



SURAT KETERANGAN  
Nomor : 3318/UN3.1.6/KP/2023

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Jabatan : Lektor Kepala

Telah melaksanakan penelitian dengan judul sebagai berikut :

No.	Judul Karya Ilmiah	Tahun Pelaksanaan Penelitian
1.	Genetic Identification of Shiga Toxin Encoding Gene from Cases of Multidrug Resistance (MDR) Escherichia coli Isolated from Raw Milk.	2021
2.	Molecular identification of blaCTX-M and blaTEM genes encoding extended-spectrum $\beta$ -lactamase (ESBL) producing Escherichia coli isolated from raw cow's milk in East Java, Indonesia	2021
3.	Incidence of Escherichia coli producing Extended-spectrum beta-lactamase in wastewater of dairy farms in East Java, Indonesia	2023
4.	Detection of Multidrug-Resistant (MDR) Escherichia coli Isolated from Raw Milk in East Java Province, Indonesia.	2020
5.	Presence of multidrug resistance (MDR) and extended-spectrum beta-lactamase (ESBL) of Escherichia coli isolated from cloacal swab of broilers in several wet markets in Surabaya.	2021
6.	A comprehensive description of the exoskeleton of six Lobster species (Genus Panulirus) in Aceh Province, Indonesia.	2023
7.	Analisis Filogenetik Gen Hemagglutinin dan Neuraminidase Avian Influenza H9N2 Asal Ayam Petelur di Jawa Timur	2020
8.	Identification of Ectoparasites in Pearl Catfish ( <i>Clarias gariepinus</i> ) with One and Three Months Age in Maclele Cultivation, Tuban District, Tuban Regency.	2022
9.	Cases of Multidrug Resistance (MDR) in <i>Klebsiella pneumoniae</i> Isolated from Healthy Pigs.	2021



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Adapun penelitian tidak perlu Uji Ethical Clearance karena **menggunakan hewan coba yang tidak disakiti**.

Demikian Surat Keterangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar**

Surabaya, 12 Juni 2023

Dekan,



Prof. Dr. Mirni Lamid, drh., MP

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# **BIOCHEMICAL AND CELLULAR ARCHIVES**

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## CASES OF MULTIDRUG RESISTANCE (MDR) IN *KLEBSIELLA PNEUMONIAE* ISOLATED FROM HEALTHY PIGS

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**ABSTRACT :** Antibiotics are commonly used in veterinary medicine throughout the world for therapeutic uses and for increasing production in pig farms. *Klebsiella pneumoniae* is one of the most important organisms clinically that has received attention in public health. *Klebsiella pneumoniae* (*K. pneumoniae*) is a group of Enterobacteriaceae which is significant for causing disease and shows frequent resistance to antibiotics in humans as well as in pigs. This study aims to determine the antibiotic resistance profile in pig farms through the identification of *K. pneumoniae*, which is one of the important bacteria involved in antibiotic resistance. This study focuses on the presence of *K. pneumoniae* bacteria in pigs carried out by rectal swabs on two pig farms in East Java, namely the pig farms in Gresik Regency and Malang Regency. The samples obtained were cultured using Mac Conkey Agar media and tested for biochemical identification and antibiotic sensitivity testing with the Kirby-Bauer method against the antibiotics ciprofloxacin, streptomycin, trimethoprim, tetracyclin and aztreonam. The *K. pneumoniae* bacteria was isolated from 7 samples of swine rectal swabs from pig farms in Gresik Regency and 4 samples from pig farms in Malang Regency. Of the 11 positive samples of *K. pneumoniae*, almost all isolates were resistant to tetracyclin and trimethoprim antibiotics. There were 4 *K. pneumoniae* isolates that were resistant to 3 antibiotics (MDR). It can be concluded that *K. pneumoniae* has potential to become a serious problem on public health.

**Key words :** Antibiotic resistance, *Klebsiella pneumoniae*, multidrug resistance, pigs, public health.

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### INTRODUCTION

Antibiotic resistance cases have increased significantly, indicating a mortality rate of up to 50,000 people each year (WHO, 2017). Problems associated with the development and spread of antibiotic resistance are major health problems that are spreading rapidly around the world and are currently seen as a major threat to public health at a global level (Riwu *et al*, 2020). The widespread and inappropriate use of antibiotics has resulted in the emergence of strains of bacteria that are resistant to antibiotics (Widodo *et al*, 2020).

