



Original article

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Microbact™ 24E system identification and antimicrobial sensitivity pattern of bacterial flora from raw milk of apparently healthy lactating cows in Gwagwalada, Nigeria

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ABSTRACT

Objective: To determine the prevalence of bacterial organisms from raw milk of cows in Gwagwalada and determine their susceptibility to commonly used antimicrobial agents.

Methods: A total of 120 samples of milk were obtained from lactating cows that were at different stages of postpartum from six different locations in Gwagwalada metropolis. Samples were subjected to Microbact™ 24E system identification, isolation and characterization of isolates, and antibiotics susceptibility test.

Results: The most prevalent organisms were *Staphylococcus aureus* (34.1%), *Escherichia coli* (27.3%) and *Bacillus* species (18.2%) while the least isolated were *Salmonella* species (11.4%) and *Pseudomonas aeruginosa* (9.0%). The highest resistance patterns were shown by *Staphylococcus aureus* which displayed resistance to five drugs: amoxicillin, ampiclox, levofloxacin, norfloxacin, chloramphenicol and streptomycin. The least resistance was displayed by *Bacillus* species which were resistant to only two drugs, norfloxacin and chloramphenicol. *Pseudomonas aeruginosa* dissipated the highest pattern susceptibility to ciprofloxacin, norfloxacin, gentamicin and streptomycin while *Salmonella* species showed the lowest pattern of susceptibility to ciprofloxacin only. Other microorganisms dissipated susceptibility patterns ranging from 16.6%–100.0%.

Conclusions: This study documented the occurrence of bacterial flora in raw milk of apparently healthy lactating cows in the Gwagwalada area. The variation in patterns of multidrug resistance and susceptibilities in our studies may lead to possibility of transfer of antibiotic resistance from raw milk consumers. More studies are required using higher molecular techniques to expose different species of microorganisms causing milk borne illness and their antibiotic resistant genes.

1. Introduction

Milk is a complex biochemical fluid synthesized in specialized cells of the mammary gland of mammals which functions as the major nourishment of infant offspring from which milk is derived. It is also used as an agricultural product for humans. Over the years, milk and milk products constitute important nutritional components for human diet and play a prominent role in human nutrition[1]. Milk is a colloid of butterfat globules within a water-based fluid that contains dissolved carbohydrates and protein aggregates with minerals[2]. Bacteria are unicellular, mostly cell-walled microorganisms living in symbiotic and parasitic relationships with plants and animals. They are widely distributed in nature and may be introduced into milk from several sources and unhealthy practices. Bacterial contamination can generally occur from three main sources[3]: from within the udder, from the exterior of the udder, and from the surface of milk handling

and storage equipment. Consequently, a broad spectrum of bacteria such as *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Micrococcus* species, *Salmonella* species, *Pseudomonas* species, *Enterobacter* species, *Klebsiella* species, *Listeria monocytogenes*, *Brucella* species, *Proteus* species and *Yersinia enterocolitica* have been recovered from raw milk[4-7] and these organisms have been determined to be potentially pathogenic and toxicogenic, and implicated in milk-borne illnesses including gastroenteritis[6]. Furthermore, the organisms frequently isolated in milk are bacteria and many bacteria organisms tend to develop a resistance, and become less treatable with one or more antimicrobial medications previously used to treat or prevent infection[8]. The discovery of antimicrobial agents had a major impact on the rate of survival from infections. However, the changing patterns of antimicrobial resistance caused a demand for new antibacterial agents. Antimicrobial resistance is a well-known clinical and public health problem[9,10]. The effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections[11]. Antimicrobial resistance is described as a phenomenon in which microbes are less treatable with one or more antimicrobial medication. This can occur in three ways, by natural resistance in certain types of bacteria, by

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genetic mutation, or by one species acquiring resistance from another. Bacteria can acquire antibiotic resistance either by mutation or through exchange of genetic material among same or closely related species. The sudden acquisition of resistance to antibiotics poses difficulties in treating infections. Resistance can occur spontaneously via mutation, to a buildup of resistance over a period of time, or to misuse of antimicrobial agents (especially as feed additives)[10]. This study helped in providing knowledge to epidemiology of bacteria organisms prevalent in milk from cattle in Gwagwalada. It also exposes drug use and their susceptibility patterns to common antibiotics. This will assist in controlling the challenges of antibiotic therapy in Nigeria.

