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DETERMINATION OF ANTIMICROBIAL DRUG RESIDUES AND THE ROLE OF BACTERIOPHAGES IN FALSE POSITIVE MICROBIAL RESIDUE DETECTION TEST IN MILK IN KADUNA STATE, NIGERIA

OGALA O., KABIR J., LAWAN M.K., MORU N.H., MISHRA V.K., ABARI J.A.

Ahmadu Bello University, Faculty of Veterinary Medicine, 810106, Samara, Zaria, Kaduna State, Nigeria.

Abstract: Antimicrobial residue in milk causes financial losses to dairy farmers due to milk rejection or low pricing. Detection of antimicrobial residues can be a bit challenging due to the presence of bacteriophages in milk, which could cause false-positive results. The aim of this study was to determine the occurrence of antimicrobial residues and lytic bacteriophage in fresh bulk cow milk from peri-urban dairy centers and the influence of phages on antimicrobial residue detection in milk. Antimicrobial residues in fresh bulk cow milk were determined using the standard microbiological methods, the disc diffusion assay and the Delvotest Sp-Nt. The plaque assay method was used for the isolation of lytic bacteriophages. Out of the overall milk sample of 204, antimicrobial residues were detected in 43.6% and 42.2% of the samples using the Delvotest and the disc diffusion tests, respectively. The inter-rater reliability (Kappa) for rating the performance between the Delvotest SP-Nt and Disc diffusion assay test was found to be 0.890 and statistically significant (P-value = 0.00). Lytic bacteriophages were isolated from 30% of all milk samples collected (204 fresh bulk cow milk samples). Ten percent of the antimicrobial positive milk samples were confirmed to be false positives after conducting the two tests in parallel. The false-positive samples were confirmed for the presence of lytic bacteriophage, and 55.5% were found to be contaminated with lytic bacteriophage. Thus, the overall prevalence of antimicrobial residues recorded in this study using the disc diffusion test exceeded the maximum residue levels. Raw milk harbors natural inhibitory agents such as bacteriophages, which often cause the misinterpretation of antibiotic contamination in milk during antimicrobial detection since tests are not specific on the types of antimicrobial residues. It is recommended that more advanced detection kits be used for antimicrobial detection to limit the levels of false positives.

Keywords: Antimicrobial, residue, bacteriophage, cow

Introduction

Milk and milk products are important sources of animal protein which contribute greatly to human growth and development worldwide (43). Milk contains all the essential food constituents for all age groups and meets the nutritional needs of the body better than any single food (29). Fermented dairy products, such as cheese, kefir, cultured buttermilk, cultured cream, koumiss, Kindirmo, Nono, Warankasi and yoghurt, are products of high value known as functional food and widely consumed due to their positive health impact. Fermented milk are known to represents a distinct food culture in every community in the world, symbolizing the heritage and socio-cultural aspects of the people (39).

Starter cultures are those microorganisms (bacteria, yeasts, and molds or their combinations) that initiate and carry out the desired fermentation essential in manufacturing cheese and other fermented dairy products (40). Lactic acid bacteria (LAB) starters can be affected by many factors, such as temperature, pH, strain capability, growth medium, antibiotics and bacteriophage (2).The growth and activity of this starter cultures in milk are unfavorably influenced because of the presence of residual antibiotics and sanitizers in milk. Additionally, the production of antibiotic-like substances (bacteriocins) by certain wild strains of *Lactococcus lactis* subsp. *lactis* and other lactic cultures in raw milk also contributes (12).

In dairy animals, mastitis is the most reason for antibiotics use (28, 32). Antibiotic residues in milk exceeding tolerance levels not only present potential public health risks when consumed, but also interfere with fermentation processes during milk processing (4). Antibiotic residues in animal derived foods in many African countries have mostly been found to exceed the WHO maximum residue levels (11). Though, common antimicrobial residues detection methods are qualitative, thus, having a drawback. The limitation of these methods is inability to specify types of antimicrobial residues that can inhibit starter culture i.e., bacteriocins and bacteriophage, this can result in false positive microbial residues detection.

Bacteriophages are viruses that infect and kill bacteria without any negative effect on both human and animal host, and they are ubiquitous in nature (14). Bacteriophage infection of dairy starter cultures remains one of the causes of fermentation failures in the dairy industry and can lead to substantial economic losses due to manufacturing delays, waste of ingredients, lower quality product, or even total production loss (34). It is, therefore, crucial to identify the sources or reservoirs of bacteriophages within a factory in order to introduce corrective action to limit their propagation (38). The presence of bacteriophages in milk can also makes detection of antimicrobial residues a bit challenging leading to a false-positive results.

