Nutrient concentrations affect the antimicrobial resistance profiles of cattle manures

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Abstract
Antimicrobial resistance (AMR) in cattle is widespread because of the increased use of antibiotics to combat microbial diseases and enhance milk production. The cattle excreta released into the environment can be a potent source of contamination in spreading antibiotic resistance, especially upon its application in agriculture. However, the correlation of AMR profile of manure with other physico-chemical parameters is limited. Therefore, the study aimed to generate AMR profiles for manure samples collected from 25 different sites of two agriculturally important states in India, Madhya Pradesh and Uttar Pradesh. Samples were tested for physico-chemical parameters, viz., electrical conductivity, pH, total nitrogen (N), total phosphorus (P), and total potassium (K). Bacterial community analysis was done by culture-dependent and culture-independent methods. The influence of feeding practices, nutrient concentration, and bacterial abundance on antibiotic resistance profiles was observed in collected manure samples. Manures of intensive feeding animals harbored highly resistant profiles of bacteria as compared to natural grazing cattle.

Keywords Antimicrobial resistance · Animal manure · Bacterial community · Environment · Nutrient concentration

Introduction

According to the World Health Organization, AMR is considered one of the 10 major health threats globally in 2019 (WHO 2019). Antibiotics are used to combat various microbial infections in both humans and animals. The current COVID-19 pandemic has further deepened the global implications for antimicrobial resistance through the unintended proliferation of AMR (Murray 2020; Rawson et al. 2020; Lai et al. 2021). There are several factors contributing to this, one of which is the high rate of antimicrobials administered to COVID-19 patients who have a moderate to low rate of secondary or co-infection(Lai et al. 2021). In a recent report, a significant increase in multidrug-resistant organisms (MDROs), Klebsiella pneumoniae—producing extended-spectrum lactamase (ESBL), carbapenem-resistant Klebsiella pneumoniae, Acinetobacter baumannii, methicillin-resistant Staphylococcus aureus (MRSA), pan-echinocandin-resistant Candida glabrata, and multi-triazole-resistant Aspergillus fumigatus was observed in response to the pandemic (Lai et al. 2021).

The incidence of AMR in livestock, which is generally caused due to the high dosage of antibiotics to combat diseases, increased production of milk and meat, and for improved performance of cattle is threatening (Jayarao et al. 2019). The annual intake of total antibiotics in livestock farming is estimated at around 14.8 g per kg for chicken, 17.2 g per kg for pig, and 4.5 g per kg for cattle (van Boeckel et al. 2015). The antimicrobials used in animal feeding operations are mostly water-soluble. They are not completely absorbed by the animals. A significant amount of antibiotics is excreted by animals in manure and urine (Manyi-Loh et al. 2018; Quaik...
et al. 2020). The rate and the amount of antibiotics released into the environment have been reported to be dependent on the type of antibiotic, on the dosage administered, and on the animal species and its age (Zhao et al. 2010). The excreta of the livestock can serve as a direct source of the spread of AMR in the environment (Wichmann et al. 2014).

Development of resistance due to mutation and transfer of resistance genes (horizontal gene transfer, mobile genetic elements), through plasmids, phages, transposons, or integron gene cassettes under the selection pressure of antimicrobials, is a common phenomenon (Woodford and Ellington 2007; Fondi and Fani 2010; Checucci et al. 2020). These mechanisms of transfer of antibiotic resistance contribute significantly to AMR diffusion in the livestock. Overuse of antibiotics in the production of livestock is generally thought to be a source of AMR in the animal excreta. However, animals raised without antibiotic supplementation (natural grazers) have also shown a large incidence of antibiotic (cefotaxime)-resistant bacteria (Markland et al. 2019). High nutrient concentration in manure supports microbial load (Lima et al. 2020). Subsequently, in the presence of antimicrobials in the manure, horizontal gene transfer is triggered (Blau et al. 2018). Thus, these reports suggest that nutrients present in animal excreta can exhibit an important effect on antimicrobial resistance profiles of manure from livestock.

