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Molecular Surveillance Reveals Widespread Colonization by Carbapenemase and Methicillin Extended Spectrum Beta-Lactamase Producing Organisms in Neonatal Sepsis at Kitale County Hospital, Kenya

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Abstract

Neonatal sepsis, a major cause of death amongst infants in Kenya, is often gut derived. Gut colonisation by *Enterobacteriaceae* producing Extended Spectrum Beta-Lactamase (ESBL) or carbapenemase enzymes can lead to Antimicrobial-Resistant (AMR) or untreatable infections. The aim of this study was to determine the antimicrobial and resistant gene characterisation of bacterial pathogens among preterm neonates at Kitale County Hospital (KCH) new-born unit. This was a descriptive cross-sectional study conducted between May 2018 to June 2020 in which a total of 181 mothers who consented into the study for the sample collection from their children. Blood samples were drawn for microbial and biochemical laboratory analysis. Our molecular analysis findings reveal that the cycle thresholds for *Staphylococcus epidermidis* (3) were 28.87, 34.38 and 38.43 while *Staphylococcus warneri* and *Staphylococcus hominis* were 37.34 and 32.76 respectively. *Enterococcus faecalis* had cycle threshold of 26.76. Gram negative bacteria were further screened for bla OXA-48 and bla KPC genes with four turning positive. *Salmonella spp.* (2) had a cycle threshold of 30.61 and 30.69, *Escherichia coli* had 33.64 and *Pseudomonas aeruginosa* had 35.43. These findings provide crucial insights into multidrug resistance in neonatal sepsis among children attending KCH, thus the importance of creating awareness and emphasis on responsible antibacterial use in neonatal sepsis management and shedding light on the need to developing more potent antibiotics to manage chronic neonatal sepsis effectively.

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Introduction

Sepsis is a major cause of neonatal morbidity and mortality, with an estimated 1.7 million cases globally in 2010, and 203,000 sepsis-attributable deaths [1]. Neonatal sepsis has a higher incidence and mortality in sub-Saharan Africa than in other regions [1,2]. It is often gut derived, with compromised immunity and an impaired gut barrier allowing colonisation by opportunistic pathogens such as *Enterobacteriaceae* to progress to blood stream infection [3]. Preterm and low birth weight infants are at the greatest risk. Colonisation by Antimicrobial Resistant Organisms (AROs) is particularly problematic and can lead to infections that are difficult or impossible to treat.

Neonatal Sepsis (NS) is defined as an infant blood infection with systematic signs and symptoms of infection in the first four weeks of life [1,2]. About 2.5 million preterm neonates die before the first month of life and around 1 million new-born deaths occur daily in developing and middle-income countries. The incidence is highest in neonates and children which accounts to 47% of total infant deaths among the under-five due to preterm related complications [3,4]. NS is characterized by symptoms like respiratory distress, septicaemia, pneumonia, and meningitis [2]. It is more common in premature infants with a low birth weight [5] and has been attributed to poor neurodevelopment disorders [6].

Currently, emergence of drug and Multi-Drug Resistant (MDR) bacterial strains causing neonatal sepsis are on the increase globally despite paucity of data from developing countries including Kenya. Effective antimicrobial agents which can improve the immune system by preventing and treating neonatal sepsis such as intrapartum antibiotic prophylaxis are available; however, these measures are not documented in the guidelines for the management of neonatal sepsis [7]. Early exposure to antimicrobial agents lead to Antimicrobial Resistance due to lack of confirmatory diagnosis hence interfering with future treatment options for the infants a fact that is quite prone to developing countries [8]. This has been attributed to the wide availability of over-the-counter antibiotics, the inappropriate use of broad-spectrum antibiotics in the community and mutations that have been observed in microbes contributing to prolonged treatment which ends up increasing costs of management resulting to impairment of mental and physical development [9,10]. Globally, about 2.5 million neonates die within the first month of life in which around 1 million new-born deaths occur daily in low-income and middle-income countries [4]. For instance, in the United States the incidence of culture proven early-onset sepsis of approximately 0.3-2 per 1000 live births [2].

In another study from Eastern Europe, it was deduced that out of the 1426 neonates admitted, 107(18.9%) were diagnosed with proven sepsis in which 68(63.6%) had Early-Onset Sepsis and 37(34.6%) had Late-Onset Sepsis with 75 cases having Coagulase Negative Staphylococcus, while in Greece, incidence rate of 8.6 per 1000 live births was reported following culturing of samples proven to be from neonatal sepsis cases [11-13].

In African continent most so Kenya few studies on NS are available, therefore there is a need for this study to enhance policy marking on the early detection, management and treatment of neonatal sepsis. The current study, therefore, sought to use molecular surveillance tools to characterize antimicrobial and resistant gene of bacterial isolates obtained from preterm in new-born unit at Kitale County Hospital, in Trans Nzoia County in Western Kenya.

Materials and methods

Study site

The study was conducted in Trans Nzoia County (1.0219^0 N, 35.0015^{0}E) at Kitale County Hospital (KCH) New-born Unit (NBU). Trans Nzoia County is in Western part of Kenya as shown in Figure 1 below.

Figure 1: Map of Kenya showing Trans Nzoia County.

Study population

Inclusion criteria

Preterm neonates with a gestation age of less than 36 weeks presenting with signs and symptoms of Neonatal Sepsis (NS), admitted in the new-born unit at KCH and had consented through their parents/guardians during the study period were included to this study. NS was determined based on criteria by Lutsar *et al.,* [20] and included the presence of at least 3 out of the following four presentations:

Presence of risk factors of sepsis e. g. prematurity and chorioamnionitis.

Presence of two or more clinical signs of sepsis such as poor reflexes, lethargy, respiratory distress, bradycardia, apnea, convulsions.

Low haemoglobin (haematocrit) levels.

Abnormal core temperature (>38°C).

Exclusion criteria

Preterm neonates whose mothers did not give consent for their participation and those who were on antibiotic treatment were excluded from the study.

Sample size calculation

Sample size was determined using Cochran's formula of 1997 [21] giving a 181 as the minimum number of participants required in the study.

Study design

Descriptive cross-sectional study was conducted from May 2018 to June 2020. A total of 181 parents/guardians consented into the study and were interviewed using well-structured questionnaires to obtain data on demography and medical history. Blood samples were drawn for microbial and biochemical laboratory analysis.

