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Antibiotic Resistant Bacteria in Lactating Cows Infected with Bovine Mastitis

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Abstract

Milk quality and quantity is greatly affected by the high incidence of bacterial infection, bovine mastitis (BM) in lactating cows. Bacteria were isolated from mastitis infected cow. The efficacy of antibiotics against the bacteria isolates were determined by agar-well diffusion technique. Eight of the isolates were Gram-negative – *Escherichia coli* (2), *Citrobacter freundii* (3), *Citrobacter diversus* (1), *Enterobacter aerogenes* (1), *Klebsiella pneumoniae* (1), while ten were Gram-positive – *Staphylococcus* spp. (8), and *Micrococcus* spp. (2). *Staphylococcus* spp. (44.44%) had the highest percentage occurrence. Gentamicin (25±1.41 mm) and ofloxacin (27.5±0.71 mm) showed the highest zone of inhibition (ZI) against the Gram-positive isolates, but the organisms were 100% resistant to ceftazidime, cefuroxime, augmentin and cloxacillin. Ofloxacin (26.5±2.12 mm) and ciprofloxacin (30±0 mm) showed the highest ZI against the Gram-negative isolates and the organisms were 100% resistant to ceftazidime, cefuroxime, cefixime, and augmentin. *Staphylococcus* spp., *C. freundii, E. coli* and *Micrococcus* spp. were the predominant pathogens associated with BM in the study area. Ofloxacin is very effective against BM but all the organisms were resistant to ceftazidime, cefuroxime and augmentin. This study confirms that antibiotic resistant bacteria are present in BM infection and the antibiotics (ceftazidime, cefuroxime, cefixime, cloxacillin, and augmentin) are not effective therapies for treating BM. Therefore, indiscriminate use of these antibiotics should be discouraged in veterinary medicine.

Keywords: Antibiotic resistance, bacteria, bovine mastitis, cow

Introduction

Milk as a rich source of vitamins and nutrients enhances the proper functioning of the body system (Pfeuffer *et al.,* 2017; Bechthold *et al.,* 2019). It is a rich source of calcium which is usually produced by all mammals to feed their young ones. In other instances, it can be taken as a beverage, and can be used to make cream, yogurt, and butter. Adequate consumption of milk and its products enhances strong and healthy bones, immunity boost for the body, promotion of muscular growth (Malmir *et al.,* 2020) good source of protein and minerals (Arise *et al*., 2019). More than 80% of the global milk production is supplied by dairy cattle, while the rest are from goats, sheep, buffalo, reindeer, and camels (FAO, 2022), but one of the problems affecting dairy milk production is bovine mastitis (Gomes and Henriques, 2016; Ameen *et al.,* 2019).

Mastitis is a serious threat in the dairy farm and is often characterized with the inflammation of the udder and teats of lactating cows. This infection can be attributed to poor hygiene and sanitation within the animal ranch. It causes physical, chemical and biological changes in the mammary gland of the cows (Gera and Guha, 2011). Mastitis can easily be transmitted from an infected cow to healthy ones until it is endemic within a ranch (Rinaldi *et al.,* 2010). Mastitis causes low milk yield and poor quality and is responsible for serious economic loss in dairy production (Halasa *et al.,* 2007; Huijps *et al.,* 2008). It also poses zoonotic threats that are associated with shedding of bacteria and their toxins in the milk (Abebe *et al.,* 2016). Some of the bacterial pathogens associated with bovine mastitis include members of the genera: *Escherichia, Staphylococcus, Micrococcus, Streptococcus* and *Corynebacterium* (Verraes *et al.,* 2015).

Antibiotics are frequently used by herders in the treatment and prevention of bovine mastitis and this is usually done without prescription from qualified veterinary doctor. The abuse and overuse of antibiotics has contributed to antibiotic resistance in the environment (Srinivasan *et al.,* 2007). Antibiotics resistance develops when bacteria develop mechanisms against antibiotics, thus reducing the potency of those drugs in curing infections (WHO, 2023). Although some researchers have advocated the use of some useful plants in animal breeding (Adesina *et al.,* 2013; Oyelere et al., 2016) but antibiotics remains the most common in veterinary medicine.

