phytophthora capsici</i> DIAN SAFITRI, SURYO WIYONO, BONNY POERNOMO WAHYU SOEKARNO, ACHMAD	1895-1901
Species composition, diversity and traditional uses of plants in homegardens in Kampung Masjid Ijok, Perak, Malaysia MOHD RAZNAN RAMLI, POZI MILOW, SORAYYA MALEK	1902-1911
The activity budgets of captive orangutan ( <i>Pongo pygmaeus</i> ) in two different Indonesian zoos NURZAIDAH PUTRI DALIMUNTHE, HADI SUKADI ALIKODRA, ENTANG ISKANDAR, SRI SUCI UTAMI ATMOKO	1912-1919
Effect of single and mixed inoculation of arbuscular mycorrhizal fungi and phosphorus fertilizer application on corn growth in calcareous soil LILY ISHAQ, A.S.J. ADU TAE, MORESI A. AIRTHUR, PETERS O. BAKO	1920-1926
Mucin-1 expression in endometrial tissue of <i>Macaca nemestrina</i> during mid-luteal phase after controlled-ovarian hyperstimulation NURHUDA SAHAR, PONCO BIROWO, KUSMARDI, DIYAH KRISTIANTY, KARINA RAHMANINGRUM, ADRIANA VIOLA MIRANDA, AFIF RASYAD, VIVITRI DEWI PRASASTY	1927-1933
Genetic identification of hydrocarbons degrading bacteria isolated from oily sludge and petroleum contaminated soil in Basrah City, Iraq ENTISAR MUHSON ABOUD, AHMED ABD BURGHAL, ABDULLAH HAMAD LAFTAH	1934-1939
Flower development, pollen viability and pollen storage test of Aeschynanthus radicans FRISCA DAMAYANTI, R. VITRI GARVITA, HARY WAWANGNINGRUM, SRI RAHAYU	1940-1945

Coleoptera of the Penza region, Russia based on fermental crown trap A.B. RUCHIN, L.V. EGOROV, O.A. POLUMORDVINOV				
Taxonomic investigation of the Xanthium strumarium L. complex (Asteraceae) distributed in Iran inferred from morphological, palynological and molecular data FARIBA NOEDOOST, JAMIL VAEZI, SEDIGHEH NIKZAT SIAHKOLAEE	1961-1974			
The infection of ectoparasitic protozoa on farmed Nile tilapia ( <i>Oreochromis niloticus</i> ) at three reservoirs in Central Java, Indonesia WALEED SULIMAN KRPOS KOLIA, SUNARTO, TETRI WIDIYANI	1975-1980			
The potential of cellulose-degrading fungi at various peat maturities in Teluk Bakung Peat Area, Kubu Raya District, Indonesia SITI KHOTIMAH, SUHARJONO, TRI ARDYATI, YULIA NURAINI	1981-1990			
Morphological characteristics of <i>Phaius</i> spp. orchids from Indonesia SRI HARTATI, SAMANHUDI, IDA RUMIA MANURUNG, ONGKO CAHYONO	1991-1995			
Population, habitat characteristic, and modelling of Endangered Orchid, <i>Paphiopedilum javanicum</i> in Mt. Lawu, Java, Indonesia MUH. ARIF ROMADLON, FATIMAH AZZHARA, GILANG DWI NUGROHO, ARI PITOYO	1996-2004			
<b>Evaluation of diversity in some genotypes of Algerian durum wheat using agronomical and biochemical markers</b> AICHA ATOUI, LEILA BOUDOUR, GHANIA CHAIB, BOUDERSA NABIL	2005-2011			
Physicochemical and microbiological properties of yogurt made with microencapsulation probiotic starter during cold storage EVY ROSSI, FAJAR RESTUHADI, RASWEN EFENDI, YOSSIE KHARISMA DEWI	2012-2018			
Compendium of plants used for preparation of traditional alcoholic beverages by different major ethnic communities of Assam, northeast India DIPANKAR BORAH, TRIDIP GOGOI, JINTU SARMA, PUNAM JYOTI BORAH, BICHITRA GOHAIN, CHIRANJIB MILI, ANKUR UPADHYAYA, JENIMA BASUMATARY, KASTURI NEOG, TONLONG WANGPAN, SUMPAM TANGJANG	2019-2031			
The structure of permaculture landscapes in the Philippines JABEZ JOSHUA M. FLORES, INOCENCIO E. BUOT JR.	2032- 2044			
Traditional market, social relations, and diversity of edible plants traded in Beringharjo Market, Yogyakarta, Indonesia BUDIAWATI SUPANGKAT ISKANDAR, JOHAN ISKANDAR, DEDE MULYANTO, RAHMAN LATIF ALFIAN, SUROSO	2045-2057			
Effects of copper on the leaf morpho-anatomy of <i>Rhizophora mucronata</i> : Implications for mangrove ecosystem restoration KERSTIN LEI DJ. PEREZ, MARILYN O. QUIMADO, LERMA SJ. MALDIA, CRUSTY E. TINIO, JONATHAN O. HERNANDEZ, MARILYN S. COMBALICER	2058-2065			
Species diversity and composition, and above-ground carbon of mangrove vegetation in Jor Bay, East Lombok, Indonesia ZULHALIFAH, ABDUL SYUKUR, DIDIK SANTOSO, KARNAN	2066-2071			
Short Communication: Assessing the state and change of forest health of the proposed arboretum in Wan Abdul Rachman Grand Forest Park, Lampung, Indonesia RAHMAT SAFE'I, FRANSINA S. LATUMAHINA, BAINAH SARI DEWI, FERDY ARDIANSYAH	2072-2077			
The ethnobotany of <i>Ngusaba</i> ceremonial plant utilization by Tenganan Pegringsingan community in Karangasem, Bali, Indonesia DEWA AYU SRI RATNANI, I KETUT JUNITHA, ENIEK KRISWIYANTI, I NYOMAN DHANA	2078-2087			
Projecting expansion range of <i>Selaginella zollingeriana</i> in the Indonesian archipelago under future climate condition AHMAD DWI SETYAWAN, JATNA SUPRIATNA, NISYAWATI, ILYAS NURSAMSI, SUTARNO, SUGIYARTO, SUNARTO, PRAKASH PRADAN, SUGENG BUDIHARTA, ARI PITOYO, SAPTA SUHARDONO, PRABANG SETYONO, MUHAMMAD INDRAWAN	2088-2103			