Isolation and characterisation of bacterial isolates

Blood obtained from 181 neonates suspected to be suffering from NS based on criteria by Lutsar *et al.,* [20] under inclusion

criteria were immediately sent to the laboratory for culturing on Blood Agar (BA), Chocolate Blood Agar (CBA) and MacConkey Agar (MCA) using established procedures [22]. Gram staining and relevant biochemical tests were done on the growth obtained on the media for confirmation purposes of the isolates of interest using established methods as presented in our previous published work [23-25].

Antimicrobial assays (Disc diffusion assay and Minimum Inhibitory Concentration)

Disc diffusion test:

Susceptibility testing was done on the 41 isolates obtained from our previous study [25] using Kirby-Bauer disk diffusion method as used previously [26] on Muller Hinton media (Himedia, India) and incubated at 37^0C for 18 hours to evaluate the antimicrobial activity of commonly used antibiotics {Penicillin G (10 Units), Oxacillin (1µg), Gentamicin (10µg), Levofloxacin (5µg), Moxifloxacin (5µg), Clindamycin (2µg), Erythromycin (15µg), Linezolid (30µg), Tetracycline (30µg), Vancomycin (30µg), Ampicillin (10µg), Amoxicillin/ Clavulanic acid (20/10µg), Piperacillin/ Tazobactam (100/10µg), Cefazolin (30µg), Ampicillin/ Sulbactam (10/10µg), Cefuroxime (30µg), Cefepime (30µg), Cefoxitin (30µg), Aztreonam (30µg), Meropenem (10µg), Trimethoprim/Sulfamethoxazole (1.25/23.75µg), Amikacin (30µg), Gentamicin (10µg), and Ciprofloxacin (5µg)} [27]. Antimicrobial agents were chosen based on specific organism group for routine testing and reporting as per Clinical and Laboratory Standards Institute (CLSI) guidelines 32st ed. CLSI supplement M100 [26]. Already manufactured discs impregnated with antibiotics were sourced from established suppliers (Biomerieux-France) and were used in this study. The agar plate surface was inoculated with the bacteria of interest (100µl) by spreading plate method. Then the sterile discs were placed gently and aseptically on the surface of the agar with inoculated bacteria (100µl) before the agar plates were incubated under suitable conditions depending upon the test microorganism. The zone of inhibition was read and compared with the standards set for various antibiotics and microorganisms as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. CLSI performance standards for Antimicrobial Susceptibility Testing 32st ed. CLSI supplement M100 [26]. The zones of inhibition (measured in mm) around every antibiotic plate indicated the lethality of the antibiotic on the bacteria. *S. aureus* ATCC 33592 and *E. coli* ATCC 25922 were used as standard microorganisms for quality control.

Minimum inhibitory concentration (MIC):

Minimum Inhibitory Concentration (MIC) for individual antimicrobial agents were determined by Epsilometer test (E test) method which involves the dilution and diffusion of the antibiotic into the medium. Antimicrobial agent concentrations (Oxacillin, Gentamicin, Clindamycin, Erythromycin, Linezolid, Vancomycin and Tetracycline had MIC concentration range of 0.016-256 µg/ml each, while Levofloxacin and Moxifloxacin had an MIC concentration range of 0.002-32 µg/ml) were immobilized along the test strips in a continuous and exponential gradient. Drop shaped inhibition zones intersected the graded test strip at the inhibitory concentration of the antibiotics after 18 hours of incubation. The intersections of the lower parts of the ellipse shaped growth with the test strips indicated the MIC values (µg/ml). Dilutions of 0.5 MacFarland standard of individual test strains were prepared in tubes of saline, sterile cotton swabs were used to streak the prepared inoculums onto the entire surface of the agar plates (Muller Hinton) and were left for 5 minutes to allow absorption of excess moisture. The strips were applied to agar surfaces using sterile forceps while the E end of the strips were placed at the edges of the plates with the scales facing upwards. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as controls. Plates were incubated at 37^0C for 18 hours while MIC values were read at the points where ellipses intersected the scales and were interpreted as Susceptible, Intermediate and Resistant, while breakpoints values were compared with the criteria recommended by CLSI [26].

Resistance genes profiling

Screening for carbepenemase and methicillin genes from isolates responsible for neonatal sepsis:

Gram negative isolates were screened for carbepenemase genes namely: blaOXA-48, using forward primer (5´-TTACGGCCTGGGAAGTGTTC-3´) and reverse primer (5´-AAGGGATTCTCCCAAGCTGC-3´) [28]. BlaKPC using forward primer (5´-GATACCACGTTCCGTCGTG-3´) and reverse primer (5´-GCAGGTTCCGGTTTTGTCTC-3´) as used previously [29]. Gram positive isolates were screened for methicillin MecA gene using forward primer (5´-GTTGTAGTTGTCGGGTTTGG-3´) and reverse primer (5´-CCACCCAATTTGTCTGCCAGTTTCTCC-3´) as used previously [30]. Test results were interpreted as positive at the intersection between threshold line and the start of amplification curve (exponential phase) at fixed signal threshold in number of cycles. DNA that was produced in each cycle was represented by fluorophore dyes produced on amplification plot. Fluorescence was released every time a new DNA copy was synthesized; therefore, the amount of fluorescence was proportional to quantity of DNA produced.

Statistical analysis

Demographic data was analysed using IBM®SPSS® (version 21). While anti-microbial susceptibility data was analysed using World Health Organization WHONET software ®, Descriptive vs 2022 and inferential statistics were used to analyse quantitative data such as prevalence of NS. Univariate and multivariate test analyses were used according to variable characteristics at the significance level of $p \leq 0.05$.

Ethical approvals

This study was approved by the instructional research and ethical committee of MU/MTRH *FAN: IREC 3174*, The National Commission for Science, Technology and Innovation NACOSTI/P/19/80813/27724. Additional approvals were obtained from Ministry of Education Science and Technology State Department of early learning and basic Education TNZ/CNT/CDE/R.GEN/1/VOL.II/14 and from County Government of Trans Nzoia, State Department of Health CGTN/HS/COHCS/2018. Confidentiality of information obtained from the mother/ guardian was maintained.