Studies have shown variation in bacteria associated with bovine mastitis in different regions of the world, but there is paucity of such information in Nigeria. Bacteria were isolated from mastitis infected cow and their susceptibility to antibiotics were determined.

Materials and Method

Sample Collection

Milk sample was collected from a *white-fulani* lactating cow of about ten years old with inflamed mammary gland from a cattle ranch beside Bowen University, Iwo, Osun State, Nigeria. The cow has had four parities and was at the late lactation stage. The sample was taken immediately to the Biological Laboratory and was processed within 30 minutes.

Isolation and Identification of Bacteria

Spread plate technique as described by Sanders (2012) with some modification was used to isolate bacteria from the milk sample. Using a sterile syringe, 0.1 mL of raw milk sample was introduced onto the surface of sterile agar plates and incubated overnight at 35-37˚C. Discrete colonies found on the plates were transferred into sterile agar plates using sterile inoculating loop. Further sub-culturing was carried out until pure cultures were obtained. Gram-staining and biochemical tests were carried out on the isolates, which include: catalase, methyl red (MR), Voges-Proskauer (VP), citrate utilization, indole, blood heamolysis, starch, and sugar fermentation (glucose, lactose, mannitol and sucrose) tests. The tested isolates were identified using Bergey's Manual of Systematic Bacteriology (Garrity *et al.,* 2004).

Antibiotic Susceptibility Test

Agar-well diffusion technique was used to determine the antibiotic susceptibility patterns of the bacterial isolates. Pure colony of 24 h old bacterial culture was introduced into sterile distilled water and spread onto the Muller-Hinton Agar (MHA) plates with the aid of swab sticks. Gram-positive and Gram-negative antibiotic discs were placed aseptically on the agar plates and incubated at 37˚C for 18 - 24 h. Clear zones around the discs were measured with the aid of millimetre rule from one edge of a clear zone to the other edge. The susceptibility or resistance of each isolate to the antibiotics was determined according to the Clinical Laboratory Standards Institute guidelines (2019). This is a standard laboratory guideline for comparing results of the microbial analysis.

Statistical Analysis

The antimicrobial sensitivity test was conducted in triplicates and the results presented as mean and standard deviation using Excel 2010 version.

Results

Diverse bacterial colonies of distinct morphological characteristics were seen on the agar plates (Plate 1). A total of eighteen (18) isolates were gotten from the milk sample, of which ten were Gram-positive cocci, while eight were Gram-negative rods (Table 1). The isolates were identified as *Staphylococcus* spp. (8), *Citrobacter freundii* (3), *Escherichia coli* (2), *Micrococcus* spp. (2), *Enterobacter aerogenes* (1), *Citrobacter diversus* (1), and

Klebsiella pneumoniae (1). All the organisms were catalase, methyl-red, glucose and lactose positive. Three of the isolates, which were identified as *Micrococcus* spp., *Citrobacter freundii,* and *Staphylococcus* sp. were positive for β blood-hemolysis, while others showed γ blood-hemolysis.

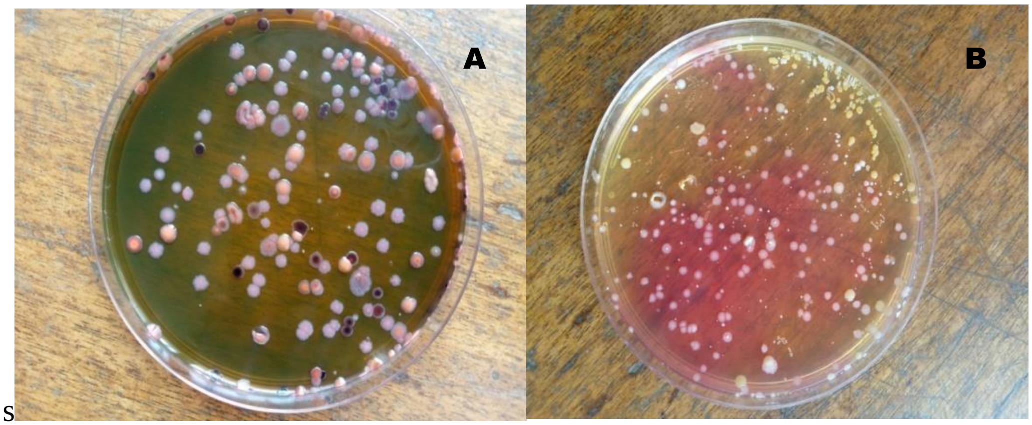

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Plate 1: Bacterial colonies on eosin methylene blue agar [A] and mannitol salt agar [B]