Morphological and anatomical characters variation of <i>Indigofera</i> accessions from Java, Indonesia MUZZAZINAH, SURATMAN, NURMIYATI, SRI RETNO DWI ARIANI	2104 <b>-2116</b>
Exogenous acetic acid pre-treatment increases drought tolerance of two Indonesian foxtail millet (Setaria italica) accessions CHOIROTIN NISA, NURUL JADID	2117-2124
Effect of termite activity on soil chemical properties using baiting systems at an arboretum area in Pontianak, West Kalimantan, Indonesia YULIATI INDRAYANI, SOFWAN ANWARI	2125-2130
Feeding habits of Tinfoil barb, <i>Barbonymus schwenenfeldii</i> in the Tasik River, South Labuhanbatu, North Sumatra, Indonesia DESRITA, FANNI K. HASUGIAN, ERI YUSNI, VINDY R. MANURUNG, RIDAHATI RAMBEY	2131-2135
Diversity of sea cucumber from intertidal area of Pacitan and Bangkalan, East Java, Indonesia ELSA DIANITA AULIA, FARID KAMAL MUZAKI, DIAN SAPTARINI, EDWIN SETIAWAN, DAVIN SETIAMARGA, ISWATUL DIAH LUTVIANTI, SANIAH KUSNATUR ROSYIDAH, NUR ALI MUHAMMAD	2136-2141
Carbon emissions as impact of mangrove degradation: A case study on the Air Telang Protected Forest, South Sumatra, Indonesia (2000-2020) SYAIFUL EDDY, NORIL MILANTARA, MOHAMMAD BASYUNI	2142-2149

# Molecular identification of *bla<sub>CTX-M</sub>* and *bla<sub>TEM</sub>* genes encoding extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from raw cow's milk in East Java, Indonesia

