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Characterization and phylogenetics of beta-lactamase *Temoneira* gene in *Escherichia coli* of the Bali cattle on Lombok island, Indonesia

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Abstract

This study aims to determine the character and phylogenetics of the *β-lactamase Temoneira* (*blaTEM*) gene in *Escherichia coli* isolated from the reproductive tract of Bali cattle with repeat breeder cases. This study was conducted in June 2021 that used 16 female Bali cattle with repeat breeder cases to take their reproductive tract fluid using a plastic sheet gun. Isolation of *Escherichia coli*, using Eosin Methylene Blue Agar was identified by biochemical tests. Antibiotic susceptibility test on *E. coli* samples was carried out by disc diffusion method. The screening test for the presence of extended-spectrum β-lactamase was done using the double-disk approximation test method. Characterization of the *blaTEM* gene in *E coli*, using the PCR method and sequences of the *blaTEM* gene, were phylogenetically analyzed. The results showed that 4 *E. coli* isolates were obtained from 16 samples of reproductive tract fluid. The sensitivity test to antibiotics of 4 isolated samples of *E. coli* showed that 50% were resistant to penicillin and cefotaxime. Samples of *E. coli* resistant to penicillin and cefotaxime showed synergy results in the double-disk approximation test. The PCR results showed that the samples of *E coli* encoded the *blaTEM* gene which was located at the 560 bp position on gel electrophoresis. The phylogenetic tree analysis found that the *blaTEM* gene encoded by *E. coli* was related to *E. coli* encoding the *blaTEM*-206 gene. The character *blaTEM* gene in samples of *E. coli* showed the character of *blaTEM-206* gene of extended-spectrum β-lactamase-producing *E. coli. Escherichia coli* from the reproductive tract of Bali cattle showed the character of *E. coli* S2.2-EK pEC-S2.2 *blaTEM* gene for class A broad-spectrum *B-lactamase TEM-206* with a high degree of kinship.

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Introduction

Cases of repeat breeder are still high in Bali cattle and have become a problem in livestock development in West Nusa Tenggara. Wodaje and Mekuria (1) state that cows experiencing repeat breeder are female cows that had normal cycles and periods of lust and had been mated twice or more times with fertile males or inseminated with fertile male semen but were not pregnant. The incidence of repeat breeder in Bali cattle in West Nusa Tenggara was 24.9% of the 2,127 cases of reproductive disorders (2). The uncontrolled use of antibiotics, especially β-lactam antibiotics will pressure bacteria to mutate so that it will cause the gene encoding Extended-spectrum β-lactamase (ESBL) which has implications for the emergence of antimicrobial resistance (AMR) and has an impact on failure

