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Prevalence of Antibiotic Resistance Bacteria From Cow Manure in Kota Tinggi & Batu Pahat, Johor

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Abstract

Antimicrobial resistance (AMR) has emerged as one of the most significant global public health concerns, with livestock manure recognised as an important environmental reservoir of antibiotic-resistant bacteria (ARB). This study investigated the prevalence of antibiotic-resistant bacteria isolated from cow manure collected from selected cattle farms in Kota Tinggi and Batu Pahat, Johor, Malaysia. Fresh manure samples were subjected to serial dilution and bacterial isolation using Nutrient Agar. Representative isolates were purified and characterised based on colony morphology and Gram staining before antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines. A total of 36 bacterial isolates were recovered from both sampling locations. The isolates exhibited varying resistance profiles against seven antibiotics. Ampicillin demonstrated the highest resistance frequency (77.78%), followed by tetracycline (36.11%), rifampicin (27.78%), chloramphenicol (25.00%), sulphamethoxazole (16.67%), gentamicin (13.89%), and ciprofloxacin (2.78%). Multiple Antibiotic Resistance (MAR) index analysis showed that 72.22% of the isolates possessed MAR values greater than 0.20, indicating that most bacterial isolates originated from environments subjected to considerable antibiotic selective pressure. Comparable multidrug resistance patterns were observed in both Kota Tinggi and Batu Pahat, suggesting that antibiotic-resistant bacteria are widely distributed in cattle manure irrespective of geographical location. These findings highlight cow manure as a potential environmental reservoir for antimicrobial resistance and emphasise the importance of continuous surveillance, prudent antibiotic stewardship, and sustainable manure management practices within the One Health framework.

Keywords: antimicrobial resistance, antibiotic-resistant bacteria, cow manure, Kirby–Bauer assay, Multiple Antibiotic Resistance index, livestock

Introduction

Antimicrobial resistance (AMR) has become one of the most serious global public health threats affecting humans, animals, and the environment (Murray et al., 2022). The widespread emergence of antibiotic-resistant bacteria (ARB) has reduced the effectiveness of antimicrobial therapy, resulting in prolonged illnesses, increased mortality, and higher healthcare costs. Consequently, the World Health Organization has recognised AMR as a priority issue requiring coordinated action through the One Health approach (WHO, 2023).

The extensive use of antibiotics in livestock production has contributed significantly to the emergence and dissemination of antimicrobial resistance (Manyi-Loh et al., 2018). A considerable proportion of administered antibiotics is excreted through animal faeces and urine, allowing livestock manure to serve as a reservoir of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Zhang et al., 2021). These resistant microorganisms may subsequently spread into soil and

water through manure application, posing potential risks to both environmental and public health (Bava et al., 2024).

Although antimicrobial resistance associated with livestock has been widely reported worldwide, information regarding antibiotic-resistant bacteria in cattle manure remains limited in Malaysia, particularly in Johor (Ho et al., 2021). Most local studies have focused on poultry farms or clinical settings, while comparatively little information is available on bacterial resistance in cattle farming systems. Therefore, baseline data are required to improve the understanding of antimicrobial resistance in cattle manure and to support future surveillance programmes.

Therefore, this study investigated the prevalence of antibiotic-resistant bacteria isolated from cow manure collected from selected cattle farms in Kota Tinggi and Batu Pahat, Johor. Bacterial isolates were characterised based on colony morphology and Gram staining, followed by antibiotic susceptibility testing using the Kirby–Bauer disc diffusion method. The Multiple Antibiotic Resistance (MAR) index was subsequently determined to evaluate multidrug resistance and compare resistance patterns between the two sampling locations. The findings provide baseline information on antimicrobial resistance in cattle manure and contribute to ongoing efforts to promote prudent antibiotic use and One Health-based antimicrobial resistance surveillance in Malaysia.

Materials and methods

Fresh cow manure samples were collected from selected cattle farms in Kota Tinggi and Batu Pahat, Johor, Malaysia. Approximately 10 g of each homogenised manure sample was serially diluted from 10^{-1} to 10^{-4} using sterile distilled water. Aliquots (0.1 mL) of each dilution were spread onto Nutrient Agar (NA) plates and incubated aerobically at 37°C for 24 h. Distinct bacterial colonies were selected based on colony morphology and repeatedly streaked on fresh NA plates to obtain pure cultures (Cappuccino & Welsh, 2017).