India has the highest livestock worldwide of ~535.78 million, with 71.6 cattle per sq. km of land (Global livestock populations 2020). In a country like India, which has also become the highest antibiotic consumer in recent years (6.3 billion defined daily doses) (Klein et al. 2021; Mutua et al. 2020), limited reports related to AMR in livestock increase the concern to a great extent. Considering the above situation, it becomes pertinent to understand the pattern of antibiotic resistance in cattle manure, and its correlation, if any, with various physico-chemical and biological characteristics of manure, both culture-dependent and culture-independent approaches were adopted. Properties, viz., electrical conductivity (EC), pH, total nitrogen (N), total phosphorus (P), and total potassium (K), of the manure samples were analyzed using the standard methods (Moore 1968; Rana et al. 2014; Wenscher et al. 2015). To target the total bacterial abundance, DNA was extracted from the manure samples using FastPrep-24™ bead beater (MP Biomedicals, USA) for bead homogenization and lysis, and cetyl trimethylammonium bromide (CTAB) was used as extraction buffer (Sharma et al. 2012). The V3-V4 hypervariable region of the 16S rRNA gene was used as a marker for quantification by qPCR using CFX96TMReal-Time PCR C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) (López-Gutiérrez et al. 2004). All experimental analyses were performed in triplicates. One-way analysis of variance (ANOVA) was performed with SPSS statistical system (SPSS 20.0 for windows) to determine significant changes in the attributes between the manure samples from different locations. For a comparison between the values for the 50 locations, multiple Tukey’s HSD was performed at a significance level of $P < 0.05$ (Little and Hills 1978).

2) Antimicrobial resistance profiling of manure samples

For profiling of antibiotic resistance of manure samples, eight antibiotics were selected for disc diffusion assay (Davis and Stout 1971). These included ampicillin (AMP 10 mcg), azithromycin (AZM 30 mcg), chloramphenicol (CHL 30 mcg), clindamycin (CLI 2 mcg), doxycycline (DOX 30 mcg), nalidixic acid (NAL 30 mcg), norfloxacin (NOR 30 mcg), and sulfadiazine (SUL 100 mcg). One gram of manure sample was serially diluted, followed by plating of 100 μL of the final dilution on nutrient agar medium containing fluconazole (50 mg L$^{-1}$) as antifungal agent (Kunova and Piecková 2002). Antibiotic discs were placed on plates and then incubated for 2 days at 30°C. Subsequently, the plates were examined for the zone of inhibition, to determine the antibiotic sensitivity of the bacterial load.

Materials and methods

1) Sampling and characterization of manure samples

Cattle manure samples were collected in duplicate from 25 different geographical locations in Northern and Central India (Bundelkhand region) (Supplementary Table 1, Supplementary Figure 1). Out of the 50 manure samples thus collected, 18 samples were from intensive farming sites and 32 were from natural grazing sites. To gain an in-depth understanding of the pattern of antibiotic resistance in cattle manure, and its correlation, if any, with various physico-chemical and biological characteristics of manure, both culture-dependent and culture-independent approaches were adopted. Properties, viz., electrical conductivity (EC), pH, total nitrogen (N), total phosphorus (P), and total potassium (K), of the manure samples were analyzed using the standard methods (Moore 1968; Rana et al. 2014; Wenscher et al. 2015). To target the total bacterial abundance, DNA was extracted from the manure samples using FastPrep-24™ bead beater (MP Biomedicals, USA) for bead homogenization and lysis, and cetyl trimethylammonium bromide (CTAB) was used as extraction buffer (Sharma et al. 2012). The V3-V4 hypervariable region of the 16S rRNA gene was used as a marker for quantification by qPCR using CFX96TMReal-Time PCR C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) (López-Gutiérrez et al. 2004). All experimental analyses were performed in triplicates. One-way analysis of variance (ANOVA) was performed with SPSS statistical system (SPSS 20.0 for windows) to determine significant changes in the attributes between the manure samples from different locations. For a comparison between the values for the 50 locations, multiple Tukey’s HSD was performed at a significance level of $P < 0.05$ (Little and Hills 1978).
Results and discussion