to treat repeat breeder cases. One of the resistance genes that is widely discussed is the β-lactamase Temoneira *(blaTEM*) gene in *Escherichia coli* which can spread to animals, humans, and the environment either by gene transfer or contamination. Antibiotic resistance genes from *E. coli* can occur through horizontal gene transfer. The antibiotic resistance gene's spread can be influenced by environments with rich nutrients (3). The AMR profile of *E. coli* almost reflects the use of antimicrobials in food-production animals, so the emergence of the *blaTEM* gene in *E. coli* and its transmission needs attention (4). Resistance genes such as TEM (Temoneira) in *Escherichia coli* have been found in cattle and humans. The *blaTEM* in *E. coli* has been found in the urine of dairy cows with uterine infections in India (5). *Escherichia coli* producing the TEM-type ESBL gene has been found in cattle feces and the environment in Peninsular Malaysia (6). *Escherichia coli* that can carry two ESBL-coding genes with TEM and CTX-M types have been found in cattle farmed in Florida of the 59 *E. coli* isolated (7). *E. coli*, which is resistant to beta-lactam antibiotics such as penicillin and cefotaxime derived from cow feces, has also been reported in Lombok, West Nusa Tenggara province, but the gene encoding it has not been reported. The province of West Nusa Tenggara, which consists of the islands of Lombok and Sumbawa, is one of the provinces that are rich in smallholder livestock and is the national supplier of Bali cattle. Data from the BPS Province of West Nusa Tenggara until 2019 stated that the total population of cattle in West Nusa Tenggara was 1,234,357 heads (8). Since 2013 in East Lombok Province, there have been around 504 livestock farmer groups, and 238 of 504 livestock farmer groups were livestock groups. The high number of beginner livestock groups on the island of Lombok because there is a lack of education and knowledge about sanitation management and handling related to reproduction such as post-partum care, detection of lust, post-handling care for dystocia cases, post-treatment care for retained placenta, and uterine prolapse. It will trigger contamination of *E. coli* in the reproductive tract. The previous study stated that predisposing factors for repeat breeder were bacterial infection when handling cases of dystocia, retained placenta, uterine prolapse, and ovarian disorders (9). Kholik *et al.* (10) reported that *E. coli* from feces of Bali cattle with repeat breeder cases were 100% resistant to Penicillin G, and 25% were resistant to Cefotaxime of 4 samples of *E. coli that have been isolated.* Data stating that the number of repeat breeder cases in Bali cattle is quite high with a beginner livestock breeding system makes it very possible for the use of uncontrolled antibiotics that can cause the emergence and spread of *E. coli* which encodes the *blaTEM* gene in animals and humans. These resistance genes can spread in the human food chain either directly or indirectly because *E. coli* encoding the *blaTEM* gene may be excreted from the reproductive tract fluid, then microbial resistance genes will be disseminated to the environment and transfer-resistant genes to other bacteria (3), so research on gene character of the *β-lactamase* Temoneira (*blaTEM*) in *E. coli* isolated from the reproductive tract of Bali cattle is needed in anticipation of the spread of these bacteria.

This study aimed to determine the character and phylogenetic of the *β-lactamase Temoneira* (*blaTEM*) gene in *E. coli* isolated from the reproductive tract of Bali cattle with repeat breeder cases.

Materials and methods

Study site

This study is a descriptive cross-sectional study conducted in June 2021 on smallholder farms located in Central Lombok Regency and East Lombok Regency. In this study, 16 Bali cattle, that had repeat breeder, were used to take samples of the reproductive tract fluids of 30 Bali cattle with repeat breeder cases. The criteria for Bali cattle that experienced repeat breeder in this study were female cows with normal estrus cycles that have bred two or more times but have not conceived.

Isolation and identification of *Escherichia coli*

The reproductive tract fluid of Bali cattle as samples were taken using a plastic sheet gun of artificial insemination. The reproductive tract fluid of Bali cattle was collected by a qualified veterinarian from the Faculty of Veterinary Medicine, Universitas Pendidikan Mandalika, Mataram, Indonesia based on the sampling protocol of Andani *et al.* (11). Samples were put in Brain Heart Infusion (BHI) media and incubated for 24 hours at 37°C at the Health Testing and Calibration Laboratory of West Nusa Tenggara Province. The samples were then planted on Eosin Methylene Blue Agar (EMBA) media and incubated for 24 hours at 37°C to grow *E. coli*. The growing *E. coli* were then identified by Gram staining and biochemical tests. Biochemical tests include SIM, and fermentation of lactose, glucose, fructose & mannitol which refers to Basic laboratory procedures in clinical bacteriology (12).