Table 1: Bacteria Isolated from the Raw Milk Sample

S/N	Isolate code	Shape	Gram's reaction	Indole	Citrate	Catalase	Methyl red	Voges Proskauer	Blood Haemolysis	Starch hydrolysis	Glucose	Lactose	Mannitol	Sucrose	Identity of organism
1	MA	Rod	-	+	+	+	+	-	Γ	-	+	+	+	+	*Citrobacter diversus*
2	MB	Rod	-	+	-	+	+	-	Γ	-	+	+	+	+	*Escherichia coli*
3	MC	Rod	-	+	-	+	+	-	Γ	-	+	+	+	-	*Escherichia coli*
4	MD	Cocci	+	-	+	+	+	-	Γ	+	+	+	+	+	*Staphylococcus* sp.
5	ME	Cocci	+	-	-	+	+	-	Γ	-	+	+	-	+	*Micrococcus* sp.
6	MF	Rod	-	-	+	+	+	+	Γ	-	+	+	+	+	*Enterobacter aerogenes*
7	MG	Rod	-	-	+	+	+	-	Γ	-	+	+	+	+	*Citrobacter freundii*
8	MH	Cocci	+	-	-	+	+	-	B	-	+	+	-	+	*Micrococcus* sp.
9	MI	Cocci	+	-	-	+	+	-	Γ	-	+	+	+	+	*Staphylococcus* sp.
10	MJ	Rod	-	-	+	+	+	-	Γ	-	+	+	+	+	*Citrobacter freundii*
11	MK	Cocci	+	-	+	+	+	-	Γ	-	+	+	+	+	*Staphylococcus* sp.
12	ML	Rod	-	-	+	+	+	+	Γ	-	+	+	+	+	*Klebsiella pneumoniae*
13	MM	Cocci	+	+	+	+	+	-	Γ	+	+	+	+	+	*Staphylococcus* sp.
14	MN	Cocci	+	-	+	+	+	+	Γ	-	+	+	+	+	*Staphylococcus* sp.
15	MO	Rod	-	-	+	+	+	-	B	+	+	+	+	+	*Citrobacter freundii*
16	MP	Cocci	+	-	+	+	+	-	Γ	-	+	+	+	+	*Staphylococcus* sp.
17	MQ	Cocci	+	-	+	+	+	-	Γ	-	+	+	+	+	*Staphylococcus* sp.
18	MR	Cocci	+	-	+	+	+	-	B	+	+	+	+	+	*Staphylococcus* sp.

Table 2 represents the percentage occurrence of bacteria in the milk sample. The most abundant was *Staphylococcus* spp. (44.44%), while the least were *Citrobacter diversus* (5.56%), *Klebsiella pneumoniae* (5.56%), and *Enterobacter aerogenes* (5.56%).

Table 2: Percentage Occurrence of Bacteria Isolated from the Raw Milk Sample

S/N	Isolate	Number	Occurrence (%)
1	*Staphylococcus* spp.	8	44.44%
2	*Citrobacter freundii*	3	16.66%
3	*Escherichia coli*	2	11.11%
4	*Micrococcus* spp.	2	11.11%
5	*Enterobacter aerogenes*	1	5.56%
6	*Citrobacter diversus*	1	5.56%
7	*Klebsiella pneumonia*	1	5.56%
	Total	**18**	**100**

The zones of inhibition of the antibiotics against the Gram-positive isolates are presented in Table 3. Ceftazidime and cefuroxime were not effective against any of the bacterial isolates, but the isolates showed susceptibility to gentamicin (14.5±2.12 - 25±1.41 mm) and ofloxacin (23±0 - 27.5±0.71 mm). Table 4 shows the zones of inhibition of the antibiotics against the Gram-negative bacteria that were isolated from the milk sample. The results showed that the Gram-negative bacteria did not respond to ceftazidime, cefuroxime, cefixime and augmentin. The results showed that ciprofloxacin and ofloxacin were the very effective antibiotics against the bacterial isolates. Ofloxacin inhibited the bacteria (22.5±0.71 - 26.5±2.12 mm) and ciprofloxacin (25±0.00 mm to 30±0.00 mm). Gentamicin and ofloxacin are still very efficacious against the Gram-positive bacteria isolates. Similarly, ofloxacin and ciprofloxacin are very effective against all the Gram-negative isolates. All the isolates were resistant to ceftazidime and cefuroxime.