Manure samples were observed to be rich in nutrients with varying concentrations across the sampling locations (Supplementary Table 2). In the manure samples, the total nitrogen, phosphorus, and potassium contents ranged between 1.5–36, 2–95, and 5.2–20 g kg⁻¹, respectively. Several studies around the globe have reported the application of manure for building nutrient (N, P, K) status in soil (Schlegel et al. 2017; Ozlu et al. 2019; Chatzistathis et al. 2020). Apart from the quantification of major nutrients, pH and EC were measured as both play crucial roles in affecting the nutrient concentrations and microbial activity (Azeez and van Averbeke 2012; Hill et al. 2021). The pH of the manure samples varied from acidic to basic (5.79–9.05), while electrical conductivity was found to be in the range of 0.01–0.06 dS m⁻¹. Distinct differences have been reported in the pH and EC values upon comparison of different types of manure, viz., poultry, cattle, and goat, and it is believed to reflect upon the differences in animal feeding practices (Azeez and van Averbeke 2012). Also, a strong connection has been found among the type of nutrients present and bacterial community dynamics by Zhang et al. (2017). 16S rRNA gene copies in the manure samples ranged from 5.58 × 10⁸ to 2.04 × 10¹¹ gene copy number per gram dry manure (Supplementary Table 2). Pearson correlation (r) between bacterial abundance and the nutrient concentrations revealed significant correlation with nitrogen (r=0.48, P=0.01), phosphorus (r=0.33, P=0.05), and potassium (r=0.32, P=0.05) (Table 1). The pH (r=0.04) of manure samples did not show any significant correlation with bacterial abundance, whereas a significant negative relationship was seen with EC (r=−0.31, P=0.05).

The result of the disc diffusion assay showed diverse resistance profiles of the bacterial community present in manure samples (Figure 1, Table 2). Most of the manure samples exhibited resistance against multiple antibiotics. Out of the 50 samples, only nine samples, viz., S1, S7, S18, S19, S23, S24, S26, S34, and S36, were found to be resistant against only a single antibiotic, viz., sulfadiazine, clindamycin, nalidixic acid, ampicillin, and norfloxacin. Cultivable bacteria from manure samples showed the maximum percentage of resistance against clindamycin (24%) followed by nalidixic acid (22%), ampicillin (14%), sulfadiazine (13%), norfloxacin (10%), doxycycline (9%), chloramphenicol (5%), and azithromycin (3%).

Clindamycin belongs to the macrolide group of antibiotics and is one of the most commonly administered as veterinary medicines (Wang et al. 2016). A spike of clindamycin-resistant coliforms was reported in the soil after the application of manure (Wind et al. 2018). Clindamycin was observed to enrich the cultivable Proteobacteria on the surface of tomatoes (Lee et al. 2016). Nalidixic acid belongs to the quinolone group of antibiotics and has been popularly used in veterinary consumption for treating intestinal infections in livestock (Boothe 2015). Quinolones have been reported to be persistent in manure with dissipation time (DT₉₀) ranging from 100 to 5800 days (Berendsen et al. 2018). In a recent study, nalidixic acid–resistant E. coli was found in beef cattle farmyard manure, which contributed to 1.2% of the total resistant isolates (Huygens et al. 2021). In the current study, the cultivable bacteria present in the manure samples showed significant resistance (22%) against nalidixic acid. Out of the total AMR recorded in manure samples, ampicillin and sulfadiazine resistance contributed to 14% and 13%, respectively. Sulfonamides are widely present in human and animal wastes (Adekanmbi et al. 2020; Huygens et al. 2021). A recent study reported that 75% of sulfonamide-resistant bacteria were found to be resistant to ampicillin (Adekanmbi et al. 2020), which is in accordance with the data obtained in the present study. Pearson correlation (r) was drawn among profiles of antibiotic resistance bacteria (ARB), physico-chemical parameters, and bacterial abundance in the manure samples (Table 1).