Sensitivity test to antibiotics

An antibiotic susceptibility test was carried out to determine the resistance of *E. coli* samples to several antibiotics by disk diffusion (Oxoid LTD) on Mueller Hinton Agar using the Kirby-Bauer method. *E. coli* colonies were taken from the EMB medium and then put into a tube containing 0.9% NaCl and homogenized to reach the McFarland standard of 0.5. The E. coli suspension, which had reached the standard McFarland 0.5, was then swabbed on Mueller Hinton Agar and 5 types of antibiotics were planted, including penicillin G 10 U, oxytetracycline 30 ug gentamicin 10 ug, tetracycline 30 ug and cefotaxime 30 ug and incubated for 24 hours at 37°C. Sensitivity to antibiotics was carried out by measuring the diameter of the inhibition zone formed. The screening test for the presence of ESBL,

using the double-disk approximation test method, used cefotaxime (CTX) 30 ug, and amoxicillin-clavulanate (AMC) 30 ug. *E. coli* ATCC 25922 as a negative control was used in this study. Sensitive (S), intermediate (I), and resistant (R) assessments were determined by the size of the inhibition zone formed based on the Clinical and Laboratory Standards Institute (13).

Characterization of Beta-Lactamase Gene

The molecular detection of ESBL *E. coli* genes was carried out by polymerase chain reaction (PCR) which was previously extracted from *E. coli*. Genomic DNA extraction of *E. coli* was performed using the *QIAprep® Spin Miniprep* Kit procedure (Qiagen). The purity and DNA concentration was estimated using a spectrophotometer

The extracted DNA of *E. coli* was subjected to a polymerase chain reaction (PCR) assay by a specific primer. The specific primer was designed based on NCBI Reference Sequence: NG_050238.1 (14). The reagents for PCR were done with a total volume of 51 µL consisting of 25 µL PCR mix Dream Taq HS (Thermo®), 1.5 µL primer, 20 µL ddH2O, and 3 µL extracted DNA of *E. coli* as a template. The mixture was processed in the Biorad i-cycler PCR machine (Biorad system, USA). The specific primers were used in this study, for *blaTEM* genes forward (*blaTEM*F) 5' TCCTTGAGTTTTCGCCCC-3' and *blaTEM* genes reverse (*blaTEM*R) 5' CAGTGCTGCAATGATACCGC-3" with an amplicon of 581 bp (Table 1).

The PCR conditions used were as follows: Pre-denaturation temperature 95 ºC for 3 minutes, denaturation 94ºC for 30 seconds, annealing 57 ºC for 30 seconds, elongation 72 ºC for 1 minute, and post-elongation 72 ºC for 5 minutes with 35 cycles. The PCR amplicon from the *blaTEM* gene was electrophoresed on 2% agarose gel. The result of electrophoresis was read using the Gel Imager Biorad. American Type Culture Collection (ATCC) *E. coli* isolate type 25922 was used as a negative control in this study. Sequencing of PCR results was carried out at Genetic Science Indonesia.

Phylogenetic tree analysis of *blaTEM* gene in *E. coli* samples was used to determine the genetic relationship with various *E. coli* encoding *blaTEM* as references in GenBank data using the Mega X 10 software algorithm with the Neighbor-Joining method (15-17).

Table 1: Primers in detecting *blaTEM* genes in *Escherichia coli*

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Reference
blaTEM	Primer Forward	5- TCCTTGAGTTTTCGCCCC -3	581	(14)
	Primer Reverse	5- CAGTGCTGCAATGATACCGC -3		

Results

The results of isolation of *E. coli* on Eosin methylene blue Agar (EMBA) medium obtained 4 (25%) of 16 reproductive tract fluid samples of Bali cattle which were collected. The results of the research on the sensitivity test to antibiotics on 4 isolated *E. coli* showed that 100% *E. coli* is resistant to penicillin G, 75% *E. coli* is resistant to oxytetracycline and gentamicin, 50% *E. coli* is resistant to cefotaxime and 25% *E. coli* is resistant to tetracycline. In this study, it was also found that 50% (2/4) of *E. coli* was resistant to penicillin and cefotaxime (Table 2). *E. coli*, which was resistant to penicillin and cefotaxime showed positive synergy results by an expansion of the cefotaxime (CTX) disc diameter zone around the Amoxicillin-Clavulanate (AMC) disc in the screening test for the presence of ESBL with the double-disk approximation test method (Figure 1).