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**Abstract.** Ansharieta R, Ramandinianto SC, Effendi MH, Plumeriastuti H. 2021. Molecular identification of  $bla_{CTX-M}$  and  $bla_{TEM}$  genes encoding extended-spectrum  $\beta$ -lactamase (ESBL) producing Escherichia coli isolated from raw cow's milk in East Java, Indonesia. Biodiversitas 22: 1600-1605. The emergence of extended-spectrum  $\beta$ -lactamase (ESBL) producing bacteria and its increasing level has become public health issue. The presence of these bacteria in food of animal origin is quite alarming. The objective of this study was to detect and characterize *Escherichia coli* producing ESBL encoding genes, isolated from 200 raw cow milk samples in East Java, Indonesia. The results of this study showed that 70.5% of isolates were confirmed as *E. coli*, based on the morphological growth of colonies on the EMB Agar and biochemical IMViC tests. In this study, the double-disc synergy test (DDST) method was used to confirm the ESBL, and previously sorted out presumptively by using Aztreonam antibiotic disc. The antibiotics used were amoxicillin-clavulanate, ceftazidime, and cefotaxime for DDST. In addition, ESBL confirmation with Multiplex PCR method for bla<sub>CTX-M</sub> and bla<sub>TEM</sub> genes were done. The presence of ESBL-producing by *E. coli* isolated from raw cow's milk in East Java were 2.12% (3/141). The PCR results showed that the double bla<sub>CTX-M</sub> and bla<sub>TEM</sub> gene harbored by 2 ESBL isolates and one bla<sub>TEM</sub> gene as many as 1 ESBL isolate. Thus, the findings of our study indicate that milk can be a good reservoir of bacteria carrying bla<sub>CTX-M</sub> and bla<sub>TEM</sub> ESBL resistance genes with the potential to affect human health.

Keywords: blacTX-M gene, blaTEM gene, ESBL, Escherichia coli, human health, raw cow's milk

## **INTRODUCTION**

Extended-spectrum  $\beta$ -lactamase (ESBL) producing bacteria are a major threat to public health today. The presence of ESBL bacteria is often caused by inappropriate use of antibiotics in handling infections and their irrational administration (Abayneh et al. 2018). *Escherichia coli* (*E. coli*) is a major pollutant in the environment which is often associated with ESBL encoding genes, namely bla<sub>CTX-M</sub> and bla<sub>TEM</sub> (Jena et al. 2017). Milk is a food source of animal origin that can act as a reservoir in transmitting infectious bacterial diseases. The presence of *E. coli* bacteria in raw milk is often reported with regard to sources of food-borne disease (Odenthal et al. 2016).

The prevalence of ESBL-producing *E. coli* in animal origin food products is very high (Geser et al. 2012; Wibisono et al. 2020a). The existence of livestock and livestock products such as milk can be a mode of transmission and circulation of ESBL producing bacteria and act as a potential new threat because they are directly related to the food chain in humans. Livestock and livestock products are one of the main sources of animal protein, including the most commonly consumed sources of meat and milk and being one of the main elements in the

food chain in humans. Livestock manure in the form of large-capacity feces has the potential to transmit bacterial infectious agents to humans through contamination. Most intestinal diseases in humans come from animals that are transmitted directly from animals to humans or indirectly through food of animal origin or water contaminated with feces (Widodo et al. 2020).

The distribution pattern of ESBL enzymes globally in humans and animals can be a reference and consideration in efforts to prevent and treat ESBL bacterial infections. The type of CTX-M enzyme is a variant of the enzyme found in isolates from humans, animals and the environment. The CTX-M-15 strain has been widely reported on all continents and has been detected in all major ecological aspects including humans, animals and the environment. Treatment of ESBL-producing bacterial infections in clinical trials has rarely been successful (Widodo et al. 2020). This study aimed to detect the presence of ESBL-producing bacteria from dairy cows milk by double-disc synergy test (DDST) and molecular tests to identify the encoding bla<sub>CTX-M</sub> and bla<sub>TEM</sub> genes for ESBL producing *E. coli*.

#### MATERIALS AND METHODS

#### **Research design, location and sampling**

Raw milk was taken from dairy farm with a total of 200 samples, located in Probolinggo, Pasuruan, Batu, and Blitar districts, East Java Province, Indonesia, in the period between September 2019-January 2020. The farms selected to take as research samples have belonged to farmers who had an average of 3 to 5 cows. About 10 mL of milk sample from dairy cows milk was taken and placed in a sterile tube. Raw milk samples contained in wrapped sterile plastic were taken to the laboratory using a cool box container at 4°C.

# Isolation and identification of E.coli

Each 100  $\mu$ L milk sample was cultured into 10 mL enrichment media of Brilliant Green Bile Lactose Broth (Merck, 105454) and incubated at 37 °C for 18-24 hours. The positive results are characterized by change in media from green color to cloudy green and presence of gas in Durham tube (Putra et al. 2019). Then 100  $\mu$ L of the resulted samples were streaked onto Eosin Methylene Blue (EMB) agar (Merck, 101347) and incubated at 37 °C for 18-24 hours (Effendi et al. 2018). Presumptive 3-5 colonies of *E. coli* that showed metallic green color was purified and continued to identification with the IMViC test (Wibisono et al. 2020b).