Results

Isolated and characterised bacterial isolates from samples obtained from NS neonates

Based on our previous study findings [25], out of the 41 iso-

lates on samples obtained from neonates with NS, Gram positive bacteria were 35 (85.4%) majority and Gram negative bacteria were 6 (14.6%). From the Gram-positive isolates Coagulase Negative *Staphylococcus* (CoNS) were 31 (75.6%). Among the CoNS, *Staphylococcus epidermidis* were predominant with 19 (46.3%) followed by *Staphylococcus hemolyticus at* 5 (12.2%), *Enterococcus ssp.* 3 (7.3%), *Staphylococcus hominis, Staphylococcus lentus* and *Staphylococcus warneri* both at 2 (4.9%) and lastly *Staphylococcus saprophyticus* and *Staphylococcus aureus* both at 1 (2.4%). The Gram-negative isolates comprises of *Salmonella spp.* at 3 (7.6%) while *Escherichia coli, Pseudomonas aeruginosa* and *Acinetobacter spp* at 1 (2.4%) each [25].

Table 1: Distribution of microbial profiles.

	n	Isolates N= 41
Profile of isolates		**Percent (%)**
i. Gram positive N = 35 (85.4%)		
• *Staphylococcus aureus*	1	2.4
• *Enterococcus ssp.*	3	7.3
• Coagulase negative Staphylococcus (CoNS) N = 31 (75.6%)		
- *Staphylococcus epidermidis*	19	46.3
- *Staphylococcus haemolyticus*	5	12.2
- *Staphylococcus hominis*	2	4.9
- *Staphylococcus lentus*	2	4.9
- *Staphylococcus saprophyticus*	1	2.4
- *Staphylococcus warneri*	2	4.9
ii. Gram negative N = 6 (14.6%)		
• *Escherichia coli*	1	2.4
• *Pseudomonas aeruginosa*	1	2.4
• *Acinetobacter spp.*	1	2.4
• *Salmonella spp.*	3	7.3
Total	41	99.8

Antimicrobial susceptibility and resistant patterns of Gram-positive isolates

Out of 35 Gram positive isolates, 1(2.9%) *Staphylococcus aureus,* 3(8.6%) *Enterococcus spp.,* 19(54.3%) *Staphylococcus epidermidis,* 5(14.3%) *Staphylococcus haemolyticus*, 1(2.9%) *Staphylococcus saprophyticus, 2(5.7%) Staphylococcus hominis*, *2(5.7%) Staphylococcus lentus, and 2(5.7%) Staphylococcus warneri* were expressed to antimicrobial sensitivity test which shows susceptibility to different drugs at different zones as summarized in Table 2 below.

From the findings both Staphylococcus aureus and Staphylococcus saprophyticus shows resistant to Penicillin G producing average zones of inhibition of 6±0.577 mm and 7±0.00 mm respectively while Enterococcus spp. Shows resistant to Erythromycin and Tetracycline with an average zone of inhibition of 8.11±1.05 mm and (7.78±0.66 mm) respective. Staphylococcus epidermidis (only shows resistant to Penicillin G (8.84±2.41 mm).

All the 5(100%) Staphylococcus haemolyticus shows high multidrug resistance in which it shows resistant to Penicillin G (6.20±1.35 mm), Oxacillin (8.2±1.095 mm), Gentamicin (9.6±0.548). Erythromycin (6.8±0.837 mm) and tetracycline (6±0.00 mm), while 3(60%) were resistant to Levofloxacin, Moxifloxacin and Clindamycin. Staphylococcus hominis were

Table 2: Antimicrobial susceptibility and resistant patterns (Average Zones of inhibition-mm) of Gram-positive bacterial isolates responsible for neonatal sepsis.

		Microorganism (N=35)							
Antibiotics (Concentration used)	**Break points (mm)**	***Staphylococcus aureus (n=1)***	***Enterococcus spp (n=3)***	***S. epidermidis (n=19)***	***S. haemolyticus (n=5)***	***S. saprophyticus (n=1)***	***S. hominis (n=2)***	***S. lentus (n=2)***	***S. warneri (n=2)***
Penicillin G (10 Units)	S ≥29	6±0.577 mm(R)	NT	8.84±2.41 mm (R)	6.20±1.35mm (R)	7±0.00 mm(R)	6.5±0.548mm(R)	6.83±0.41mm (R)	8±0.894mm(R)
Oxacillin (1µg)		24±0.00 mm (S)	NT	23.33±0.58mm(S)	8.2±1.095 mm (R)	7.67±0.577mm(R)	8±0.632 mm(R)	7.17±0.75 mm (R)	7.67±0.52mm(R)
Gentamicin (10µg)	13-14	17±1 mm (S)	NT	21.09±0.70 mm(S)	9.6±0.548 mm (R)	19.67 ±0.577mm (S)	20.33±0.516mm(S)	19.3±0.58 mm(S)	20±1.169 mm(S)
Levofloxacin (5µg)	16-18	19.33±0.58 mm(S)	19.67±0.58 mm(S)	22.54±1.33 mm(S)	21.33±0.816 mm(S)	19.33±0.577 mm (S)	20 ±0.00 mm (S)	20±0.00 mm (S)	21.5±1.378 mm(S)
Moxifloxacin (5µg)	21-23	24.67±1.53mm (S)	NT	24±0.816 mm(S)	24.5±0.816 mm (S)	24.67±0.577mm (S)	24±0.00 mm (S)	23.67±0.58mm(S)	24.33±0.516mm(S)
Clindamycin (2µg)	15-20	21±0 mm (S)	NT	21.6±0.737 mm(S)	20.5±0.548 mm (S)	22±0.00 mm(S)	20.83±0.41 mm (S)	20.33±0.58mm(S)	22.6±0.516 mm(S)
Erythromycin (15µg)	14-22	23.33±1.16 mm (S)	8.11±1.05 mm(R)	25.38±0.75 mm(S)	6.8±0.837 mm (R)	24±0.00 mm (S)	24.67±0.516mm(S)	8±0.632 mm (R)	24.67±0.516mm(S)
Linezolid (30µg)	S ≥21	23.67±0.58 mm (S)	21.67±0.58 mm(S)	24.32±0.48 mm(S)	23±0.00 mm (S)	22.67±0.577 mm (S)	24±0.00 mm (S)	23±1.00mm (S)	23±0.00 mm (S)
Vancomycin (30µg)		NT	20.67 ±0.58 mm(S)	NT	NT	NT	NT	NT	NT
Tetracycline (30µg)	15-18	18±0.00 mm (S)	7.78±0.66(R)	25.25±0.96 mm(S)	6±0.00 mm (R)	25±0.00 mm (S)	24.17±0.41mm (S)	6.33±0.516 mm(R)	25.83±0.983mm(S)