The results of the electrophoresis of the *blaTEM* gene PCR product on *E. coli* using specific primers showed that 2 samples in no. (2 and 4) of *E. coli* encode the *blaTEM* gene American Type Culture Collection (ATCC) *E. coli* type 25922 was used as a negative control. The samples were at position 560 bp on agarose gel 2 %, while negative control did not code for the *blaTEM* gene (Figure 2).

The results of the sequencing of the *blaTEM* gene from the *E. coli* samples in Figure 3 were then analyzed phylogenetically to determine the relationship with several *E. coli* encoding the *blaTEM* gene originating from humans, animals, and the environment in the data in GenBank. The data in GenBank used include Code (NG_050238, MT387477.1, MG653169.1, MT789719.1, and code MW1838931). The results of the phylogenetic analysis for the *blaTEM* gene in *Escherichia coli* from the Bali cattle reproductive tract had a genetic relationship with the reference in GenBank, namely Code NG_050238.1 with bootstraps 97% and also with Code MW183897.1 and Code MT789719.1. Bootstraps' value of 97% indicates a high value and a strong relationship (Figure 3). Code NG_050238.1 is *E. coli* S2.2-EK pEC-S2.2 *blaTEM* gene for class A broad-spectrum beta-lactamase *TEM*-206. The codes NG_050238.1 and MT789719.1 are *E. coli blaTEM-1* isolated from pigs. Code MW183897.1 is *E. coli* strain U-10 *TEM* family beta-lactamase from human feces and urine.

Table 2: The result of the antibiotics sensitivity test of isolated *E. coli*

No.	Inhibition Zone Diameter (mm)				
	cefotaxime	penicillin	oxytetracycline	gentamycin	tetracycline
1	40 S	0 R	0 R	18S	16 S
2	36 S	0 R	24 S	0 R	20 S
3	10 R	0 R	0 R	0 R	0 R
4	12 R	0 R	0 R	0 R	0 R

S= susceptible, I= Intermediate, R= Resistant.

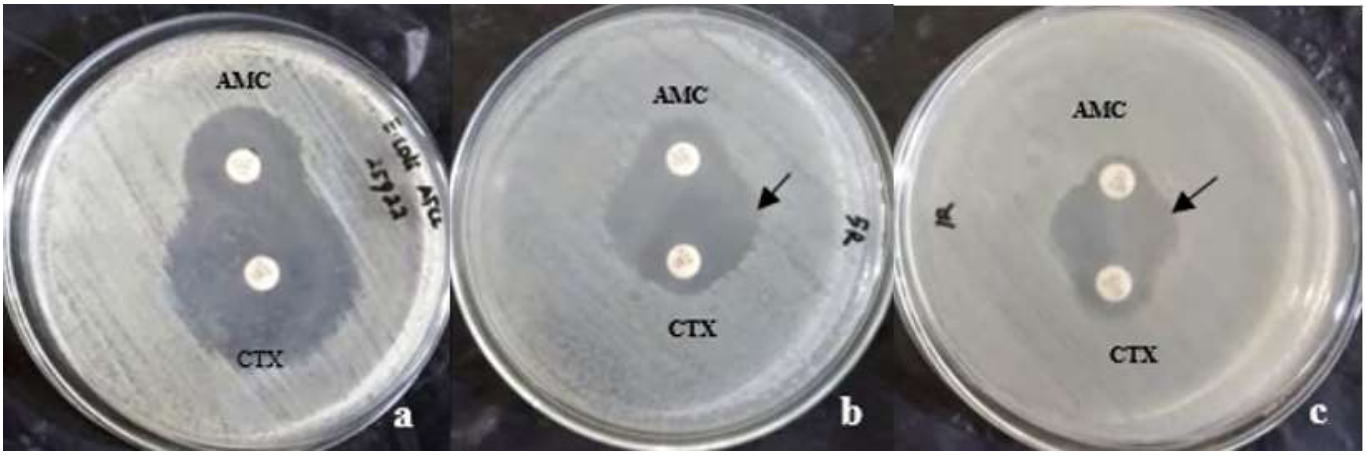

Figure 1: Double-disk approximation Synergy test on *Escherichia coli*. a= *E. coli* ATCC 25922. b= sample 1, c= sample 1, AMC= amoxicillin-clavulanate; CTX= cefotaxime;(arrow) = Synergy.