#### Antibiotic sensitivity test

Antibiotic sensitivity testing was done using Kirby-Bauer disc diffusion assay on Mueller-Hinton agar medium (Oxoid, CM0337). Firstly, screened for presumptive ESBL using Aztreonam and other antibiotic discs for checking the multidrug resistance profile. Antibiotics discs used were Tetracycline 30  $\mu$ g (Oxoid, CT0054), Streptomycin 10  $\mu$ g (Oxoid, CT0047), Chloramphenicol 30  $\mu$ g (Oxoid, CT0013), Trimethoprim 5  $\mu$ g (Oxoid, CT0057), Aztreonam 30  $\mu$ g (Oxoid, CToid02). Antibiotics discs used for Double Disc Synergy Test (DDST) were Amoxicillin-Clavulanate

Table 1. Details of primers used in this study

20/10  $\mu$ g (Oxoid, CT0223B), Cefotaxime 30  $\mu$ g (Oxoid, CT0166), Ceftazidime 30  $\mu$ g (Oxoid, CT0412). Interpretation of results was done by measuring the diameter of the inhibitory zone formed, after overnight incubation at 37 °C, based on Clinical and Laboratory Standards Institutions (CLSI 2018, Putra et al. 2020).

# Preparation for polymerase chain reaction

The initial step of DNA extraction from bacterial culture was referred to Kristianingtyas et al. (2020) and tested by specific primers for the bla<sub>CTX-M</sub> and bla<sub>TEM</sub> genes (Table 1) as described in Ali et al. (2016), with slight modifications in cycling conditions. Taq DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers used in the PCR mixture were obtained from Thermo Fisher Scientific Inc. (Massachusetts, USA). Thermocycling reaction was conducted for initial denaturation at 94°C for 2 minutes followed by 30 cycles of: denaturation at 94°C for 1 minute, annealing for 52°C for 30 sec, extended at 72°C at 45 sec, and final extension at 72°C for 5 minutes. PCR products were visualized in mini gel electrophoresis and documented in the UV Reader/ Gel Documentation System.

# **RESULTS AND DISCUSSION**

#### Isolation and identification of Escherichia coli

The results of the isolation and identification of 200 raw milk samples obtained as many as 200 (100%) isolate suspected of *E. coli* on the basis of macroscopic characters, on the Brilliant Green Lactose Broth medium, the color is cloudy green and all of them cause gas in the Durham tube after incubation at  $37^{\circ}$ C for 24 hours. A total of 141 (70.5%) samples on EMB Agar medium with two times purification showed the growth of convex colonies in metallic green with a round black center (Figure 1) after incubation at  $37^{\circ}$ C for 24 hours, can be seen in Table 2.

Primers	Sequences (5' to 3')	Target gene	Amplicons size	References
CTX-MA	CGC TTT GCG ATG TGC AG	blactx-м	550-bp	Villegas et al. (2004)
CTX-MB	ACC GCG ATA TCG TTG GT		_	
TEM-F	ATA AAA TTC TTG AAG ACG AAA	blatem	1086-bp	Yao et al. (2007)
TEM-R	GAC AGT TAC CAA TGC TTA ATC			

Table 2. Results for detection multidrug-resistant (MDR) and ESBL producing Escherichia coli

Location	Sample size	Confirmed E. coli	Multidrug- resistant	Presumptive ESBL (Aztreonam disc)	DDST positive	bla <sub>CTX-M</sub> gene	bla <sub>TEM</sub> gene
Probolinggo (A)	50	36	2	0	0	0	0
Pasuruan (G)	50	30	4	1	1	1	1
Batu (H)	50	37	6	0	0	0	0
Blitar (S)	50	38	2	2	1	1	2
Total	200	141	14	3	2	2	3



Figure 1. EMBA with Escherichia coli colonies in metallic green



Figure 3. Sample code of S-25 showed Aztreonam resistant isolate as presumptive ESBL

Gram staining was performed and stained bacterial smear was viewed under a microscope with a magnification of 1000x. to ensure that the bacteria are Gram-negative rod-shaped bacteria. Microscopically, *E. coli* bacterial cells appeared in the form of short rods and were red in Gram stain. The next test was a biochemical test with IMViC and then incubated at 37 °C for 24 hours. Isolates used for the IMViC test came from separate colonies on EMBA media. It was found that 141(70.5%) isolates tested positive for *E. coli*. Isolates were stated as *E. coli* with negative Sulphide test results, positive indole, positive motile, positive MR, negative VP, and negative citrate (Figure 2).