Table 3: Zones of Inhibition of Antibiotics against Gram-positive Bacteria Isolated from the Raw Milk Sample

Isolate code	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG
MD	0.00	0.00	23±2.83	0.00	15±2.83	0.00	25.5±0.71	0.00
ME	0.00	0.00	21±1.41	0.00	10.5±0.71	0.00	23±1.41	0.00
MH	0.00	0.00	22±0.00	0.00	9.5±0.71	0.00	23.5±0.71	0.00
MI	0.00	0.00	24.5±2.12	0.00	12.5±0.71	0.00	27.5±0.71	0.00
MK	0.00	0.00	25±1.41	0.00	10±1.41	8±1.41	25±0.71	8±0.71
MM	0.00	0.00	23.5±2.12	0.00	11.5±0.71	0.00	24±0.00	0.00
MN	0.00	0.00	14.5±2.12	24.5±0.71	0.00	0.00	23±0.00	0.00
MP	0.00	0.00	21.5±2.12	22.5±0.71	0.00	0.00	23±0.00	0.00
MQ	0.00	0.00	15±0.00	0.00	0.00	0.00	24.5±0.71	0.00
MR	0.00	0.00	16.5±2.12	0.00	0.00	0.00	26.5±2.12	0.00

Key: CAZ – ceftazidime (30µg), CRX – cefuroxime (30µg), GEN – gentamicin (10µg), CTR – ceftriaxone (30µg), ERY – erythromycin (5µg), CXC – cloxacillin (5µg), OFL – ofloxacin (5µg), AUG – augmentin (30µg); all readings in millilitre (mm)

Table 4: Zones of Inhibition of Antibiotics against Gram-negative Bacteria Isolated from the Raw Milk Sample

Isolate code	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR
MA	0.00	0.00	13.5±3.54	0.00	22.5±0.71	0.00	19.5±0.71	27.5±3.54
MB	0.00	0.00	11±0.00	0.00	24±2.12	0.00	19.5±0.71	25±0.00
MC	0.00	0.00	14.5±0.71	0.00	26±0.00	0.00	22±1.41	29.5±0.71
MF	0.00	0.00	13.5±2.12	0.00	23±1.41	0.00	18±2.83	26±0.00
MG	0.00	0.00	12.5±0.71	0.00	26.5±2.12	0.00	14.5±3.54	30±0.00
MJ	0.00	0.00	13±1.41	0.00	24.5±0.71	0.00	21±1.41	29.5±0.71
ML	0.00	0.00	15.5±0.71	0.00	22.5±0.71	0.00	21.5±0.71	27.5±3.54
MO	0.00	0.00	21.5±0.71	0.00	22.5±1.41	0.00	23±1.41	25±0.00

Key: CAZ – ceftazidime (30µg), CRX – cefuroxime (30µg), GEN – gentamicin (10µg), CXM – cefixime (5µg), OFL – ofloxacin (5µg), AUG – augmentin (30µg); NIT – nitrofurantoin (300µg), CPR – ciprofloxacin (5µg); All readings in millilitre (mm)

Figures 1 and 2 show the percentage susceptibility of the Gram-positive and Gram-negative bacteria to antibiotics respectively. The Gram-positive isolates were 100% susceptible to gentamicin and ofloxacin, 20% susceptible to ceftriaxone and no susceptibility to the remaining tested antibiotics. The result shows that the Gram-negative isolates were 100% susceptible to ofloxacin and ciprofloxacin, 87.5% and 37.5% susceptibility were observed for nitrofurantoin, and gentamicin respectively.