The purified isolates were characterised based on colony morphology and Gram staining. Colony characteristics, including colour, shape, elevation, margin, and surface texture, were recorded. Gram staining was performed using crystal violet, Gram's iodine, 95% ethanol, and safranin before microscopic examination under oil immersion (1000×) to differentiate Gram-positive and Gram-negative bacteria according to the standard staining procedure described by Claus (1992).

Antibiotic susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2025). Fresh bacterial cultures were adjusted to approximately 0.5 McFarland standard before inoculation onto Mueller–Hinton agar. Commercial antibiotic discs containing ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), rifampicin (5 µg), sulphamethoxazole (300 µg), and tetracycline (30 µg) were placed on the inoculated agar surface. Following incubation at 37°C for 18–24 h, inhibition zone diameters were measured and interpreted as susceptible, intermediate, or resistant according to CLSI breakpoints (CLSI, 2025).

The Multiple Antibiotic Resistance (MAR) index was calculated by dividing the number of antibiotics to which each isolate was resistant by the total number of antibiotics tested, as described by Krumperman (1983). Isolates with MAR index values greater than 0.20 were considered to originate from environments with high antibiotic selective pressure, whereas values of 0.20 or below indicated relatively lower antibiotic exposure. The MAR index was subsequently used to compare multidrug resistance patterns between bacterial isolates obtained from Kota Tinggi and Batu Pahat.

Results and discussion

Figure 1 shows representative bacterial colonies isolated from cow manure samples. The isolates exhibited diverse colony morphologies, including differences in colour, size, shape and margin, suggesting the presence of multiple bacterial species. Following repeated streaking, pure colonies with distinct morphological characteristics were successfully obtained. Gram staining further demonstrated that the isolates consisted of both Gram-positive and Gram-negative bacteria, indicating a heterogeneous bacterial community commonly associated with cattle manure. Such

morphological and Gram reaction diversity has been widely reported in livestock-associated bacterial populations and reflects the complex microbial composition of manure.

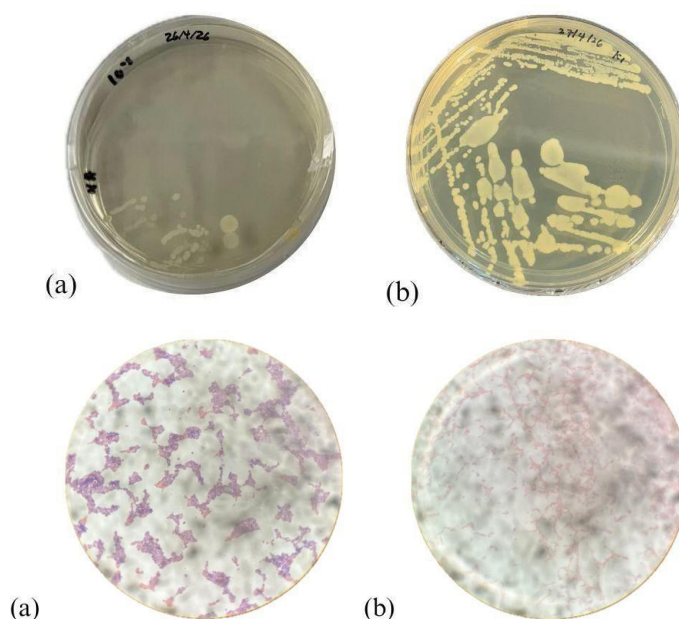


Figure 1 Representative bacterial isolates obtained from cow manure samples. (a) Primary bacterial colonies cultured on nutrient agar, (b) purified bacterial colonies following sub-culturing, (a) Gram-positive bacterial cells, and (b) Gram-negative bacterial cells observed after Gram staining under oil immersion (1000×).

Referring to Figure 1, representative bacterial isolates recovered from cow manure samples collected from Kota Tinggi and Batu Pahat exhibited diverse colony morphologies, including differences in colour, size, shape, elevation and colony margin, indicating the presence of multiple bacterial populations. Following repeated streaking, well-isolated colonies were successfully obtained (Figure 1b), confirming the establishment of pure cultures for subsequent antibiotic susceptibility testing. Gram staining further revealed the presence of both Gram-positive and Gram-negative bacteria (Figure 1c–d), demonstrating the heterogeneous bacterial community present in cow manure. The observed differences in colony morphology and Gram reaction suggest that the isolates represented different bacterial groups, although species-level identification would require molecular techniques such as 16S rRNA gene sequencing (Bava et al., 2024).