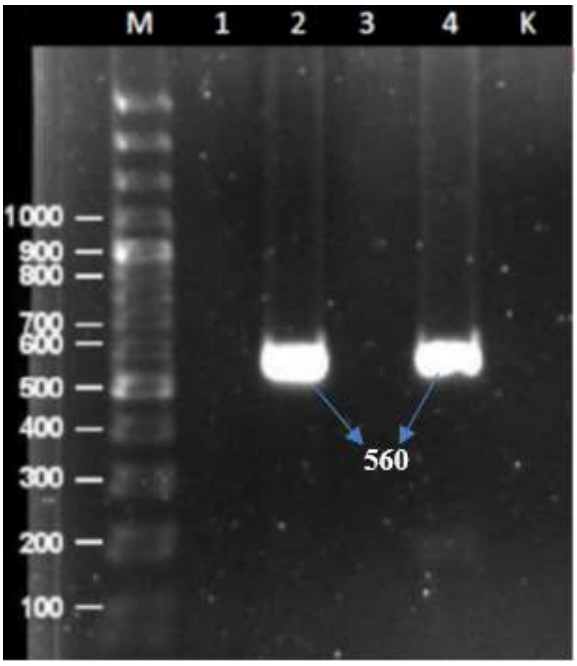

Figure 2: 2% of agarose gel electrophoresis shows 2 samples in no. (2 and 4) of *E. coli* encode the *blaTEM* gene. M is the DNA marker, the sample is in lanes: 1-4, and K is *E. coli* ATCC 25922.

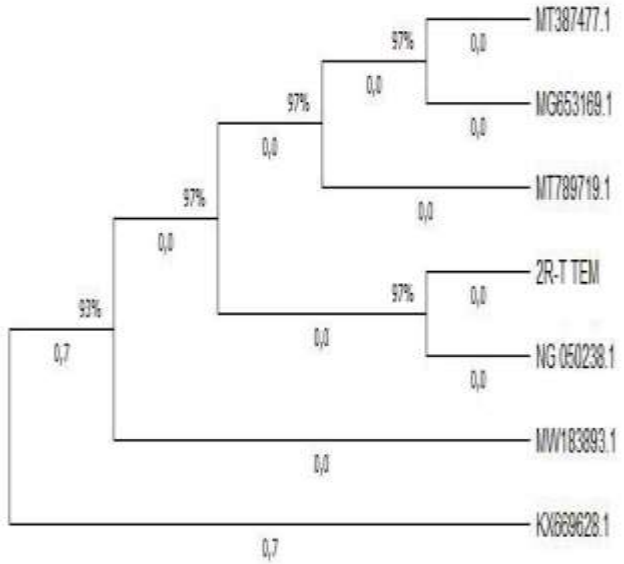

Figure 3: Phylogenetic analysis of the *blaTEM* gene on *E. coli* samples with data from GenBank. 2R-TTEM: Sample of *E. coli*; (NG_050238.1, MT387477.1, MG653169.1, MT789719.1, MW183893.1, and KX871187.1): Data reference in GenBank.

Discussion

The results of the isolation of *E. coli* on eosin methylene blue agar (EMB) obtained 4 (25%) of 16 reproductive tract fluid samples of Bali cattle that had been collected. These results are close to the results of the previous study, which stated that 33.33% (3/10) of *E. coli* were found in 10 isolates of Gram-negative bacteria in repeat breeder cattle in Aceh (9). Research on Bali cattle in Lombok also documented the presence of *E. coli*, *Citrobacter freundii*, and *Proteus vulgaris bacteria* in 4 samples of reproductive tract fluid from Bali cattle that experienced repeat breeder (18).