NT (Not Tested), µg (Micrograms disk content), mm (millimeters), R (Resistant), S (Susceptible). Break points (mm): are as per CLSI 32nd Edition, 2023 guidelines

resistant to Penicillin G (6.5±0.548 mm) and Oxacillin (8±0.632 mm) while Staphylococcus lentus also shows resistant to Penicillin G (6.83±0.41mm), Oxacillin (7.17±0.75 mm), Erythromycin (8±0.632 mm) and tetracycline (6.33±0.516 mm).

Lastly, Staphylococcus warneri were found also to be resistant to Penicillin G (8±0.894mm) and Oxacillin (7.67±0.52 mm) as shown in Table 3 below. This clearly demonstrate that AMR is really becoming a menace in the current society as these antibiotics may not offer any medicare against the NS condition if it is caused by these pathogens.

Antimicrobial susceptibility and resistant patterns of Gram-negative isolates

From the total six Gram negative isolates isolated, the predominant was *Salmonella spp* at 3(50%) followed by *Pseudomonas aeruginosa, Escherichia coli* and *Acinetobacter spp* both at 1(10.7%) each. *Pseudomonas aeruginosa* was subjected to six antimicrobial agents and proved to be sensitive/susceptible to (Piperacillin/ Tazobactam (21.33±0.77 mm), Cefepime (24.33±0.577 mm), Meropenem (25.33±0.577 mm), Amikacin (20.67±1.155 mm), Gentamicin (17.67±0.577 mm) and Ciprofloxacin (31.67±0.577 mm). while *Escherichia coli* isolate proved to be susceptible to some antimicrobial agents bioassayed (Amoxicillin/ Clavulanic acid (17.67±0.577 mm), Piperacillin/ Tazobactam (20±0.00 mm), Cefazolin (23.33±0.577 mm), Cefuroxime (23.67±0.577 mm), Cefepime (24±0.00 mm), Cefoxitin (18.33±1.15 mm), Aztreonam (24.33±0.577 mm), Meropenem (25±0.00), Amikacin (21±1.00 mm), Gentamicin (18±0.00 mm), Ciprofloxacin (32±0.00 mm) and Trimethoprim/ Sulfamethoxazole (16.67±0.577). *Salmonella* spp. shows susceptible to Ampicillin (16.67±1.00 mm), Amoxicillin/ Clavulanic acid (19.56±0.527 mm), Ampicillin/ Sulbactam (15.44±0.527 mm), Piperacillin/ Tazobactam (21.67±0.50 mm), Cefuroxime (23±1.00 mm), Cefepime (25.67±0.866 mm), Aztreonam (23.44±0.527 mm), Meropenem (24.44±0.527 mm), Ciprofloxacin (31.56±0.27 mm) and Trimethoprim/ Sulfamethoxazole (15.67±0.5 mm). Finally, *Acinetobacter* spp. shows susceptible to Ampicillin (17.67±0.577 mm), Amoxicillin/ Clavulanic acid (20.67±0.577 mm), Ampicillin/ Sulbactam (16±0.00 mm), Piperacillin/ Tazobactam (21.67±0.577 mm), Cefepime (25±0.00 mm), Meropenem (26.33±0.577 mm), Ciprofloxacin (33.33±0.577 mm) and Trimethoprim/ Sulfamethoxazole (16±1.00 mm) as shown in Table 3 below.

Pseudomonas aeruginosa was found to be resistant to only Cefazolin (9±0.00 mm), while *Escherichia coli* was resistant against Ampicillin (6.33±0.577 mm) and Ampicillin/ Sulbactam (6.67±0.577 mm). *Salmonella* spp. strains proved to be resistant to most of the drugs such as Cefazolin (7.78±0.441mm), Cefoxitin (9.89±0.333 mm), Amikacin (7.89±0.333 mm) and Gentamicin (7.33±0.50 mm) and *Acinetobacter* spp. Was only resistant to Amikacin (7±0.00 mm) and Gentamicin (7.67±0.577 mm) as shown in Table 3.3.

Table 3: Antimicrobial susceptibility and resistant patterns (Average Zones of inhibition-mm) of Gram-negative bacterial isolates responsible for neonatal sepsis.

		Microorganism N=6			
Antibiotics (Concentration used)	**Break points (mm)**	***Pseudomonas aeruginosa (n-1)***	***Escherichia coli (n=1)***	***Salmonella spp (n=3)***	***Acinetobacter spp (n=1)***
Ampicillin (10µg)	14-16	NT	6.33±0.577mm(R)	16.67±1.00 mm(S)	17.67±0.577mm (S)
Amoxicillin/ Clavulanic acid (20/10µg)	14-17	NT	17.67±0.577mm(S)	19.56±0.527 mm(S)	20.67 ±0.577 mm (S)
Ampicillin/Sulbactam (10/10µg)	12-14	NT	6.67±0.577mm(R)	15.44±0.527 mm (S)	16 ±0.00 mm (S)
Piperacillin/ Tazobactam (100/10µg)	18-20	21.33±0.577 mm (S)	20±0.00 mm (S)	21.67±0.5 mm (S)	21.67 ±0.577 mm (S)
Cefazolin (30µg)	20-22	9 ± 0.00 mm(R)	23.33±0.577 mm (S)	7.78±0.44mm (R)	NT
Cefuroxime (30µg)	15-22	NT	23.67±0.577 mm (S)	23±1.00 mm (S)	NT
Cefepime (30µg)	19-24	24.33±0.577 mm (S)	24±0.00 mm (S)	25.67±0.866 mm (S)	25±0.00 mm (S)
Cefoxitin (30µg)	15-17	NT	18.33±1.15 mm (S)	9.89±0.33mm(R)	NT
Aztreonam (30µg)	18-20	NT	24.33±0.577 mm (S)	23.44±0.527 mm (S)	NT
Meropenem (10µg)	20-22	25.33±0.577 mm (S)	25±0.00 mm (S)	24.44±0.527 mm (S)	26.33±0.577 mm (S)
Amikacin (30µg)	15-16	20.67±1.155 mm (S)	21±1.00 mm (S)	7.89±0.33mm (R)	7±0.00mm (R)
Gentamicin (10µg)	13-14	17.67±0.577 mm (S)	18±0.00 mm (S)	7.33±0.50 mm (R)	7.67±0.57mm(R)
Ciprofloxacin (5µg)	21-30	31.67±0.577 mm (S)	32 ±0.00 mm (S)	31.56±0.27 mm (S)	33.33±0.577 mm (S)
Trimethoprim/ Sulfamethoxazole (1.25/23.75µg)	11-15	NT	16.67±0.577 mm (S)	15.67±0.50 mm (S)	16±1.00 mm (S)