Antibiotics have been commonly used in veterinary medicine throughout the world for therapeutic uses and

to increase production in livestock (Widodo *et al*, 2020; Khairullah *et al*, 2020). The use of antibiotics in pig farms in Indonesia is still widely practiced by pig breeders themselves, where the level of farmer knowledge about antibiotics and antibiotic resistance is generally still low. Many farmers determine their own use of antibiotics on their farms based on their own experience and input from other breeders (Arief *et al*, 2016). Therefore, 90% of the digested dose can be excreted unmodified or partly metabolized directly through urine and feces. As a result, the feces of pigs given antibiotic treatment becomes an important reservoir in terms of antibiotic residues and bacteria that are resistant to many classes of antibiotics or multidrug resistance (MDR) (Jury *et al*, 2010;

Hidayatullah *et al*, 2020).

Bacteria that have MDR properties will be more difficult and take longer to treat, and may even require new antibiotics as treatment (WHO, 2017). It has been reported in the World Health Organization's (WHO) global surveillance of bacterial resistance to antibiotics, *Klebsiella pneumoniae* (*K. pneumoniae*) is classified as one of nine bacteria involved in antibiotic resistance. *K. pneumoniae* is one of the most important organisms clinically that has received attention in public health (Effendi *et al*, 2018). *K. pneumoniae* is a group of Enterobacteriaceae which is significantly considered as an opportunistic pathogen as a cause of disease and shows frequent resistance to antibiotics (Effah *et al*, 2020).

*K. pneumoniae* becomes resistant to several classes of antibiotics because it can produce beta-lactamase enzymes, which can deactivate the effectiveness of the antibiotics. There have been many *K. pneumoniae* that is resistant to several classes of antibiotics or multidrug resistance (MDR) in pig farms in European and Asian countries, but there are no reports of MDR incidence in pig farms in Indonesia (Sofiana *et al*, 2020). *K. pneumoniae* is known to be pathogenic and cause respiratory problems in pigs, which can lead to death (Bidewell *et al*, 2018). *K. pneumoniae* bacteria detected in animals are increasing, this allows the beta-lactamase-producing *K. pneumoniae* to contribute to the increased incidence of infection with beta-lactamase-producing bacteria in humans (Effendi *et al*, 2018; Mobasser *et al*, 2019).

The high pig population in Indonesia and the lack of application of biosafety and biosecurity in the pig farming sector are among the factors for the spread of resistant bacteria (Arief *et al*, 2016). Animals spread bacteria that are resistant to antibiotics through feces. Resistant bacteria contained in feces can re-enter the human population through direct contact between animals and humans or vice versa, through water, food and the surrounding environment (Ansharieta *et al*, 2020; Widodo *et al*, 2020). Pigs are agents of the spread of *K. pneumoniae* bacteria which have MDR properties to other animals, the environment and humans (Yang *et al*, 2019; Sofiana *et al*, 2020).

Today awareness of human and animal health is inseparably linked to their environment leading to an integrated One Health approach, especially focusing on food safety, zoonotic surveillance and antibiotic resistance control (Rahmahani *et al*, 2020; Effendi *et al*, 2021). The discovery of many *K. pneumoniae* that is resistant to

antibiotics in animals has a negative impact on public health and has an impact on the economy of a country, so there needs to be a control or prevention so that this problem can be resolved (Permatasari *et al*, 2020). Therefore, study aims to determine the antibiotic resistance profile in pig farms through the identification of *K. pneumoniae*, which is one of the important bacteria involved in antibiotic resistance. This study focuses on the presence of *K. pneumoniae* bacteria in pigs carried out by rectal swabs on two pig farms in East Java, namely the pig farms in Gresik Regency and Malang Regency.

## MATERIALS AND METHODS

### Sample collection and preparation

In this study, a total sample of 130 rectal swabs were obtained from 2 locations in East Java. Sampling was carried out using transport medium of amies viscosa, swab was carried out aseptically on pigs in Gresik Regency farms as many as 80 samples and 50 samples in farms in Malang Regency. The transportation process of all the rectal swab sample specimens obtained were put into a cool box which also equipped with an cool pack (Effendi *et al*, 2019). A total of 130 samples were cultured using inoculating loop and on MCA media then incubated at 37°C for 24 hours (Wibisono *et al*, 2020; Permatasari *et al*, 2020).