2. Materials and methods

2.1. Experimental design and sample collection

A total of 120 samples of milk were obtained from lactating cows that were at different stages of postpartum from six different locations in Gwagwalada metropolis. The cow was properly restrained and the udder was cleaned with a sterilized damp cloth, after which the teat of the udder was held gently and squeezed to express about 5–10 mL of milk unto clean bijou bottles. They were arranged in Ice Park cooler and transported immediately to the Microbiology Central Laboratory, University of Abuja for analysis. The samples were collected for a period of 6 weeks from May to July, 2016. The locations for the sample collection sites were designated as follows: Tunganmaje was designated as Area A, Dukpa as Area B, Paiko town as Area C, Gwako town as Area D, Kutunku as Area E and Baure as Area F.

2.2. Isolation and characterization of isolates

2½ mL of milk sample was added to 5 mL sterile TSB (tryptone soy both containing 6.5% NaCl as enrichment) in bijou bottles, mixed thoroughly and incubated overnight at 37 °C. A loopful of the broth was inoculated at 37 °C for 24 h and sub-cultured onto prepared nutrient agar, blood agar and MacConkey agar (LAB-M) Oxoid, UK plates to obtain discrete colonies. The suspected colonies were Gram stained and tested for catalase as described by Cheesbrough[5]. On mannitol salt agar, colonies that appeared yellowish were presumptively identified as *S. aureus*, eosin methylene blue agar was used for *E. coli*, *Salmonella-Shigella* agar was used for isolation of *Salmonella* while MacConkey agar for isolation of other members of the Enterobacteriaceae. Colourless colonies with black spots on *Salmonella-Shigella* agar were identified as *Salmonella* species[12-14]. The isolates were subsequently confirmed using the commercially prepared biochemical test kits (Microbact Oxoid). The Microbact™ identification kits (Oxoid) were inoculated as described by the manufacturer. The following biochemical test reactions were used: oxidase, catalase, coagulase, Gram staining, lysine, ornithine, H₂S, glucose, mannitol, hylose, indole, urease, VP, citrate, gelatin, inositol, sorbitol, rhamnase, sucrose, lactose, arabinose, adonitol, raffinose, salicin, arginine and nitrate. The results obtained were interpreted to identify the isolates using the Microbact™ computer aided identification package (Oxoid) supplied along with the kits in combination with the Cowan and Steel's Manual for the Identification of Medical Bacteria[14,15].

2.3. Microbact™ 24E system identification

The Microbact 24E system is a new miniaturized identification system for the identification of microorganisms. The Microbact™ is a commercially used microsystem for the identification of common clinical isolates of Enterobacteriaceae and non-fermenting Gram-negative bacilli and consists of dehydrated substrates distributed in wells of microtitre trays. The specified Microbact system used for this study was: Oxoid™ Microbact™ GNB 24E System kit, manufactured

by Thermo–Fisher Scientific, Waltham, Massachusetts, USA. This system assists in the final identification of fresh isolates from fresh cow's milk; the system is easy to use and comes with complete computerized profile registers to assist with final identification of the isolates. This system proves to be very accurate and convenient in the identification of microorganisms.