AMR in manure samples showed a significant positive correlation with nitrogen and phosphorus content of the manure (r=0.69 and r=0.54, respectively, P=0.01). The

| Parameters | N | P | K | pH | EC | 16S RNA gene copies | AMR |
|------------|---|---|---|----|----|---------------------|-----|
| N          | 1 |  0.558** |  0.330* |  0.181 |  0.185 |  0.486** |  0.690** |
| P          | 0.558** | 1 |  0.225 |  0.160 |  0.358* |  0.329* |  0.543** |
| K          | 0.330* | 0.225 | 1 |  0.062 | -0.080 |  0.320* |  0.283* |
| pH         | 0.181 | 0.160 | 0.062 |  1 |  0.256 |  0.040 |  0.099 |
| EC         | 0.185 | 0.358* | -0.080 |  0.256 |  1 | -0.312* | -0.034 |
| Total bacterial abundance | 0.486** | 0.329* |  0.320* |  0.040 | -0.312* |  1 |  0.629** |
| ARB        | 0.690** | 0.543** |  0.283* |  0.099 | -0.034 |  0.629** |  1 |

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). N nitrogen, P phosphorus, K potassium, EC electrical conductivity, AMR antimicrobial resistance profiles of manure.
The correlation coefficient between AMR profiles of manure and potassium was $r=0.28$ ($P=0.05$). The pH profiles of manure did not correlate with AMR ($r=0.09$), while a negative relationship was seen in the case of electrical conductivity ($r=-0.034$). pH of manure is dependent on the integral composition and nature of the supplements given to livestock (Lin et al. 2020). Lin et al. (2020) reported the effect of acidic pH in the reduction of sulfonamide-resistant bacteria. However, the fate of AMR in manure was unclear. In the current study, no such significant correlation was found between antibiotic resistance and the pH of the manure samples. The 16S rRNA gene copy number in the manure samples showed a significant positive correlation with antibiotic resistance profiles of manure ($r=0.63$, $P=0.01$). However, in the study, the antibiotic resistance profiles generated using cultivation-dependent approach represents only a small fraction of the total bacterial community that are culturable (Hofer 2018). Positive correlations have been reported between the abundance of 16S rRNA gene copies and antibiotic resistance in manure-amended soil using both cultivable and uncultivable methods (Udikovic-Kolic et al. 2014; McKinney et al. 2018). Moreover, manure treatment has been found to specifically enhance the bla_{CEP-04} gene copies, as estimated by qPCR. Manure has the potential...
for the emergence and proliferation of antibiotic resistance in native microbiota (Yuan et al. 2019; Chen et al. 2016; Zhang et al. 2019). Udikovic-Kolic et al. (2014) described the presence of *Pseudomonas* and *Janthinobacterium* spp. as a major component of manure microbiota containing β-lactamase genes. *Janthinobacterium* belongs to the order *Burkholderiales* that uses ammonium as the sole nitrogen source (Gillis and Logan 2015). This is in accordance with the present study as positive correlations were observed between nutrients present in the manure and the population of antibiotic-resistant bacteria in the manure.

Principal component analysis of antibiotic resistance profiles of manure reflected clustering based on the cattle feeding practices (Figure 2). Samples collected from natural grazing animals were clustered together, whereas those from intensive farming zones were grouped separately, though some outliers clustered differently as they shared similar antibiotic resistance profiles. Similar correlation was reported in a study where swine under artificial feeding resulted in higher antibiotic residues in manure as compared to natural feed (Pan et al. 2011).

**Conclusions**

The present study attempted to comprehend the patterns of antimicrobial resistance prevalent in manure samples collected from different geographical locations of north and central India. A positive correlation was observed between the AMR profiles and nutrient concentrations of manure samples. A strong impact of feeding strategies was also evident on AMR profiles. This outcome serves as a basis for carrying out future studies in surveillance and in policy-making for the use of important veterinary antibiotics. However, the study was conducted with cattle manure collected from selected regions of India. Further studies are needed to comprehend the antibiotic footprints in the agricultural and veterinary sectors across different regions of the country and beyond.

![Component Plot in Rotated Space](image)
Supplementary Information  The online version contains supplementary material available at https://doi.org/10.1007/s11356-021-16700-6.

Author contribution  VLS performed the experiments and wrote the first draft of the manuscript, AKC procured the samples, VLS and PH analyzed the data, AKC and SS procured the funding and designed the experiments, and SS and PH supervised the study; all authors reviewed and approved the manuscript.

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Data availability  All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate  Not applicable

Consent for publication  Not applicable

Competing interests  The authors declare no competing interests.

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