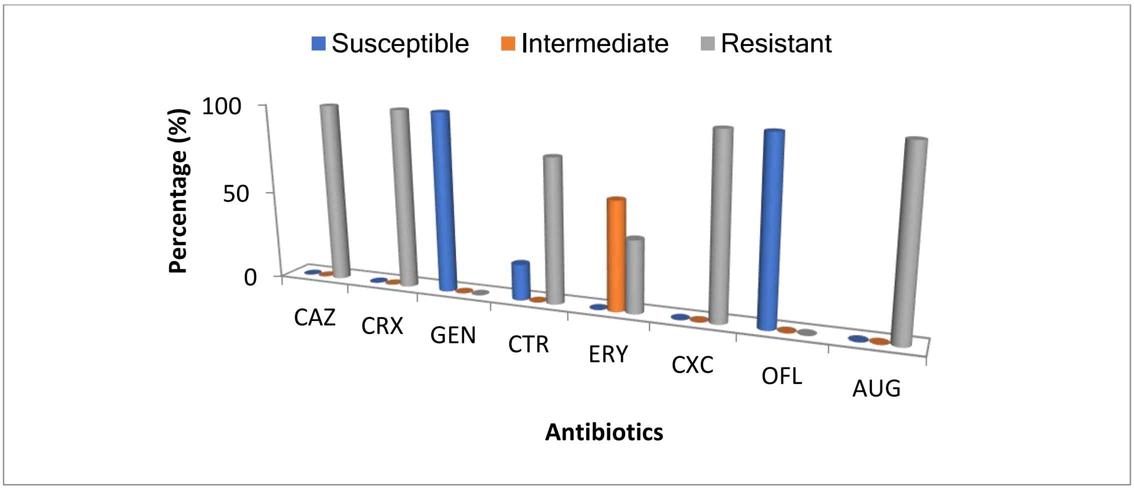

Figure 1: Percentage susceptibility of Gram-positive bacteria to antibiotics
Key: S: Susceptibility, I: Intermediate, R: Resistant
Key: CAZ - ceftazidime (30µg), CRX - cefuroxime (30µg), GEN - gentamicin (10µg), CTR - ceftriaxone (30µg), ERY - erythromycin (5µg), CXC - cloxacillin (5µg), OFL - ofloxacin (5µg), AUG - augmentin (30µg)

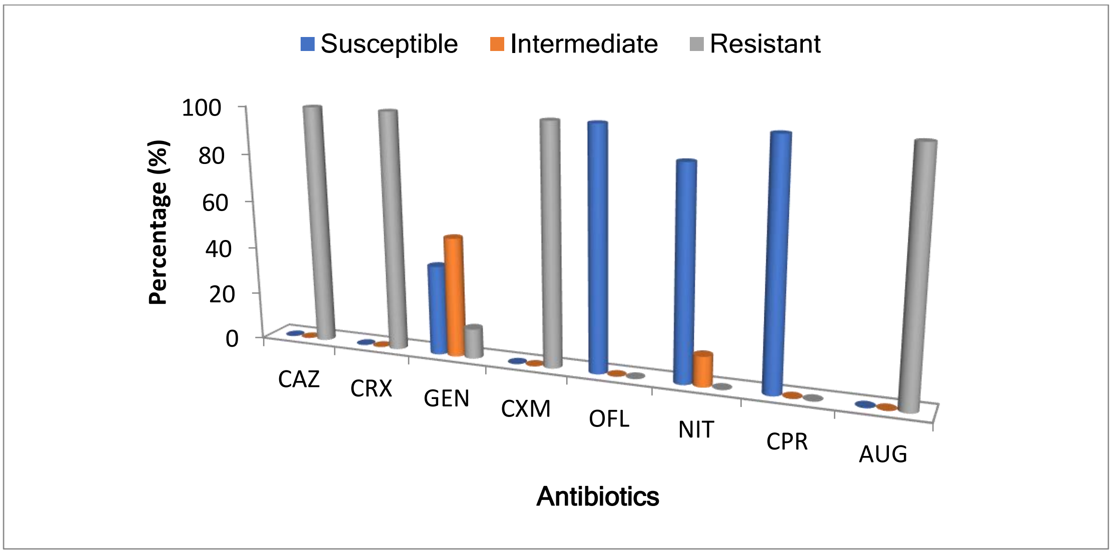

Figure 2: Percentage susceptibility of Gram-negative bacteria to antibiotics
Key: S: Susceptibility, I: Intermediate, R: Resistant
Key: CAZ - ceftazidime (30µg), CRX - cefuroxime (30µg), GEN - gentamicin (10µg), CPR - ciprofloxacin (5µg), CXM - cefixime (5µg), NIT - nitrofurantoin (300µg), OFL - ofloxacin (5µg), AUG - augmentin (30µg