Spp. (species), NT (Not Tested), µg (Micrograms disk content), mm (millimeters) R (Resistant), S (Susceptible). Break points (mm): are as per CLSI 30th Edition, 2020 guidelines.

Minimum inhibitory concentrations (MIC) of antimicrobial agents on gram positive isolates

Nine antimicrobial agents (Oxacillin, Gentamicin, Levofloxacin, Moxifloxacin, Clindamycin, Erythromycin, Linezolid, Vancomycin and Tetracycline.) were used to determine MIC values of the isolated Gram-positive isolates responsible for neonatal sepsis. *Staphylococcus aureus* (1) had an average MIC of 0.25±0.00 µg/ml for Moxifloxacin, Clindamycin and Erythromycin. Also, an average MIC of 0.5±0.00 µg/ml for Oxacillin and Gentamicin were reported from this study. Additionally, against this isolate an average MIC of 1±0.00 µg/ml for Linezolid and Tetracycline were reported while Levofloxacin had an average MIC of 0.12±0.00 µg/ml.

For *Enterococcus spp* (3) the average MICs of 3.5±1.146 µg/ml for Levofloxacin, 1±0.00 µg/ml for Linezolid, and 1.67±1.333 µg/ml for Vancomycin were also documented.

Staphylococcus epidermidis (19) bacterial isolate also had an average MICs 3.4±0.324 µg/ml for Oxacillin, 3.83±0.903 µg/ml for Gentamicin, 2.79±0.831 µg/ml for Levofloxacin, 0.722±0.174 µg/ml for Moxifloxacin, 0.25±0.00 µg/ml for Clindamycin, 4.986±0.916 µg/ml for Erythromycin, 1±0.00 µg/ml for Linezolid, and 5.833±0.749 µg/ml for Tetracycline., Similarly, *Staphylococcus haemolyticus* (5) isolates had an average MICs 2.726±1.309 µg/ml for Levofloxacin, 0.958±0.350 µg/ml for Moxifloxacin, of 0.291±0.416 µg/ml for Clindamycin, and 1±0.00 µg/ml for Linezolid. *Staphylococcus saprophyticus* (1) isolate also had varying average MICs across the antibiotics bioassayed. For instance, it did produce an average MIC of 2±0.00 µg/ml for Oxacillin and Tetracycline, 0.5±0.00 µg/ml for Gentamicin, Levofloxacin, and erythromycin, 0.25±0.00 µg/ml for Moxifloxacin and Clindamycin, and 1±0.00 µg/ml for Linezolid. *Staphylococcus hominis* (2) also did produce varying average MICs against the antibiotics bioassayed: - 0.5±0.00 µg/ml for Gentamicin, Levofloxacin and Erythromycin, 0.25±0.00 µg/ml for Moxifloxacin and Clindamycin, and 1±0.00 µg/ml for Linezolid. Similar findings were recorded with *Staphylococcus lentus* (2) that had an average MIC of 4.25±1.677 µg/ml for Gentamicin, 4.06±1.76 µg/ml for Levofloxacin, 1.125±0.39 µg/ml for Moxifloxacin, 0.37±0.055 µg/ml for Clindamycin, 8±0.00 µg/ml for Erythromycin, 1±0.00 µg/ml for Linezolid. Lastly *Staphylococcus warneri* (2) isolate also produced varying average MICs against the antibiotics bioassayed: - 0.25±0.00 µg/ml for Levofloxacin, Moxifloxacin, Clindamycin and Erythromycin, 0.375±0.06 µg/ml for Gentamicin, 1±0.00 µg/ml for Linezolid, 0.5±0.00 µg/ml for Tetracycline. This varying average MICs also clearly demonstrates that the isolates do react differently to various antibiotics and these findings are summarised and presented in Table 4.

Table 4: Minimum inhibitory concentration (MIC) of Gram-positive isolates responsible for neonatal sepsis.

		Organism N=35							
		***Staphylococcus aureus* (n=1)**	***Enterococcus spp.* (n=3)**	***S. epidermidis* (n=19)**	***S. hemolyticus* (n=5)**	***S. saprophyticus* (n=1)**	***S. hominis* (n=2)**	***S. lentus* (n=2)**	***S. warneri* (n=2)**
Antibiotics	**Concentration range (µg/ml)**	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
Penicillin G		R	NT	R	R	R	R	R	R
Oxacillin	0.016-256	0.5±0	NT	3.4 ±0.324	R	2±0	R	R	R
Gentamicin	0.016-256	0.5±0	NT	3.83 ±0.903	R	0.5±0	0.5±0	4.25±1.677	0.375±0.06
Levofloxacin	0.002-32	0.12±0	3.5±1.146	2.79 ±0.831	2.726 ±1.309	0.5±0	0.5±0	4.06±1.76	0.25±0
Moxifloxacin	0.002-32	0.25±0	NT	0.722 ±0.174	0.958 ±0.350	0.25±0	0.25±0	1.125±0.39	0.25±0
Clindamycin	0.016-256	0.25±0	NT	0.25 ±0	0.291 ±0.416	0.25±0	0.25±0	0.37±0.055	0.25±0
Erythromycin	0.016-256	0.25±0	R	4.986 ±0.916	R	0.5±0	0.5±0	8±0	0.25±0
Linezolid	0.016-256	1±0	1±0	1±0	1±0	1±0	1±0	1±0	1±0
Vancomycin	0.016-256	NT	1.67 ±0.333	NT	NT	NT	NT	NT	NT
Tetracycline	0.016-256	1±0	R	5.833 ±0.749	R	2±0	R	R	0.5±0
MIC Range		0.12-1	0.25-8	0.12-8	0.12-8	0.25-2	0.12-2	0.12-8	0.25-1