### Characterization of isolates

Pure bacterial isolates were identified based on colony morphology, cell morphology and biochemical tests. Colony morphology observations were seen from the shape, color and edges of bacterial colonies on MCA media. Observation of cell morphology includes the shape and arrangement of cells selected through Gram stain. Furthermore, each isolate was characterized biochemically. Biochemical tests were carried out to see the characteristics of *Klebsiella pneumoniae* bacteria through biochemical reactions on Simon's Citrate Agar, Semi Solid Agar media, MR-VP media, Kligler's Iron Agar, peptone water which is then dropped by kovach reagent for Indol test and urea media (Leber, 2016; Permatasari *et al*, 2020).

### Antibiotic sensitivity test

Antibiotic sensitivity test was performed using the Kirby-Bauer agar diffusion method. The antibiotics used are those that are already on the disc. The clear zone formed is then grouped into sensitive (S), intermediate (I) or resistant (R) groups. The procedure in the antibiotic sensitivity test is first of all the bacteria culture obtained from the colony contained in the MCA media dissolved in a test tube containing 8 ml of physiological NaCl, homogenized using a vortex until turbidity is obtained

which is the same as the Mc Farland standard of 0.5 (Putra *et al*, 2019). Physiological nacl that has been tested for its recurrence with the Mc Farland standard of 0.5 is then applied to a sterile cotton swab and then gently rubbed on the entire surface of the Mueller Hinton Agar medium (Putra *et al*, 2020; Wibisono *et al*, 2020). Sensitivity test and resistance profiling were carried out using 5 types of antibiotics, namely ciprofloxacin 5 µg, streptomycin 10 µg, tetracyclin 30 µg, trimethoprim 5 µg and aztreonam 30 µg. Bacterial cultures were incubated at 35-37°C for 18-24 hours (CLSI, 2018).

After the test results were obtained, the bacteria were grouped into the Multi drug Resistance (MDR) or non MDR groups. MDR bacteria are bacteria that are resistant to 3 or more classes of antibiotics (Magiorakos *et al*, 2012; Harijani *et al*, 2020).

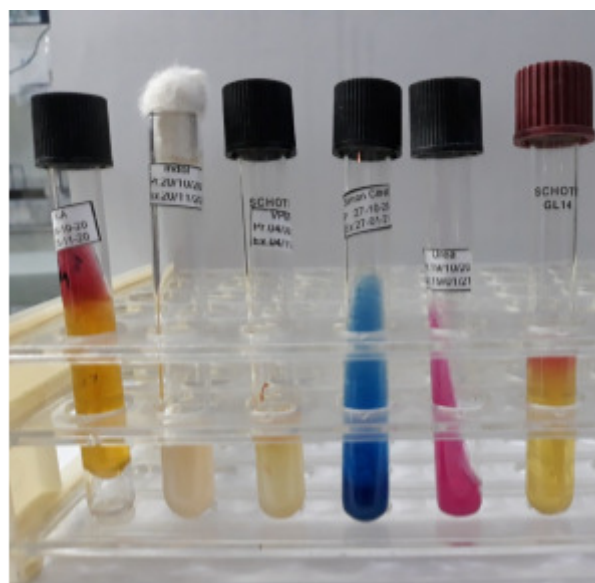
## RESULTS AND DISCUSSION

The results showed 11 out of 130 samples isolated from the pig rectal swab were positive for *K. pneumoniae*. The results of colony morphological identification on Mac Conkey Agar media are shown in Fig. 1 and the results of biochemical tests will be presented in Fig. 2. The percentage of swab swab swab samples from swab rectals after identification and biochemical tests obtained positive samples of *K. pneumoniae* bacteria as much as 8.75% (7 / 80) samples from pig farms in Gresik Regency and 8% (4/50) samples from pig farms in Malang Regency, so that the total number of positive samples for *K. pneumoniae* bacteria was 8.5% (11/130) samples which is shown in Table 1.