2.4. Antibiotics susceptibility test

An overnight culture of each isolate was prepared on nutrient broth and incubated at 37 °C for 18 h. Dry sterile plates of Mueller-Hinton's agar (Oxoid, UK) were prepared and each of the isolates was uniformly and aseptically inoculated into the Mueller-Hinton agar plates. The appropriate antibiotic multi-discs were aseptically placed on the agar with the standardized inoculums of 18 h culture test bacteria isolate using sterile forceps[14]. The plates were then incubated at 37 °C for 24 h. Antibiotics impregnated disc (from OPTUDISC) used included ciprofloxacin (10 µg), norfloxacin (10 µg), gentamycin (10 µg), amoxicillin (20 µg), streptomycin (30 µg), erythromycin (30 µg), rifampicin (20 µg), chloramphenicol (30 µg), levofloxacin (20 µg), ampiclox (20 µg). After incubation, the zones of inhibition were measured to the nearest millimeter using a transparent ruler and the values were recorded[10,16]. The results were expressed as susceptible/resistant according to criteria developed by using Manual of Antimicrobial Susceptibility Testing guidelines[17] and the Clinical and Laboratory Standards Institute guidelines[18].

3. Results

Table 1 shows the results of the distribution of microorganisms from milk based on cultural and biochemical characteristics which yielded an overall prevalence of 36.7%. The highest prevalence was in week 6 with a prevalence of 55.0%. The lowest prevalence is in week 1 with a prevalence of 25.0%.

Table 1

Weekly distribution of microorganisms from milk.

Weeks	Samples collected	No. Positive	Positive (%)
Weeks 1	20	5	25.0
Weeks 2	20	8	40.0
Weeks 3	20	7	35.0
Weeks 4	20	6	30.0
Weeks 5	20	7	35.0
Weeks 6	20	11	55.0
Total	120	44	36.7

Table 2 shows the frequency of isolation of microorganisms from milk samples. Organisms isolated during the study *S. aureus*, *Bacillus* species, *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Salmonella* species. The most prevalent organism was *S. aureus* accounting for 15 of the 44 isolates (34.1%), 12 of the isolates turned out to be *E. coli* (27.3%), 8 were *Bacillus* species (18.2%) in that descending order.

Table 2

Frequency of isolation of bacterial organism from milk samples.

Organism	No. of isolates	Prevalence (%)
<i>Bacillus</i> species	8	18.2
<i>E. coli</i>	12	27.3
<i>P. aeruginosa</i>	4	9.1
<i>Salmonella</i> species	5	11.4
<i>S. aureus</i>	15	34.1
Total	44	100.0

Table 3 shows the dissipation of antimicrobial resistance to the commonly used antimicrobial agents. *Bacillus* species isolated were resistant to chloramphenicol and norfloxacin while the organism was sensitive to all the other antimicrobial agents used in the study. *E. coli* showed resistance to five antimicrobial agents including amoxicillin,

ampiclox, ciprofloxacin, erythromycin, and rifampicin. Isolated *Pseudomonas* species showed resistance to amoxicillin, ampiclox, levofloxacin, and rifampicin, while sensitive to other antimicrobial agents used in the study. *Salmonella* species isolated were resistant to ampiclox, rifampicin, levofloxacin, gentamycin and chloramphenicol while *S. aureus* was resistant to amoxicillin, ampiclox, levofloxacin, norfloxacin, streptomycin and chloramphenicol but sensitive to other antimicrobial agents used in the study.

Table 3

Bacterial resistance and sensitivity of microorganisms to antimicrobial agents

Antibiotics	<i>Bacillus</i> spp.	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>Salmonella</i> spp.	<i>S. aureus</i>
Amoxicillin	S	R	R	S	R
Ampiclox	S	R	R	R	R
Ciprofloxacin	S	R	S	S	S
Erythromycin	S	R	S	S	S
Levofloxacin	S	S	R	R	R
Norfloxacin	R	S	S	S	R
Rifampicin	S	R	R	R	S
Gentamycin	S	S	S	R	S
Chloramphenicol	R	S	S	R	R
Streptomycin	S	S	S	S	R

S: Sensitive; R: Resistance.

Table 4 shows the susceptibility pattern dissipated by the various antimicrobial agents. *Bacillus* species showed 100.0% susceptibility to amoxicillin and gentamicin. *E. coli* dissipated 100.0% susceptibility to levofloxacin, gentamicin and streptomycin while *P. aeruginosa* showed 100.0% susceptibility to ciprofloxacin, norfloxacin, gentamicin and streptomycin. *Salmonella* species dissipated 100.0% susceptibility to only ciprofloxacin. Finally, *S. aureus* showed 100.0% susceptibility to ciprofloxacin and rifampicin. All the isolates showed varying susceptibility patterns ranging from 16.6% to 87.5%.