Spp (species), NT (Not Tested), R (resistant), µg/ml (Micrograms/ milliliter)

Minimum inhibitory concentrations (MIC) of antimicrobial agents on gram negative isolates

From the Gram-negative isolates responsible for neonatal sepsis, Pseudomonas aeruginosa had varying average MICs against various antibiotics used such as 0.25±0.00 µg/ml for Meropenem and Ciprofloxacin, 4±0.00 µg/ml for Piperacillin/ Tazobactam 1±0.00 µg/ml for Cefepime and Gentamicin, 2±0.00 µg/ml for Amikacin. For Escherichia coli similar findings were obtained with varying average MICs: - 8±0.00 µg/ml for Amoxicillin / Clavulanic acid and Trimethoprim/ sulfamethoxazole, 4±0.00 µg/ml for Piperacillin/ Tazobactam, Cefazolin, Cefuroxime and Cefoxitin, 1±0.00 µg/ml for Cefepime, Aztreonam and Gentamicin, 0.25±0.00 µg/ml for Meropenem and ciprofloxacin, and 2±0.00 µg/ml for Amikacin.

Salmonella spp. had also varying average MICs against the antibiotics screened: - 2±0.00 µg/ml for Ampicillin, Amoxicillin/ Clavulanic acid and Ampicillin Sulbactam, 4±0.00 µg/ml for Piperacillin/Tazobactam and Cefuroxime, 1±0.00 µg/ml for Cefepime and Aztreonam, 0.25±0.00 µg/ml for Meropenem and Ciprofloxacin, and 8±0.00 µg/ml for Trimethoprim/ sulfamethoxazole. Acinetobacter spp. also had similar findings on the average MICs against commonly used antibiotics used to manage this isolate: - 2±0.00 µg/ml for Ampicillin, Amoxicillin/ Clavulanic acid and Ampicillin / Sulbactam, 4±0.00 µg/ml for Piperacillin/ Tazobactam and Trimethoprim/ Sulfamethoxazole, 1±0.00 µg/ml for Cefepime, and 0.25±0.00 µg/ml for Meropenem and Ciprofloxacin. All these findings are summarised and presented as shown in Table 5. From our findings it's clear that different isolates react differently to various antibiotics as some need small doses to inhibit their growth while other need higher doses.

Screening for carbapenems producing (blaOXA-48 and bla KPC genes) in Gram negative isolates

Deoxyribonucleic Acid (DNA) from Gram negative bacteria were screened for the plasmid-encoded blaOXA-48 and bla KPC genes that confer resistance to bacteria against carbapenem class of antibiotics considered to be the drugs of last resort. Six DNA templates were screened and four turned positive for the markers giving 66.7%. Two (50%) *Salmonella ssp.* had cycle threshold of 30.61 and 30.69 while 1 (25%) *Escherichia coli* at 33.64 and 1 (25%) Pseudomonas *aeruginosa* one at 33.43 as illustrated in the Figure 2 below.

Table 5: Minimum inhibitory concentration (MIC) of Gram-positive isolates responsible for neonatal sepsis.

		Organism N=6			
		Pseudomonas aeruginosa (n=1)	*Escherichia coli* (n=1)	*Salmonella spp* (n=3)	*Acinetobacter spp* (n=1)
Antibiotics	**Concentration range (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**
Ampicillin	0.016-256	NT	R	2±0.00	2±0.00
Amoxicillin/ Clavulanic acid	0.016-256	NT	8±0.00	2±0.00	2±2.0
Ampicillin/ Sulbactam	0.016-256	NT	R	2±0.00	2±0.00
Piperacillin/ Tazobactam	0.016-256	4±0.00	4±0.00	4±000	4±0.00
Cefazolin	0.016-256	R	4±0.00	R	NT
Cefuroxime	0.016-256	NT	4±0.00	4±0.00	NT
Cefepime	0.016-256	1±0.00	1±0.00	1±0.00	1±0.00
Cefoxitin	0.016-256	NT	4±0.00	R	NT
Aztreonam	0.016-256	NT	1±0.00	1±0.00	NT
Meropenem	0.002-32	0.25±0.00	0.25±0.00	0.25±0.00	0.25±0.00
Amikacin	0.016-256	2±0.00	2±0.00	R	R
Gentamicin	0.016-256	1±0.00	1±0.00	R	R
Ciprofloxacin	0.002-32	0.25±0.00	0.25±0.00	0.25±0.00	0.25±0.00
Trimethoprim/ Sulfamethoxazole	0.002-32	NT	8±0.00	8±0.00	4±0.00
MIC Range		0.25-4.00	0.25-8.00	0.25-8.00	0.25-4.00

Spp (species), NT (Not Tested), R (resistant), µg/ml (Micrograms/ milliliter).

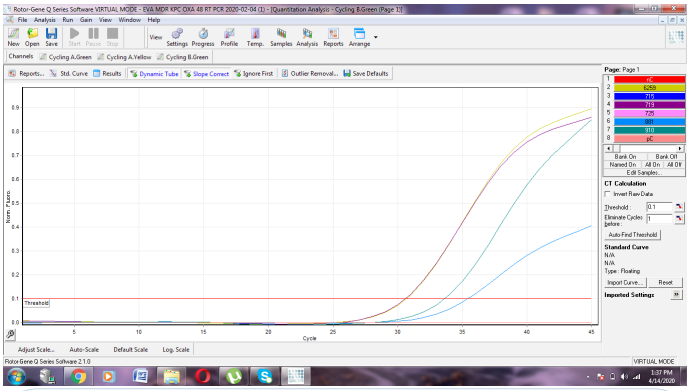

Figure 2: Carbepenemase (blaOXA-48 and bla KPC) genes determination in Gram negative isolates.
Key: *Y axis (represents visualization of fluorescence produced). X axis (represents visualization of number of cycle threshold). While lines above the threshold line are (positive results with different cut off values). The different coloured lines represent (the isolates) Purple 30.61 (Salmonella spp.), Jungle green 30.69 (Salmonella spp.), Light blue 33.43 (Pseudomonas aeruginosa), Cornflower blue 33.64 (Escherichia coli).*