Escherichia coli isolates were resistant to penicillin G, oxytetracycline and gentamicin, cefotaxime, and tetracycline and also showed positive synergy results by an expansion of the cefotaxime (CTX) disc diameter zone around the amoxicillin-clavulanate (AMC) disc in the screening test for the presence of ESBL with the double-disk approximation test. These facts show that *E. coli* resistance has occurred to various antibiotics used in the treatment of repeat breeder cases in Bali cattle. Poorly controlled administration of antibiotics will cause *E. coli* bacteria to adapt to produce genes encoding extended-spectrum β-lactamase (ESBL) found on chromosomes and plasmids. These results are in line with the statement that the AMR profile of *E. coli* almost reflects the use of antimicrobials in animals for food production (4). *E. coli* has also been isolated from Bali cattle which are resistant to erythromycin, tetracycline, and ciprofloxacin (19). Kholik *et al.* (10) also found that *E. coli* from the feces of Bali cattle with repeat breeder cases on Lombok Island were resistant to penicillin G, oxytetracycline, and cefotaxime.

The PCR results of the *blaTEM* gene on *E. coli* from samples, isolated from the reproductive tract of Bali cattle in this study, were at a position of 560 bp on the agarose band electrophoresis. The result of this study is in line with several studies documenting the position of the *blaTEM* gene in the agarose band. Previous research had stated that *blaTEM* isolated *Escherichia coli* from the urine of dairy cows with uterine infection in India at 800 bp (5). The *blaTEM* gene in *Escherichia coli* was also found in dairy cows to be at around 500 bp (6)

Based on the phylogenetic analysis in figure 3, *E. coli* encoding the *blaTEM* gene were obtained from the reproductive tract of Bali cattle and had a genetic relationship with *E. coli blaTEM-206* that isolated from pig and had a genetic relationship with *E. coli* (*Escherichia coli* strain U-10 TEM family beta-lactamase gene) from stool and urine of humans. This situation can happen because the reproductive tract is contaminated by *E. coli* through humans or the environment. The previous research documented that predisposing factors for repeat breeder were bacterial infection when handling cases of reproductive disorders in cattle like dystocia, retained placenta, uterine prolapse, and ovarian disorders (9). Uterine infection can be caused by environmental conditions during delivery, but the retained placenta is the most important predisposition (20). *Escherichia coli* contamination can also be the result of an unfavorable environment, especially postpartum. An unfavorable postpartum environment will facilitate the entry of microbes into the uterine lumen, pollute the uterine lumen environment, and interfere with embryonic life which can cause early embryonic death.

The presence of *E. coli* that encodes the antibiotic resistance gene of Bali cattle from the reproductive tract indicates that the uncontrolled use of antibiotics has caused *E. coli* to produce antibiotic resistance genes. Uncontrolled administration of antibiotics will cause the response of bacteria to become resistant by various mechanisms including enzyme production and mutation. Mutations in the gene of *E. coli* can occur through point mutations and rearrangement of DNA segments. Allcock *et al.* (21) stated that a bacterium will become resistant to an antibiotic may have one or more mechanisms. Genetic variation is critical to microbial evolution and may arise by a variety of mechanisms including point mutations, rearrangement of large segments of DNA from one chromosomal or plasmid location to another, or acquisition of foreign DNA from another bacterium by horizontal transfer of cells' genetic elements.