The level of bacterial resistance to antibiotics is obtained by measuring the diameter of the inhibition zone formed after the antibiotic disc attachment process. The standard for assessing the diameter of the inhibition zone for antibiotics based on the CLSI (Clinical Laboratory Standards Institute) is a reference for comparing the measurement results of the inhibition zone obtained in this study. In this study, there were 5 types of antibiotic discs used, namely ciprofloxacin (5 µg), streptomycin (10 µg), tetracyclin (30 µg), trimethoprim (5 µg) and aztreonam (30 µg). The results of the inhibition zone of the antibiotics formed on MHA media were then measured using a caliper (mm) in diameter. The results of antibiotic resistance tests are shown in Fig. 3.



**Fig. 1 :** Isolation of *K. pneumoniae* bacterial colonies on Mac Conkey Agar (MCA) selective media. **Note:** *K. pneumoniae* bacteria culture on MCA media looks pink, round in shape, the surface of the colony looks smooth and moist.



**Fig. 2 :** Biochemical test results of bacterial isolates identified as *Klebsiella pneumoniae*. **Note:** From left to right are biochemical media: KIA, Indol, MR-VP, Simon Citrat, Urea and Semi Solid Media.

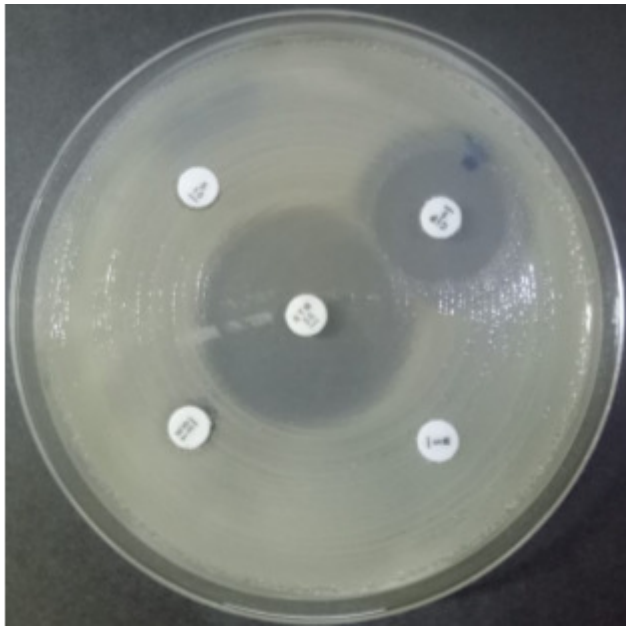
The results of the antibiotic resistance test in this study were indicated by the presence of an antibiotic inhibition zone against bacterial growth. In this study, 91%

**Table 1 :** Number of positive samples for *Klebsiella pneumoniae* bacteria.

Location	Sample size	Positive samples	Sample Code
Gresik Farm	80	7	GB24, GB29, GB30, GB32, GB33, GB37, GB41
Malang Farm	50	4	MB60, MB81, MB82, MB91

**Note:** GB (Gresik ) followed by the sample number and MB (Malang) followed by the sample number.





**Fig. 3 :** Antibiotic resistance test results against *K. pneumoniae*. **Note :** CIP (ciprofloxacin 5 µg), S (streptomycin 10 µg), TE (tetracyclin 30 µg), W (trimethoprim 5 µg) and ATM (aztreonam 30 µg).

were Multidrug Resistance (MDR) bacteria. MDR is the incidence of antibiotic resistance in isolates that are resistant to  $\geq 3$  types of antibiotics (Magiorakos *et al*, 2012; Permatasari *et al*, 2020). The incidence of MDR in *K. pneumoniae* bacteria resulted in treatment being longer and difficult to cure. By looking at the pattern of antibiotic resistance, it is hoped that it can be a guide in choosing the right antibiotic for treatment (Hayati *et al*, 2019).