The study has been carried out on 200 milk samples obtained from Probolinggo, Pasuruan, Batu, and Blitar, showed that 70.5% were contaminated with *E. coli* bacteria. From each area, *E. coli* bacteria were found which were multidrug-resistant to various antibiotics that had been tested (Table 2 and Table 3), and shown in Figure 3.

# Identification for bla<sub>CTX-M</sub> and bla<sub>TEM</sub> Genes in ESBL-Producing *E.coli*

A total of two ESBL producer isolates showed the presence of 'keyhole' in DDST testing (Figure 4.) The three isolates were tested by multiplex PCR method to find out



Figure 2. Positive biochemical test for Escherichia coli



**Figure 4.** Sample code of S-25 DDST positive result (white arrow shows positive synergy)

encoded ESBL genes. The two positive DDST isolates produced 2 double bands for the  $bla_{CTX-M}$  and  $bla_{TEM}$  genes. Whereas, one negative DDST isolate gave 1 single band for  $bla_{TEM}$ , due to resistant to Aztreonam (Figure 5).

# Discussion

Based on this study, antibiotics Trimethoprim, Chloramphenicol, and Aztreonam may still be used to treat multidrug-resistant (MDR) bacteria found because there are still some MDR of E. coli isolates that are sensitive to it. Trimethoprim is usually combined with sulfonamides which work synergistically with a broad spectrum of activities. Chloramphenicol is also a broad-spectrum antibiotic. In the case of urinary tract infections, Trimethoprim is proven to be effective in killing infections caused by E. coli bacteria (AlRabiah et al. 2018), while in the case of gastrointestinal tract and reproductive tract infections due to E. coli, Chloramphenicol is a good antibiotic (Santos et al. 2010). The antibiotic monobactam (Aztreonam) is rarely used in veterinary practice and human medicine today (Kennedy et al. 2015). The discovery of isolates that are resistant to Aztreonam, increases the possibility of finding the ESBL of E. coli bacteria in raw milk.

	Resistant to				N		
Sample code	ТЕ	S	W	С	ATM	— Multidrug resistant	ESBL producer
-	30 µg	10 µg	5 µg	30 µg	30 µg	(MDK)	
A-26	+	+	+	+	-	+	-
A-44	-	+	+	+	-	+	-
G-12	+	+	+	-	-	+	-
G-31	-	+	+	-	+	+	+
G-35	+	+	+	-	-	+	-
G-43	+	+	-	+	-	+	-
H-11	+	+	+	+	-	+	-
H-15	+	+	+	-	-	+	-
H-28	+	+	-	+	-	+	-
H-37	+	+	-	+	-	+	-
H-44	+	+	-	+	-	+	-
H-45	+	+	-	+	-	+	-
S-25	+	-	-	+	+	+	+
S-38	_	+	-	_	+	_	+

Table 3. Detail samples of MDR and ESBL of Escherichia coli isolates

Note: TE: Tetracycline, S: Streptomycin, W: Trimethoprim, C: Chloramphenicol, ATM: Aztreonam, MDR is resistant to three or more classes, ESBL producer is resistant to ATM, +: positive resistant



**Figure 5.** Electrophoresis result for 2 ESBL producer isolates and one contain blaTEM Isolate with negative DDST. Lane 1: G-31 (From Pasuruan), 2: S-25 (From Blitar), 3: S-38 (From Blitar), 4: Negative Control, 5: Positive Control for bla<sub>TEM</sub> gene, 6: Positive Control for bla<sub>CTX-M</sub> gene, 7: Marker

The occurrence of antibiotic resistance is known to originate from bacterial plasmids that are able to accommodate resistance genes and spread them to other bacteria (Ramírez-Castillo et al. 2018). Various resistance genes can accumulate in bacterial plasmids, usually in R (resistant) plasmids which is the reason for finding bacterial isolates that are resistant to various kinds of antibiotics and are able to create new gene sequences (Nikaido 2009).