Accordingly, the present study was to determine the occurrence of antimicrobial residues and lytic bacteriophage in fresh bulk cow milk and their influence on false positive results in microbial residues detection tests in milk, using Delvotest® Sp-Nt kit and disc diffusion assay and to compare their performance of the two methods.

Material and method

Study Area: The study was conducted in the peri-urban dairy centers in Kaduna State.it is located in the northern Guinea Savannah Zone of Nigeria and lies between Latitude 09^0 02'N and $11^0$32'N and between Longitude 06^0 15'E and 08^0 38'E. It is made up of twenty three local Government areas and is characterized by rain-fed agricultural activity which spans from May to October and irrigation farming in the dry season which occurs between November and April. It has a tropical climate with mean and annual rainfall of 1092.8mm and a monthly mean temperature which ranges between 13.8°c to 36.7°c (1).

Sample Collection: A total of 204 fresh bulk cow milk samples were collected. About 100mls of fresh bulk cow milk sample from each milk churn were collected from the peri-urban dairy centers. The fresh bulk cow milk were sampled once weekly between November to January from two Peri-urban dairy centers in Kaduna State, Nigeria. The samples were collected using sterile universal bottles and transported on ice to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, for laboratory analysis.

Disc Diffusion Assay Method: This was done using the method described by Kabir (16). The presence of antimicrobial residues in the milk sample was indicated by the presence of inhibition zone of the growth of *Bacillus subtilis* around the disc while absence of antimicrobial residues was indicated by the absence of inhibition zone growth around the disc.

Delvotest®SP- NT Method: The Delvotest SP-NT kit was used as described by the manufacturer (DSM, Netherlands).

Bacteriophage Isolation: Bacteriophages isolation from raw milk samples were done as described by Clokie and Kropinski. (10). Briefly, 5ml of each raw milk sample was centrifuged at 2,000rpm for 5minutes. Each supernatant was filtered through a 0.22µm sterile syringe membrane filter in to a sterile test tube. Then, 2.5mls of each filtrate were inoculated in 5ml of Luria Bertani broth and 0.lml of an overnight culture of *Bacillus substilis.* The tube was incubated at 37°c for 24hr. After 24hr of incubation, the content of the tubes was centrifuged at 2,000rpm for 5minutes. Then, each supernatant was pipetted into a new sterile test tube and 0.5ml of chloroform was added, then, filtered into a sterile test tube. Each filtrate was considered to be a clean source of phage and stored at 4°C for further analysis.

Plaque Assay: 10µl of bacteriophage filtrate sample was transferred into the test tube using micropipette and allowed to sit undisturbed for 5-10 minutes. Two Petri dishes were labeled for each sample; one and the other as control. Eight milliliters of Luria Bertani broth was pipetted and dispensed into the phage test tube and 90µl of $CaCl_2$ and 4.5ml agar solution were added using micropipette and pipette respectively. This was mixed and poured into the Petri dish labeled as test and allowed to solidify. Five milliliters agar solution was dispensed into the bottom of the Petri dish labeled as control. Both petri dishes were swirled in both directions for even coating of the agar and incubated at 37^0C for 24 hours.

Plaque Picking Method: each plaque was picked using a Pasteur pipette into a 100µl of bacteriophage buffer (1mM$CaCl_2$) in a labeled test tube. The solution in test tube was mixed well by vortexing for thirty seconds. The resulting solution containing bacteriophage sample was re-plated and incubated at 37^0C for 24 hours.

Statistical Analysis: Statistical Package for Social Science (SPSS) version 20.0 was used for data analysis. The correlation between Disc diffusion assay and Delvotest® was determined using kappa statistic. Prevalence was calculated using the formula:

$$\text{Prevalence}(\%) = \frac{\text{Number of positive samples}}{\text{Total samples collected}} \text{X} \quad 100$$

Results and discussions

The present study was undertaken to determine the presence of antimicrobial residues in fresh bulk cow milk samples from the peri-urban dairy centers in Kaduna State, Nigeria. Out of the 204 fresh bulk cow milk samples tested (Table 1) for antimicrobial residues, 43.6% had antimicrobial residues using Delvotest Sp –Nt kit, this was higher than (14.7%) report by Muhammad *et al*., (24) in Zaria, Kaduna State. Antimicrobial residues found in this study was also above the 33.8% reported by Salman *et al*., (33) in Sudan, 21 % in Kenya (36), 14% in Iran, (22), 13% in Uganda (35), and 3.1% in Ghana (3).