This study also showed that the bacterial isolates found were still sensitive to the antibiotics Ciprofloxacin and Aztreonam and some were still intermediates to Streptomycin. Bacterial resistance to antibiotics occurs due to inappropriate and excessive use of antibiotics. *K. pneumoniae* resistance to antibiotics occurs because these bacteria have the ability to produce Extended Spectrum Beta Lactamase (ESBL). Beta-lactamase enzymes can protect Gram-negative bacteria against beta-lactam antibiotics. The target of beta-lactam antibiotic attack is the cell wall. Antibiotics in this class have beta-lactam groups as well as cell walls that react with enzymes

**Table 2 :** Pattern of antibiotic resistance to the *Klebsiella pneumoniae*.

No	Antibiotics	Sample code										
		GB24	GB29	GB30	GB32	GB33	GB37	GB41	MB60	MB81	MB82	MB91
1	Ciprofloxacin	S (22)	S (21)	S (27)	S (30)	S (23)	S (27)	S (21)	S (30)	S (29)	S (30)	S (22)
2	Streptomycin	R (6)	R (5)	I (12)	I (14)	I (12)	I (13)	R (5)	R (11)	I (12)	I (13)	R (6)
3	Tetracyclin	R (6)	R (7)	R (8)	R (10)	R (6)	R (9)	R (5)	I (14)	R (6)	R (9)	R (6)
4	Trimethoprim	R (5)	R (5)	R (8)	R (10)	R (6)	R (6)	R (5)	S (26)	R (6)	R (6)	R (6)
5	Aztreonam	S (30)	S (29)	S (31)	S (31)	S (30)	S (30)	S (29)	S (32)	S (31)	S (34)	S (30)
<b>Total</b>	3	3	2	2	2	2	3	1	2	2	3	

**Note:** R (resistance), I (intermediate), S (sensitive), the number in brackets is the diameter of the drag zone in mm.

(10/11) of samples were resistant to Tetracyclin and Trimethoprim, namely the samples GB24, GB29, GB30, GB32, GB33, GB37, GB41, MB 81, MB82 and MB91 with a zone size of  $\leq 11$  mm. Antibiotic resistance testing on all positive isolates of *K. pneumoniae* showed that 4 isolates were resistant to 3 antibiotics and can be classes as Multidrug resistance (MDR). The results of the antibiotic inhibition zone measurements is presented in the Table 2.

## DISCUSSION

The results of biochemical identification showed that the *K. pneumoniae* bacteria had a positive Simon's Citrate character, a negative or non-motile motility test, negative Methyl Red, positive Voges Proskauer, the KIA test on the slant/ slant was acidic and at the bottom was acidic and produced gas but  $H_2S$  negative, negative indole and positive urea (Leber, 2016). The results showed that there were 36% (4/11) of *K. pneumoniae* isolates which

in the process of making cell walls (Ansharieta *et al*, 2021; Wibisono *et al*, 2021). Enzymes will no longer function so that the cell walls will not form completely. Cell walls that are not fully formed and bacterial cells without cell walls cause bacteria to die (Finley *et al*, 2013; Kristianingtyas *et al*, 2020).

The widespread use of antibiotics and without close supervision has led to the emergence of resistance to antibiotics. The majority of pig farms in Indonesia use antibiotics for both treatment and prevention (Arief *et al*, 2016; Faridah *et al*, 2020). As many as 82% of *K. pneumoniae* isolates were resistant to tetracycline and trimethoprim in this study. Tetracyclines and trimethoprim are the antibiotics that are most widely used by livestock in Indonesia and around the world because of their efficacy as broad spectrum antibiotics, easy to absorb, low prices and low side effects (Arief *et al*, 2016).

Tetracyclin antibiotics in certain countries are even

commonly used as additional feed ingredients in pig farms. Tetracyclin is well absorbed and has low toxicity (Michalova *et al*, 2004). This is also supported by research conducted in Malaysia, *Klebsiella pneumoniae* in pigs was found to be resistant to several antibiotics including: ciprofloxacin, aztreonam, ampicillin, tazobactam, amikacin, tetracyclin. In that study, the highest level of antimicrobial resistance to tetracyclin antibiotics. All strains from the agricultural environment and pigs show resistance to tetracyclin, which is widely used in feed supplements (HAIAP, 2013). Research conducted by Yang in China also revealed that the *Klebsiella pneumoniae* bacteria in pigs was 74.5% resistant to tetracyclin (Yang *et al*, 2019). In Kieffer's study, it was stated that swab rectals of pigs on Portuguese farms tested positive for *K. pneumoniae*, which was resistant to tetracyclin and trimethoprim (Kieffer *et al*, 2017). In Founou's study in Cameroon, ESBL-producing *K. pneumoniae* in pigs was resistant to trimethoprim. This study also reported that all *K. pneumoniae* in pigs and humans showed reduced susceptibility to trimethoprim (Founou *et al*, 2018).