Multidrug-resistant *E. coli* isolates are very common in many countries and are responsible for a series of infections with high severity and difficulty to treat. In Canada, a study of cases of urinary tract infection due to *E. coli* bacteria, 60% of which were the cases of *E. coli* infection with resistance to more than 3 classes of antibiotics. Consumption of food from raw undercooked animals, travel habits between regions, and contact with reservoir animals are associated with an increased risk of urinary tract infections caused by MDR of *E. coli* (Ukah et al. 2017).

*Escherichia coli* is a bacterium that can be a reservoir of various antibiotic resistance genes, including genes encoding beta-lactam resistance  $\beta$ -lactamase encoding genes (Effendi et al. 2021). ESBLs enzymes are produced several strains belonging to the Enterobacteriaceae family. They can hydrolyze penicillin and third-generation cephalosporins, monobactams, and other antibiotics, except carbapenems (meropenem, imipenem, and ertapenem) (Pitout 2012). These enzymes are mainly encoded by several specific genes, namely the bla<sub>SHV</sub>, bla<sub>CTX-M</sub> and bla<sub>TEM</sub> genes (Bush 2013, Wibisono et al. 2020).

The presence of  $bla_{CTX-M}$  and  $bla_{TEM}$  genes is often reported in food of animal origin. In this study, the findings of ESBL producing *E. coli* isolates were dominated by the  $bla_{TEM}$  gene. Similar to the research of Hinthong et al. (2017) stated that *E. coli* contamination found in milk from dairy farms tends to find the bla<sub>TEM</sub> gene in ESBLproducing *E. coli* bacteria. This showed that pathogenic *E. coli* sourced from milk is also exposed to antibiotics and has the potential to transfer these genes to other pathogenic bacteria under certain conditions (Effendi et al. 2019; Rahmahani et al. 2020). This study was detected ESBL and bla<sub>CTX-M</sub> gene in milk since it is rarely reported worldwide to find ESBL in milk. ESBL is mainly reported in feces of poultry and porcine (Wibisono et al. 2020c). The presence of ESBL bacteria is quite dangerous if found in food of animal origin. ESBL-producing *E. coli* strains obtained from cow's milk samples are of particular concern because these pathogens can affect human consumers and calves and lead to the spread of these antibiotic-resistant pathogens to humans and animals (Batabyal et al. 2018).

In dairy cows during lactation, ESBL producing *E. coli* can also be found in raw milk with and or without symptoms of mastitis, this indicates that the cleanliness of the cage that contaminates milk cages is also a risk factor for contamination of ESBL producing *E. coli* into raw milk products. (Su et al. 2014). In cases of mastitis due to infection with ESBL-producing *E. coli* bacteria infection, it is often associated with several antibiotics that have also been inactive against the bacteria causing it, making it difficult to find other replacement antibiotics to treat it (Ali et al. 2016).

Many sources of exposure have the potential to transmit ESBL-producing E. coli, making epidemiological investigations extremely difficult. Interactions at the microbial level in humans and animals, especially between commensal bacteria and pathogenic bacteria, facultative bacteria and obligate bacteria in the same environment and horizontal gene transfer from bacteria make the distribution of ESBL encoding genes between various bacterial species becomes wider. In order to understand and identify the possibility of preventing the spread of the ESBL encoding genes and infection in humans, an integrative approach such as 'One Health' is required (Calistri et al. 2013). The application of the concept of One Health integration is assumed to accelerate disease prevention and prediction as an effort to control ESBL-producing E. coli (Wendt et al. 2014).

Food-borne diseases are a major concern throughout the world. This is an important problem in developing countries that lack the application of high sanitation management during the collection and processing of cow's milk. E. coli contamination found in raw milk may be caused by cross-contamination of milk with impurities or the lack of hygienic measures during milk collection and processing (Tanzin et al. 2016). According to Ukah et al. (2017), one of the factors causing the occurrence of antibiotic resistance in humans is due to consuming food of animal origin in raw or undercooked form. A multi-sectoral approach to medical treatment in the field of veterinary medicine, animal food production, can realize global cooperation in controlling the ecological development of antibiotic-resistant E. coli, for public health (Landers et al. 2012).

In conclusion, molecular identification showed the bla<sub>CTX-M</sub> gene found in raw cow milk collected from several

regions in East Java, Indonesia which was used to identify ESBL producing *E.coli*, and the bla<sub>TEM</sub> for molecular identification of penicillinase-producing *E. coli*. These results showed that ESBL producing *E. coli* from raw cow's milk has a relatively low prevalence. However, ESBL producing *E. coli* showed the potential for spreading and poses a threat to public health from *E. coli* isolates.

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