Discussion

In the current study, eighteen isolates were gotten from the raw milk sample collected from lactating cow with symptoms of bovine mastitis. The identified bacteria from the infected milk sample concurs with the findings of Haftu *et al.* (2012), who also isolated *Staphylococcus* spp., *Klebsiella pneumoniae* and *Escherichia coli* from bovine mastitis in Ethiopia. Also, Ameen *et al.* (2019) isolated *Escherichia coli, Streptococcus* sp.*,* and *Pseudomonas aeruginosa* from lactating cows in Egypt. In addition, *Enterobacter aerogenes, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were found in mastitis infected cows in Cameroon (Ngu *et al.,* 2020). According to Pascu *et al.* (2022), *Enterococcus* spp. and *Enterobacter* spp. were isolated from dairy cattle in Romania, which is in concord with this study. Also, Hassani *et al.* (2022) isolated bacteria from bovine mastitis, which is in agreement with this study. Beyene *et al.* (2017) and Chandrasekaran *et al.* (2014) reported abundance of *Staphylococcus* spp. in the milk samples collected from acute mastitis cow.

The result showed *Staphylococcus* spp. (44.44%), *Citrobacter freundii* (16.66%), *Escherichia coli* (11.11%), *Micrococcus* spp. (11.11%), *Enterobacter aerogenes* (5.56%), *Citrobacter diversus* (5.56%) and *Klebsiella pneumoniae* (5.56%). Haftu *et al.* (2012) observed dominance of *Staphylococcus* spp. (36%) and *Escherichia coli* (27.3%) from mastitis infected cow in Ethiopia. Ngu *et al.* (2020) reported high occurrence of coagulase-negative *Staphylococcus* species (27.5%) in infested cows in Cameroon. Pascu *et al.* (2022) observed high occurrence of *Staphylococcus* spp. (43.19%) and a low occurrence of *Enterobacter* spp. (4.31%) in Romanian cattle ranch.

The in-vitro antibiotic susceptibility testing of antibiotics, such as ceftazidime, cefuroxime, gentamicin, ceftriaxone, erythromycin, cloxacillin, ofloxacin, augumentin, ciprofloxacin, cefixime and nitrofurantoin against the bacteria were reported in this study. The bacteria showed 100% resistance to augmentin, ceftazidime, cefuroxime and cefixime, but only 12.5% were resistant to gentamicin.

The broad and frequent application of common antibiotics in the management of udder infection may be responsible for the bacterial resistance to antibiotics. In a similar study conducted by Beyene *et al.* (2017) in Ethiopia, all the *Staphylococcus* spp. isolated were susceptible to gentamicin. The antibiotic susceptibility carried out in this study implies that the bacteria isolates are gradually getting resistant to most of the tested antibiotics, except ofloxacin and gentamicin for the Gram-positive bacteria and ofloxacin and ciprofloxacin for the Gram-negative isolates.

Conclusion and Recommendation

In conclusion, *Staphylococcus* spp., *Citrobacter freudii, Escherichia coli, Micrococcus* spp., *Enterobacter aerogenes, Citrobacter diversus, Klebsiella pneumoniae* and *Micrococcus* spp. were found in the milk sample of lactating cow showing symptoms of bovine mastitis in Iwo, Osun State, Nigeria and the predominant bacteria was *Staphylococcus* spp. (44.44%). All the bacteria isolated from the infected cow were susceptible to ofloxacin. This indicated that ofloxacin is still very effective against bacteria infesting bovine mastitis. The ineffectiveness of cefuroxime, ceftazidime and augmentin could be due to the over-use of these antibiotics. Antibiotics should not be used for cows and other lactating animals showing symptoms of mastitis, if not prescribed by a qualified veterinarian, so as to prevent antibiotic resistance in the animals and the environment.

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