Table 4

Susceptibility patterns of bacterial isolates to antimicrobial agents.

Antibiotics	<i>Bacillus</i> spp.	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>Salmonella</i> spp.	<i>S. aureus</i>
Amoxicillin	100.0	33.3	0.0	60.0	20.0
Ampiclox	62.5	0.0	0.0	40.0	26.7
Ciprofloxacin	50.0	16.6	100.0	100.0	100.0
Erythromycin	87.5	25.0	50.0	80.0	66.7
Levofloxacin	100.0	100.0	0.0	20.0	0.0
Norfloxacin	50.0	0.0	100.0	60.0	0.0
Rifampicin	62.5	16.6	0.0	0.0	100.0
Gentamycin	100.0	100.0	100.0	0.0	60.0
Chloramphenicol	0.0	60.0	50.0	40.0	20.0
Streptomycin	87.5	100.0	100.0	60.0	0.0

4. Discussion

It has been reported that the unclean hands of workers, poor quality of milk, unhygienic conditions of the manufacturing unit and water supplied for washing the utensils could be the source for accelerating bacterial contamination of milk products beside the post manufacturing contamination[19]. The prevalence of the isolated bacteria observed in this study could be due to the fact that milk being a good nutritive medium enhanced the growth of bacteria contaminant in the milk investigated[20,21]. Similar bacteria isolates from milk and products have been previously reported from milk products[22,23]. The high prevalence of *S. aureus* in the milk sample may attributed to the high frequency of mastitis in the sampled cows. The isolation of *S. aureus* is of public health importance because of its ability to produce potent enterotoxins which cause a wide range of infections in humans. Isolation of *S. aureus* from milk is therefore very significant[24]. *S. aureus* are part of normal flora, and are primarily found in the nose and skin[23]. It had also been found to cause mastitis in dairy cows. *S. aureus* have been commonly isolated in diary and meat products, and its isolations from the milk samples are not unexpected. However, several studies showed that *S. aureus* can be transmitted between

man and dairy cattle, thus posing a public health risk to consumers of such contaminated dairy products. *S. aureus* had been associated with community acquired infections in humans as well as skin abscesses in milk producing animals. It is also a major cause of gastroenteritis resulting from consumption of raw milk and other dairy products could result in outbreaks of food poisoning among human populations.

Bacillus species were frequently isolated from the raw milk samples examined with an isolation rate of 18.2%. *Bacillus* species are commonly found in soil, water, decaying vegetable matter, dust and air as commensals and they are common contaminants of open wound and contribute to food spoilage. This may be due to the ubiquitous nature of *Bacillus* spores that are well known for rapid dissemination and ability to resist heat and are able to survive for a longer periods of time in hostile environment. The *Bacillus* spores multiplies rapidly at room temperature and survives on dry surfaces, leading to highly contagious and fatal disease known as anthrax[24]. The ability of *Bacillus* species to form spores permits the organism ample opportunity to survive environmental factors, hence it has high pathogenicity potential.

Pseudomonas is a major laboratory contaminant found normally in the environment such soil, water and is commonly involved in milk spoilage[20]. These fermentative bacteria are chemo-organotrophic and microaerophilic. They are non-spore forming and lack motility and are commonly used to ferment food and as probiotics.

E. coli had the prevalence rate of 18.2%. *E. coli* is a normal commensal enteric flora inhabiting the GIT of man and animals and thus, faecal materials of humans or other animals may contaminate pasture in the environment, releasing chums of the pathogenic bacteria. This, coupled with the free range system of cattle production under poor and unhygienic conditions may be responsible for the transmission of pathogenic strains of the organism to man. *E. coli* infection may be responsible for severe gastrointestinal and diarrheal symptoms leading to subsequent dehydration in animals and humans especially immunocompromised infants[25].