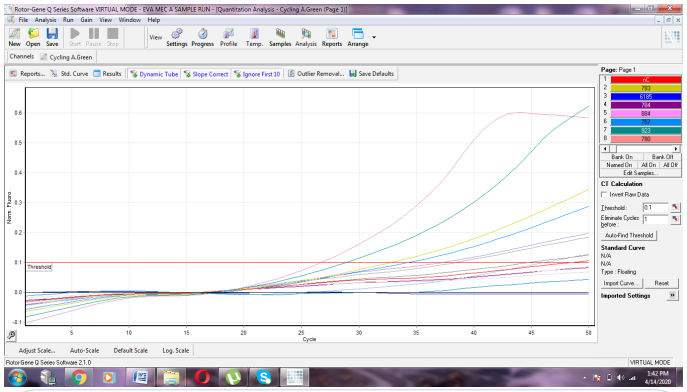

Figure 3: Methicillin resistance (Mec A) gene determination in Gram positive isolates.
Key: *Y axis (represents visualization of fluorescence produced). X axis (represents visualization of number of cycle threshold). While lines above the threshold line are (positive results with different cut off values). The different coloured lines represent (the isolates) Pink 26.76 (Enterococcus spp.), Jungle green 28.87 (Staphylococcus epidermidis), Lime green 32.76 (Staphylococcus hominis), Cornflower blue 34.38 (Staphylococcus epidermidis), purple 37.34 (Staphylococcus warneri), and Grey 38.43 (Staphylococcus epidermidis).*

Screening for methicillin resistance (Mec A gene) in gram positive isolates

Deoxyribonucleic Acid (DNA) from Gram positive bacteria were screened for MecA gene that confers resistance to bacteria against selected antimicrobial agents. Thirty-five DNA templates were screened, six turned positive for the markers giving 17.1%. For *Staphylococcus epidermidis* three (50%) isolates had cycle threshold of 28.87, 34.38 and 38.43. Also, one (16.7%) of *Staphylococcus warneri* isolate and one (16.7%) of the *Staphylococcus hominis* isolate had 37.34 and 32.76 respectively, and one (16.7%) *Enterococcus spp.* isolate had 26.76 as illustrated in the Figure 3 below.

DNA fragment analysis from bacterial isolates that had resistant genes responsible for Neonatal sepsis

Deoxyribonucleic acid (DNA) fragments of the ten bacterial isolates with resistant gene markers were separated based on their size and charge and visualized as base pair bands (bp) compared to the DNA ladder by use of Automated QIAxcel Advanced system® using QX DNA high resolution kit® and the results were visualized by QIAxcel Screen Gel software®. Out of the ten DNA fragments, four turned positive A4 *(Salmonella spp.)* at 148 bp, A6 *(Escherichia coli)* at 148 bp, A7 *(Staphylococcus epidermidis)* at 148 bp A11, *(Staphylococcus hominis*) at 296 bp while six turned negative such as A3 (*Salmonella spp*), A5 (*Pseudomonas aeruginosa*), A8 (*staphylococcus warneri*), A9 (*Enterococcus spp*), A10 (*Staphylococcus epidermidis*), A12 (*Staphylococcus epidermidis*) as illustrated on Figure 4 below.

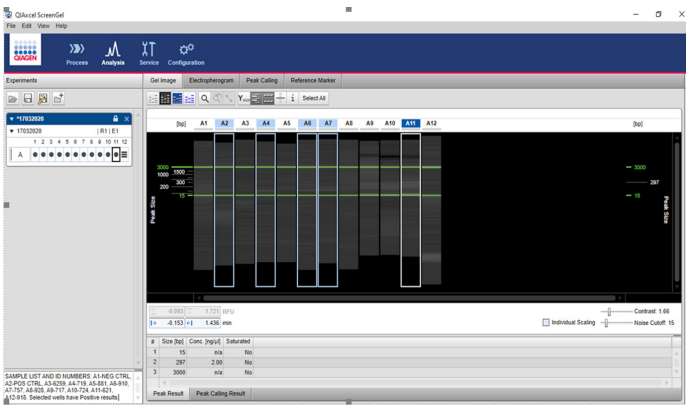

Figure 4: DNA fragments of bacterial isolates with resistant genes for neonatal sepsis
bp (base pairs). A1-A12 (Wells), A1 (Negative control), A2 (Positive control), (Positive wells A2, A4, A6, A7 and A11, A4 (Salmonella spp), A6 (Escherichia coli), A7 (Staphylococcus epidermidis) A11 (Staphylococcus hominis). Negative results A3 (Salmonella spp), A5 (Pseudomonas aeruginosa), A8 (staphylococcus warneri), A9 (Enterococcus spp), A10 (Staphylococcus epidermidis), A12(Staphylococcus epidermidis).

Discussion

From our findings it was clear that Oxazolidinones (linezolid) was the most effective antibiotic class against all Gram-positive isolates with least MIC of 1±0.00 µg/ml. The activity of this antibiotic is based on its ability to inhibit bacterial protein synthesis and it has been documented to be more effective against methicillin resistant *Staphylococcus spp.* [31]. Such pronounced activity to the Gram-positive bacteria could be attributed to its ability to binds to a site on the bacterial 23S ribosomal RNA of the 50S subunit, which prevents the formation of a functional 70S initiation complex hence stopping the translation process which ends inhibiting protein synthesis thus no bacterial multiplication [32]. However, it was noted that the Penicillin (penicillin G and Oxacillin) which belongs to the same class of antibiotics had the highest resistance against Gram positive isolates. This scenario may be attributed to the narrow spectrum of activity of the antibiotic and the resistance against the antibiotic developed over years by the bacteria. *Staphylococcus aureus* had the highest susceptibility pattern across all microbial agents tested among Gram positive isolates. It had the least variation of resistance pattern with MIC range of 0.12-1 µg/ml, a finding that concurs with the findings of a study done by [33] where *Staphylococcus aureus* had the highest susceptibility pattern with MIC range of 0.12-1µg/ml. On the other hand, *Staphylococcus epidermidis* was the only isolate that had the highest variation of resistance pattern across all the antimicrobial agents with MIC range of 0.12-8 µg/ml. This clearly demonstrates that proper intervention measures need to be put in place to manage any condition that could be caused by this isolate. Similar findings were also reported elsewhere where MIC range of 0.12-8 µg/ml against *Staphylococcus epidermidis* test isolate were documented [34]. A clear indication of the possible burden this pathogen can cause just in case it causes a pandemic more so amongst the neonates.