The results of this study indicated that 55% (6/11) of the isolates had an intermediate inhibition zone to streptomycin and 45% (5/11) of the isolates were resistant to streptomycin. The mechanisms of chromosomal resistance to aminoglycosides in *K. pneumoniae* include modification of cell permeability due to changes in the efflux pump system and due to loss of putative porin (KpnO). This may indicate a different affinity of the permeability apparatus with different aminoglycosides. Direct involvement in aminoglycoside resistance was reported in vitro for missing porin KpnO leading to resistance to tobramycin, streptomycin and spectinomycin (Amador *et al*, 2019).

Antibiotics that are often used in pig farms besides tetracyclines are the fluoroquinolones which are widely used in humans and animals as a therapy for digestive and respiratory disorders (Arief *et al*, 2016). Of the 11 *K. pneumoniae* isolates tested, all isolates still showed sensitivity to ciprofloxacin. Since 1998, fluoroquinolones have been classified by WHO as critically important in human medicine because of their importance in treating *Campylobacter*, *Salmonella* and *E. coli* infections. To prevent further resistance, fluoroquinolone treatment is limited to individual, not group treatment. Even in European countries, the use of this antibiotic has been banned for use on farms (Hayati *et al*, 2019).

Another  $\beta$ -lactam group, aztreonam, is not used in pig farms, but is commonly used in the treatment of bacterial infections in humans, especially in cases of

infections that are resistant to ampicillin and amoxicillin. Resistance of this type of antibiotic can make treatment in humans difficult and takes longer. Bacteria resistant to aztreonam indicated ESBL-producing bacteria, but the results of this test had low sensitivity. Research conducted by Sanguinetti, showed that as many as 58% of bacterial isolates declared as non-ESBL bacteria had ESBL coding genes. This biased result could be due to the fact that ESBL bacteria can go undetected or the bacteria produce other enzymes that hydrolyze other  $\beta$ -lactam antibiotics such as cefpodoxime and cefepim (Sanguinetti *et al*, 2003).

The sensitivity of the test can be increased by using another method, namely the Double Disc Diffusion (DDD) method which combines cefpodoxime (10 $\mu$ g) and clavulanic acid or also using the Phoenix ESBL test. In addition, ESBL bacteria can be identified by detecting the presence of the ESBL coding gene. If the coding gene is detected in a bacterial isolate that is still sensitive on the antibiotic sensitivity test, then the isolate is considered a resistant isolate (CLSI, 2018). In order to prevent the further spread of antibiotic resistant bacteria, it is hoped that breeders can apply good hygiene and sanitation in the enclosure environment and choose a veterinarian or paramedic as a person, who is considered an expert and is authorized to take medical measures for livestock (Sofiana *et al*, 2020). The isolation of this *K. pneumoniae* strain always urges the application of strict infection and control measures and constant surveillance of antibiotic resistance in the hospital. Similar rigorous interventions must be made in the food production industry if we are to successfully prevent in the spread of their clones in the food chain from livestock to the dining table (Founou *et al*, 2016; Hartadi *et al*, 2020).

In conclusion can be showed that *K. pneumoniae* bacteria was successfully isolated from swab samples of swine in 2 pig farms in East Java, namely Gresik Regency and Malang Regency by 8.5% (11/130). Almost all isolates showed resistance to tetracyclin and trimethprim, amoxicillin 91% (10/11). There were 36% (4/11) four isolates out of eleven positive isolates of *K. pneumoniae* bacteria that were isolated were resistant to 3 classes of antibiotics or Multidrug Resistance (MDR). MDR in *K. pneumoniae* is becoming a serious problem in humans and animals, increasing resistance to most of the available antibiotics and causing treatment difficulties. Farmers are expected to be more vigilant and need to apply biosafety and biosecurity to prevent further spread of these antibiotic resistant bacteria in animals, livestock environments, slaughterhouses, surrounding environments and humans.

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