Salmonella species constituted 11.4% of the organisms isolated from the milk of cattle. The prevalence of *Salmonella* species indicated in our study is as high as 11.4% which concurs with *Salmonella* organisms that are well known to be ubiquitous in the environment, more so, they are also known to inhabit the gastrointestinal tract of cattle, and may be discharged through the milk, causing variety of diseases such as food poisoning and typhoid fever in man. Also, unhygienic practices during milking such as contaminated utensils, and water could be responsible for this prevalence[26]. The implication of this finding is that man stands risk of contracting infection via close contact, handling or processing or consumption of unpasteurized milk. The public needs to be enlightened as the outbreak of *Salmonella*-related diseases could lead to millions of deaths.

All the isolates had a high multiple antibiotic resistance index greater than 0.2. This is an indication of high risk sources associated with indiscriminate use of antimicrobial drugs. There was high resistance of *S. aureus* to ampicillin and oxytetracycline which are the most abused antibiotic among cattle farmers in the area of study. The development of multiple resistances in these isolates may be associated with the transfer of R-factors borne on plasmids. This therefore poses serious concern as such are capable of transfer of resistance to other more pathogenic stains in farm animals.

These results were expressed as susceptible/resistant according to criteria developed by CLSI. *S. aureus* was susceptible to 4 (40%) and resistant to 6 (60%) of 10 antibiotics used. Of the 10 antibiotics tested against *E. coli* and *P. aeruginosa*, *E. coli* was susceptible to 5 (50%) and resistant to 5 (50%). *P. aeruginosa* was susceptible to 6 (60%) and resistant to 4 (40%). The *Bacillus* species were susceptible to 8 antibiotic (80%) while resistant to 2 (20%) and *Salmonella* species were sensitive to 5 of the organisms while resistant to 5 of test antibiotics. Resistance to ampiclox was common to all isolates except *Bacillus* species. Erythromycin-resistance was common to *E. coli*. Also,

rifampicin-resistance was common to all except *Bacillus* species and *S. aureus*. Resistance to gentamicin was only common to *Salmonella* specie while streptomycin-resistance was common to *S. aureus* only. Multi-drug resistance observed in this study implies that milk serves as an important vehicle of which drug resistance could be transferred to the human population through the food chain. Appropriate measures are therefore necessary to control this menace.

However, some organisms were highly susceptible to most of the test antibiotics. It was discovered that *S. aureus* and *E. coli* were the organisms to show the highest resistance to test antibiotics. *Salmonella* species were resistant to readily administered anti-typhoid drugs amoxicillin and ampiclox as well as levofloxacin and gentamycin, but were highly sensitive to ciprofloxacin. *Bacillus* species isolated were highly sensitive to amoxicillin, levofloxacin and gentamycin (100%) but also sensitivity to erythromycin and streptomycin. The multi-drug resistance patterns displayed in our studies lead to the suggestions that these drugs were abused, misused or administered without doctor's prescription.

From the study, it was observed that raw milk consumed in Gwagwalada is contaminated with bacteria organisms with 36.7% prevalence of which 34.1% were *S. aureus*, 27.3% *E. coli*, 18.2% *Bacillus* species, 11.4% *Salmonella* species, and 9.1% *P. aeruginosa*. The most active antimicrobial agents for *E. coli* isolates were levofloxacin, gentamycin and streptomycin, while ciprofloxacin and erythromycin were the most active against *Salmonella*. *P. asaeruginosa* was susceptible to ciprofloxacin, norfloxacin, gentamycin and streptomycin. *S. aureus* isolated had the highest susceptibility to rifampicin and ciprofloxacin while the isolated *Bacillus* species had susceptibility to all the antibiotics except norfloxacin and chloramphenicol. Generally, all other organisms show differences in the display of their patterns of susceptibility and resistance to other drugs. There is a need to minimize rampant use and misuse of different antibiotics in food animals to curtail the possible emergence of resistant genes in animals which could be transmitted to the human population.

Conflict of interest statement

We declare that we have no conflict of interest.

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