On the other hand, *Escherichia coli* had the highest susceptibility pattern with an MIC range of 0.25-8 µg/ml across all antimicrobial agents tested among the Gram-negative isolates. This is a very positive finding as this pathogen has been associated with various outbreaks and resistances globally. Additionally, it is a pathogen that can easily be isolated from most environments [35]. Contrary *Salmonella spp.* had the highest resistant pattern across all the microbial agents tested with MIC range of 0.25-8 µg/ml among the Gram-negative bacteria. This is comparable with similar results documented in a study done by Sheikh *et al.,* [34] who did report MIC of *Salmonella typhi* to ciprofloxacin to be 8 µg/ml which was upward trend among 20% of the strains [36].

Beta lactam inhibitors (Amoxicillin/ clavulanic acid and piperacillin/Tazobactam), 4th Generation cephalosporins, (Monobactams, carbapenems, quinolones) were the most effective against Gram negative isolates and this could be attributed to their ability to inhibit the formation of bacterial cell wall and DNA synthesis [37]. Aminoglycosides (Gentamicin and Amikacin) were the least effective antibiotics against *Pseudomonas aeruginosa* and *Escherichia coli* as they did show MIC of 1±0.00 and 2±0.00 respectively. This scenario could be attributed to the high exposure levels and misuse of this drug in the study population as it is easily obtained over the counter prescription [34]. Severe or high-risk bacterial infections are treated by the most effective available antimicrobial agents; however, it has evolved and developed mechanism to defeat antimicrobial agents by developing sophisticated mechanisms like production of enzymes under the influence of specific genes that neutralize drugs.

Methicillin resistant (MecA-gene) and carbepenemase (bla OXA 48 and bla KPC) genes are few of the known genes that enhances production of enzymes that deactivate antibiotics [16]. Among the Gram-negative pathogens, Extended Spectrum Beta Lactamases (ESBLs) are enzymes which can inactivate broad spectrum cephalosporins, aztreonam, penicillin's and comprises of TEM, SHV, OXA and CTX-M derivatives [38]. SHV enzymes are encoded by self-transmissible plasmids and have a hydrolysing activity to carbapenems and monobactams CTX-M enzymes are plasmid based encoded cefotaximases with an extended activity to cefotaxime antibiotic. TEM enzymes are plasmid mediated resulting from mutations by amino acid substitution around the active site. OXA enzymes have a high hydrolytic activity against cloxacillin and oxacillin antimicrobial agents [39].

Gram positive bacteria produce different enzymes that have different mechanisms of resistance to antimicrobial agents. Penicillin binding-proteins, DNA-dependent RNA polymerase and type II topoisomerases work by targeting antimicrobial drugs. Some work by modifying cellular targets of antimicrobial agents like the phosphoethanolamine, others work as antimicrobial drug-modifying enzymes like the Transferases and the Hydrolases, while some work as the antimicrobial drug-metabolizing enzymes like the Pyrazinamidase enzymes [40]. This study also found out presence of resistant genes (carbapenemes; bla OXA- 48, bla KPC and methicillin MecA gene) among bacteria that were isolated from preterm neonates. This clearly indicates presence of antimicrobial resistance among isolated bacteria against Beta-lactam antibiotics which also adds the numbers to the resistance incidences statistics globally. It further possesses a serious challenge on treatment and management of several illnesses including neonatal sepsis not only to the study site but also regionally and globally [41,42].

Methicillin resistant (MecA-gene) was found in six isolates, three *Staphylococcus epidermidis* with a cycle threshold of 28.87, 34.38 and 38.43, *Staphylococcus warneri* with a cycle threshold of 37.34, *Staphylococcus horminis* with cycle threshold of 32.76 and *Enterococcus spp* with cycle threshold of 26.76. This shows that the isolates could be resistant to various antibiotics, which might cause a challenge in terms of treatment. These findings are similar to those documented in a study done in Brazil where MecA gene was detected among Coagulase Negative Staphylococcus (CoNS) [43].

Bla, Oxa 48 and Bla KPC genes were isolated in four isolates, two *Salmonella spp.* with a cycle threshold of 30.61, *Escherichia coli* with cycle threshold of 33.64 and *Pseudomonas aeruginosa* with cycle threshold of 35.43. Bla Oxa-48 gene was also isolated in *Klebsiella pneumoniae* isolates that were carbapenem resistant at a tertiary care centre in Western Turkey [44]. Another study done in Huashan hospital, China did report that Bla KPC gene was dominant in clinical *Klebsiella pneumoniae* isolates [45]. From our findings, Gram positive isolates were more resistant especially the CoNS, this may be due to presence of virulence genes like the *icaA, icaB, icaC* and *icaD* that have been documented previously to be responsible for biofilm formation which is associated with antibiotic resistance [46]. Antimicrobial resistance is a global concern mostly in 3rd world countries where high cases have been documented (75%). From this study findings, it clearly indicates that there exist resistant genes among the isolates found in preterm neonates at Kitale county Hospital New-born Unit (NBU).

Conclusions and recommendations

There exists Multi-Drug Resistance (MDR) genes in bacterial isolates circulating in Kitale County Hospital new-born unit. Hospital based policies, guidelines, and Standard Operating Procedures (SOPS) on Management and treatment of preterm neonatal sepsis with multidrug resistant microbials should be developed and implemented.

Author declarations

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Author's contribution

EPK, EOO, GKM and SOA were involved in the conception and design of the study. EPK supervised interviews. EPK performed laboratory tests and analysis, EPK analysed the data and prepared the manuscript. EOO, GKM and SOA provided guidance and mentorship during the implementation of the study. All authors reviewed and approved the final manuscript. EPK takes the first authorship responsibilities.

Conflict of interest

The authors declare that, they have no financial, political, religious, intellectual or personal relationship that may have inappropriately influenced them in writing this article. This research received no grant from any funding agency in public, commercial or not-for-profit sectors

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