Figure 2 shows a representative Kirby–Bauer disc diffusion assay performed on Mueller–Hinton agar. Distinct inhibition zones were observed around several antibiotic discs, indicating differences in bacterial susceptibility. Two representative isolates from each sampling location were selected to illustrate the variation in inhibition-zone diameters observed among the bacterial isolates. Although both locations exhibited similar resistance patterns, differences in inhibition-zone sizes were observed between individual isolates, reflecting the heterogeneity of antimicrobial susceptibility within the bacterial populations. The measured inhibition zone diameters were interpreted according to CLSI (2023) guidelines and used to classify each isolate as susceptible, intermediate, or resistant. The overall resistance frequencies are summarised in Table 1.

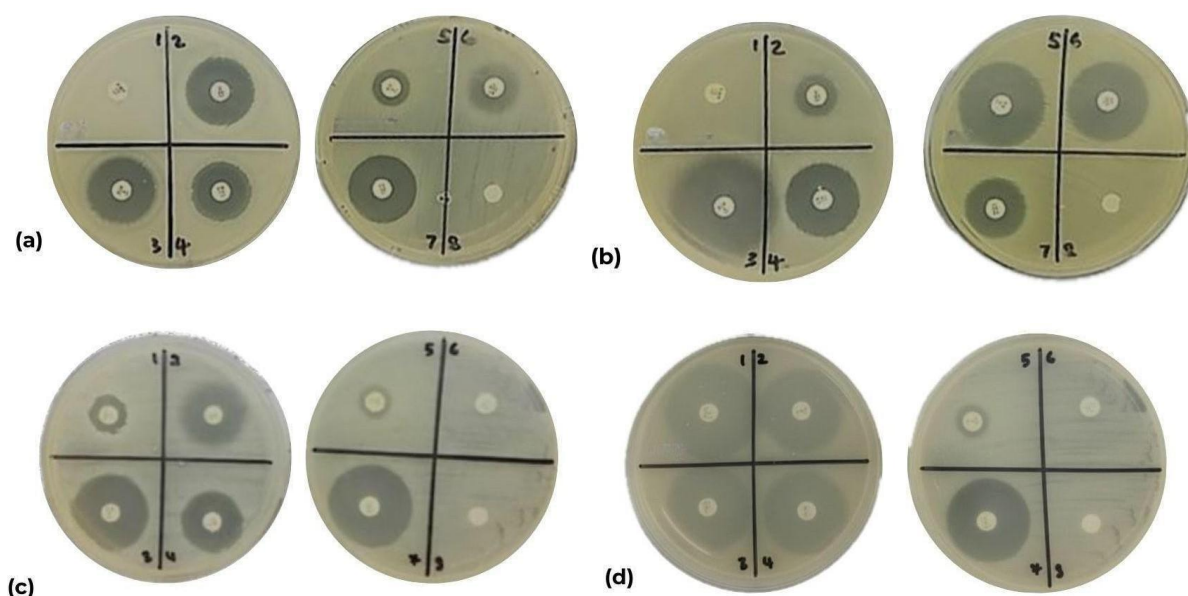


Figure 2 Antibiotic Bauer disc diffusion assay plates of bacterial isolates obtained from cow manure samples in Kota Tinggi and Batu Pahat. (a–b) Isolates from Kota Tinggi and (c–d) isolates from Batu Pahat.

Table 1: Overall antibiotic resistance percentage

Antibiotics	Resistant Isolates, n	Resistance (%)
Ampicillin	28	77.78
Tetracycline	13	36.11
Rifampicin	10	27.78
Chloramphenicol	9	25.00
Sulphamethoxazole	6	16.67
Gentamicin	5	13.89
Ciprofloxacin	1	2.78

The antibiotic susceptibility profiles of the bacterial isolates recovered from cow manure are presented in Table 1. Resistance was observed against all seven antibiotics tested, although the resistance frequencies varied considerably. Ampicillin exhibited the highest resistance rate (77.78%), followed by tetracycline (36.11%), rifampicin (27.78%), chloramphenicol (25.00%), sulphamethoxazole (16.67%), gentamicin (13.89%), and ciprofloxacin (2.78%). The high resistance to ampicillin may be associated with the widespread use of β -lactam antibiotics in livestock production, which promotes the selection of resistant bacterial populations (Ho et al., 2021). In contrast, ciprofloxacin showed the lowest resistance rate, possibly because its use in food-producing animals is more restricted due to its importance in human medicine (WHO, 2023). Similar resistance patterns have also been reported in livestock-associated bacteria by Manyi-Loh et al. (2018).