Table1

Distribution of Antimicrobial Residues in Fresh Bulk Cow Milk from the Peri-Urban Dairy centers in Kaduna State using Delvotest® SP-Nt Kit

Sampling location	Total number of Samples examined	Number of positive Samples (%)
PUC1	42	13 (31.0)
PUC2	162	76 (46.9)
TOTAL	204	89 (43.6)

PCU1 Peri-urban center 1, PCU2 Peri-urban center 2

Using disc diffusion assay (Fig 1), 42.2% (86) of fresh bulk cow milk samples out of 204 samples had antimicrobial residues, which is higher than the 40.8% reported by Olatoye *et al*., (26) in Oyo State and 76% in Delta state (37) who recorded high occurrence rate of antimicrobial residues in fresh and fermented (Nono) cow milk in Nigeria. Nevertheless, lower occurrence rates were reported in various studies from Kenya, Kosovo, and Iraq with a total occurrence range from 10% to 18.4% (6,7, 18,25, 27).

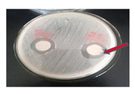

Fig.1. Inhibition zone of a positive disc diffusion assay test

The comparison between the Performance of Delvotest® and Disc Diffusion Assay Test using Cohen's Kappa statistic (K) was 0.89% (Table 2), which mean, there is almost perfect agreement between the two test methods. This finding is in line with finding of Ahmed *et al*., (5) who found 1.0% agreement between the two test in assessment of microbial loads and antibiotic residues in milk supplied in Sudan. The presence of antimicrobial residues in milk is considered as an infringement of food safety standards (42). This occurrence could be as a result of antibiotics used in dairy farming for various purposes such as treatment or prevention of numerous infectious diseases, increase milk production, increase feed efficiency, growth promotion, weight gain, and rise feed conversion ratio (9, 15, 41). The overall prevalence of 43.6% antimicrobial residues recorded in the present study which has exceeded the maximum residue levels by WHO, indicates that consumption of such milk products can be sources of potential risk to the health of the consumers, as well as interfering with fermentation processes, during milk processing (4). This problem could be due to the lack of awareness of dairy farmers on withdrawal periods of drugs among other factors.

Table 2

Comparison between the Performance of Delvotest® and Microbial Inhibition Test in Detection of Antimicrobial Residues in Cow Milk

Type of test	Total number of samples examined	Number of positive samples	Prevalence (%)	Kappa statistic (k)
Disc diffusion Assay	204	86	42.2	0.890
Delvotest SP- NT	204	89	43.6	

P-value = 0.00

Using plaque assay method (Fig 2), 30% of lytic bacteriophages were isolated from 204 fresh bulk cow milk samples that were collected from the peri-urban dairy centers (Table 4). This finding is similar to that by McIntyre *et al*., (21); who isolate lytic bacteriophages from pooled bulk raw milk in New Zealand cheese plants and Madera *et al*., (20), who also reported 10% of lytic bacteriophage isolated from raw milk in Spain. The presence of lytic bacteriophages in fresh bulk cow milk in this study indicate that raw milk could be source of milk processing failure, as determined by some researchers (13,23), that 10% of fermentation failures in the dairy sector are caused by lytic bacteriophage infections.

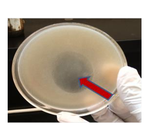

Fig 2. Positive plaque assay test, arrow indicating plaque formed

Table 4

Isolation of Lytic Bacteriophages in Fresh Bulk Cow Milk Sampled in the Peri Urban Dairy Centers in Kaduna State

Sampling location	Total Samples examined	Samples positive (%)
PUC1	42	20 (47 .6)
PUC2	162	42 (25.9)
TOTAL	204	62 (30)

PCU1 Peri-urban center 1, PCU2 Peri-urban center 2

The false positive test result in the disc diffusion assay method was found to correlate with the presence of lytic bacteriophage. Around fifty six percent of the nine false positive test results from the milk samples had lytic bacteriophages present, similar findings were reported by kang *et al*., (17) and Ahmed *et al*., (5), who recorded 2 and 3 false positive test results from raw milk samples respectively. This study has confirmed that the presences of lytic bacteriophage in raw milk which could be one of the major reason why raw milk could act as natural inhibitory agent to the test organism during microbial inhibition test because of their bactericidal nature to bacteria, hence, inhibiting the bacteria growth.

Conclusions

The overall prevalence of antimicrobial residues recorded in this study using microbial inhibition test exceeded the maximum residue levels. There is almost perfect agreement between the performance of Delvotest® Sp-Nt and disc diffussion assay in detection of antimicrobial residues in the fresh bulk cow milks, in which Cohen's Kappa statistic (K) was 0.89%. The Delvotest showed more positive test results than the disc diffusion assay, therefore, it is considered to be more sensitive than the disc assay. Raw milk harbors natural inhibitory agents such as bacteriophages, which often cause the misinterpretation of antibiotic contamination in milk during antimicrobial detection since tests are not specific on the types of antimicrobial residues..