E. coli that are resistant to antibiotics from the bovine reproductive tract will be able to transfer resistant genes to other bacteria in the bovine reproductive tract. The *E. coli* can also transfer its resistance gene to other bacteria when it is released from the reproductive tract into the environment by horizontal gene transfer. Horizontal gene transfer of *E. coli*, which encodes the resistance gene that occurs, will cause a wider spread of antibiotic-resistant genes by exchanging their genetic material to other bacteria in other animals, humans, and the environment. *Escherichia coli* which carries the material for the resistance gene may be able to transfer the gene to humans and other animals. Le Roux and Blokesch (22) stated that horizontal gene transfer (HGT) allows bacteria to exchange their genetic material including antibiotic-resistance genes among diverse species. Antibiotic resistance genes are found in various species.

Conclusion

Escherichia coli from the reproductive tract of Bali cattle showed the character of *Escherichia coli* S2.2-EK pEC-S2.2 *blaTEM* gene for class A broad-spectrum *β-lactamase TEM*-206 with a high degree of kinship and has developed multidrug resistance to antibiotics.

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Conflict of interest

There is no conflict of interest.

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التوصيف والتحليل الجيني لجين بيتا لاكتاماز تيمونيرا في الاشريكية القولونية من ماشية بالي، في جزيرة لوميوك في إندونيسيا

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الخلاصة

تهدف هذه الدراسة الى تحديد خصائص وتطور جين بيتا لاكتاماز تيمونيرا في الاشريكية القولونية المعزولة من الجهاز التناسلي لابقار بالي التي تعاني من حالات فشل الحمل. أجريت هذه الدراسة في حزيران ٢٠٢١, حيث استخدمت ١٦ من انثى الماشية في بالي والمصابة بحالات فشل الحمل لأخذ سوائل الجهاز التناسلي باستخدام مسدس بلاستيكي. جرى تحديد عزل بكتيريا الاشريكية القولونية باستخدام الايوسين والميثيلين والبلو آكارعن طريق الاختبارات الحياتية والكيميائية. تم أجراء أختبار الحساسية للمضادات الحيوية على عينات الاشريكية القولونية بطريقة الانتشار القرصي. وكذلك فحص وجود أنزيم بيتا لاكتامـاز واســع النطاق باســـتخدام طريقة اختبـار القرص

المزدوج التقريبية. وتم تحليل وجود جين بلاتيم في الاشريكية القولونية من خلال اجراء فحص التطور الوراثي ال بي سي آر واستخدام التعاقبات الوراثية لجين بلاتيم. واظهرت النتائج انه تم الحصول على ٤ عصيات من الاشريكية القولونية من ١٦ عينة ماخوذة من سائل الجهاز التناسلي.هذا وتبين من خلال فحص الحساسية للمضادات الحيويةالذي تم على ٤ عينات معزولة للاشريكية القولونية بان ٥٠% منها مقاومة للبنسيلين والسيفوتاكسيم. .واظهرت عينات الاشريكية القولونية المقاومة للبنسيلين والسيفوتاكسيم نتائج تآزر في اختبار القرص المزدوج التقريبي. أظهرت نتائج تفاعل البوليميراز االمتسلسل ان عينات الاشريكية القولونية قامت بتشفير جين البلاتيم والمتمركز في موضع ٥٦٠ بي ببي ضمن المزيج الهلامي اثناء عملية فصل الجزيئات. واظهر تحليل شجرة التطور أن جين البلاتيم الذي قامت الاشريكية القولونية بتشفيره كان على ارتباط بالاشريكية القولونية التي قامت بتشفير جين البلاتيم رقم ٢٠٦. كما اوضحت خصائص جين البلاتيم في عينات الاشريكية القولونية الخصائص التابعة لجين بلاتيم ٢٠٦ لاكتاماز بي واسع النطاق والذي اسهم في انتاج الاشريكية القولونية في الجهاز التناسلي لقطعان الماشية في بالي بأن خصائص جين البلاتيم الناشيء من الاشريكية القولونية S2.2-EK pEC-S2.2 فئة اي واسع النطاق بي لاكتاماز تيم ٢٠٦ درجة عالية من القرابة.