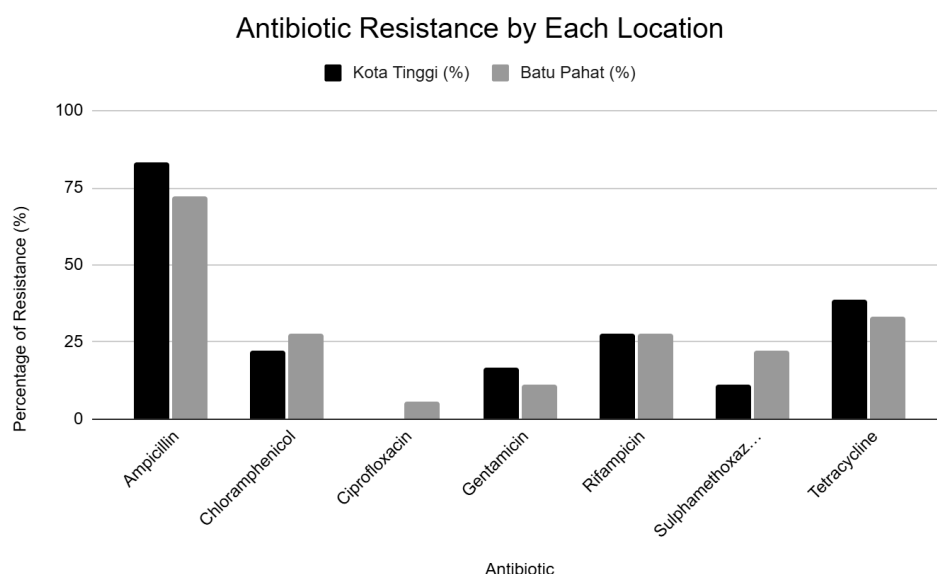


Figure 3: Antibiotic Resistance in Kota Tinggi vs Batu Pahat

Referring to Figure 2, it compares the antibiotic resistance profiles of bacterial isolates recovered from Kota Tinggi and Batu Pahat. Ampicillin exhibited the highest resistance in both locations, with 83.33% of isolates from Kota Tinggi and 72.22% from Batu Pahat showing resistance. Resistance to tetracycline was also relatively high in both districts, accounting for 38.89% and 33.33% of isolates from Kota Tinggi and Batu Pahat, respectively. Rifampicin resistance was identical in both locations (27.78%), whereas chloramphenicol and sulphamethoxazole resistance were slightly higher among Batu Pahat isolates (27.78% and 22.22%, respectively) than Kota Tinggi isolates (22.22% and 11.11%). Gentamicin resistance remained relatively low in both sampling sites, while ciprofloxacin resistance was detected only in Batu Pahat (5.56%) and was absent among isolates from Kota Tinggi.

To further evaluate the extent of multidrug resistance, the Multiple Antibiotic Resistance (MAR) index was calculated for all isolates, and the distribution of MAR index values is presented in Table 2. Among the 36 bacterial isolates, 26 isolates (72.22%) had MAR index values ≥ 0.20 , while 10 isolates (27.78%) had MAR values ≤ 0.20 . According to Krumpelman (1983), a MAR index greater than 0.20 indicates that bacteria originate from environments with frequent antibiotic exposure. The high proportion of isolates with MAR values above this threshold suggests that both cattle farms may serve as potential reservoirs of multidrug-resistant bacteria. This finding agrees with the high resistance observed for ampicillin in both sampling locations and indicates that antimicrobial selective pressure is present in both farming environments. Similar findings have been reported in livestock-associated bacteria exposed to continuous antimicrobial use.

Table 2: Distribution of MAR index among bacterial isolates

MAR Index	Number of Isolates (n)	Percentages (%)	Interpretation
≥ 0.20	26	72.22	High-risk source
≤ 0.20	10	27.78	Low-risk source

Conclusion

This study demonstrated the presence of antibiotic-resistant bacteria in cow manure collected from cattle farms in Kota Tinggi and Batu Pahat, Johor. Ampicillin exhibited the highest resistance rate, followed by tetracycline and rifampicin, whereas ciprofloxacin remained the most effective antibiotic

against the bacterial isolates. Furthermore, most of the isolates showed MAR index values greater than 0.20, indicating frequent antibiotic exposure and suggesting that both farms may serve as reservoirs of multidrug-resistant bacteria. These findings highlight the importance of continuous surveillance and prudent antibiotic use in livestock farming to reduce the spread of antimicrobial resistance and minimise its impact on environmental and public health.

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