German Journal of Veterinary Research

eISSN:2703-1322

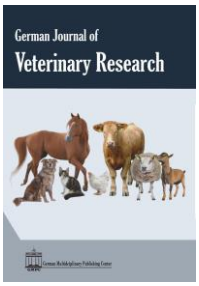
Research article

Prevalence and antimicrobial susceptibility profiles of *Staphylococcus aureus* from raw bovine milk in dairy and pastoral farms in Morogoro region, Tanzania

Nancy E. Kalee[1,2*], **Noel Gahamanyi** [1,2], **and Abubakar S. Hoza**[1]

[1] Department of Veterinary Microbiology, Parasitology and Biotechnology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3019, Chuo Kikuu, Morogoro, Tanzania

[2] SACIDS Foundation for One Health, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3015, Chuo Kikuu, Morogoro, Tanzania



Article History:

Received: 23-Feb-2021

Accepted: 04-Mar-2021

***Corresponding author:**

Nancy E. Kalee

E-mail:nancy.kalee@sacids.org

Abstract

Increased resistance of *Staphylococcus aureus* isolates to existing antimicrobials constitutes a major concern in human and veterinary medicine. This study aimed to determine the prevalence, antimicrobial susceptibility profiles, and molecular characteristics of *S. aureus* from raw bovine milk in dairy and pastoral farms in Morogoro urban and Mvomero districts, Tanzania. In a cross-sectional study, 397 raw bovine milk samples were collected and carried to the laboratory. Conventional Gram staining, colony morphology on blood agar, and mannitol salt agar, along with biochemical tests, were used for *S. aureus* identification. Antimicrobial susceptibility testing (AST) was performed using the disk diffusion method, while multiplex polymerase chain reaction (PCR) was used to detect virulence and antimicrobial resistance genes. Data were analyzed using Epi Info (Version 7). Out of the 397 samples, *S. aureus* was confirmed in 124 (31.2%). Contamination of raw bovine milk by *S. aureus* in the study area was associated with poor milking hygienic measures. The AST revealed that all *S. aureus* isolates were susceptible to chloramphenicol and cefoxitin, while the highest resistance, 116/124 (93.5%), was noticed for penicillin. Resistance to other antimicrobials varied between 1.6-28.2%. Of the 124 *S. aureus* isolates, 80 (64.5%) possessed *spa* gene, with 76/80 (95.0%) harboring more than seven tandem repeats. One of the *S. aureus* isolates, 1/124 (0.8%), harbored a *mecA* resistance gene. The presence of antimicrobial-resistant *S. aureus* isolates in raw bovine milk at the farm level is alarming and requires herd health improvement interventions to protect society.

Keywords: Milk, Morogoro, Tanzania, virulence, *mecA*, *Staphylococcus aureus*

Citation: Kalee, N. E., Gahamanyi, N., and Hoza, A. S. 2021. Prevalence and antimicrobial susceptibility profiles of *Staphylococcus aureus* from raw bovine milk in dairy and pastoral farms in Morogoro region, Tanzania. Ger. J. Vet. Res. 1 (2): 1-7. https://doi.org/10.51585/gjvr.2021.2.0007

Introduction

Staphylococcus aureus is an economically important pathogen in the dairy industry (Mekonnen et al., 2018) that causes mastitis. It can be spread to humans by consuming foods of animal origin, such as raw/unpasteurized milk (Ateba et al., 2010). Transmission of *S. aureus* can be facilitated by either a cow infected with mastitis or unhygienic handling and processing of milk and related products (Ngasala et al., 2015). The severity of *S. aureus* infections in animals or humans is enhanced by producing newly evolved virulence factors and acquiring resistance to methicillin, resulting in methicillin-resistant *S. aureus* (MRSA) (Akkou et al., 2018).

The *S. aureus* protein A is an important virulence factor encoded by the *spa* gene, which contributes to the development of diseases by evading phagocytosis (Yadav et al., 2015). Methicillin resistance is triggered by either *mecA* or *mecC* gene acquisition (Kalayu et al., 2020). The two *mec* genes code for penicillin-binding protein 2a (PBP2a), whose expression induces resistance to virtually all β-lactam antibiotics (Harrison, 2014). The dairy industry in Tanzania faces various challenges; however, milk contamination with antimicrobial-resistant (AMR) pathogens from various sources stands to be one of the major concerns.

The misuse of antimicrobials in veterinary medicine for therapeutic purposes or growth promotion accelerates the occurrence of AMR microbes in livestock and their spread to humans (Massawe et al., 2019). Easy access to antimicrobials from unauthorized drug sellers and a lack of knowledge on antimicrobial use and resistance among livestock keepers complicates the AMR issue (Kimera et al., 2020). It is estimated that without adequate actions by the year 2050, mortality rates due to AMR will reach 10 million people globally and cost $100 trillion (Burki, 2018).

The current study aimed to determine the prevalence, antimicrobial susceptibility profiles, and molecular characteristics of *S. aureus* from raw bovine milk in dairy and pastoral farms in Morogoro urban and Mvomero districts, Tanzania. The majority of dairy cattle in the Morogoro urban district dairy farms are crosses of Friesian, Ayrshire, and Jersey, which are managed in a semi-intensive system, where cattle are kept in sheds for some time and fed with cut and carry pasture. Contrary, the model of production in pastoral communities of the Mvomero district is free grazing of large herds of cattle in open grazing lands where most of the dairy cattle are indigenous Tanzanian short horn zebu and their crosses of Friesian, Ayrshire, and Jersey.

Despite the first launch of the National Action Plan on AMR in Tanzania in 2017, we hypothesized that (i) *S. aureus* strains are resistant to *β*-lactam and tetracycline antimicrobials and (ii) poor hygienic conditions are contributing to the sustained presence of AMR *S. aureus* strains. Currently, there is a scarcity of information on the prevalence and drug resistance profiles of *S. aureus* from raw bovine milk in the Morogoro region, Tanzania. Therefore, our study will be significant in informing veterinary and public health authorities on appropriate control and prevention strategies for AMR in the study area.

Materials and methods

Study area and design

A cross-sectional study was carried out in Morogoro urban and Mvomero districts in the Morogoro region, Tanzania (Figure 1), from October 2019 to January 2020.

Ethical approval and informed consent

This study was approved by the institutional review board of Sokoine University of Agriculture (Ref. SUA/ADM/R.1/8/428). Farmers also gave a verbal or a signed written consent form before sampling their cows.

Sample size and collection

A total of 397 lactating dairy cows were sampled. The milk samples were pooled from all the teats of individual cows. Briefly, the udder was cleaned with sterile water, and the first stream of milk (foremilk) was aseptically discarded using gloved hands. Ten milliliters of milk were collected from each cow into a sterile universal bottle. All samples were packed in a cool box and transported to the laboratory within 2 hours of collection for further processing. A semi-structured questionnaire was used to collect data on dairy farming practices from each farm.

Bacterial isolation and identification

S. aureus isolation was performed as previously described by Jahan et al. (2015). Briefly, 10 µl of milk sample was streaked onto 5% horse blood agar (BA) (HiMedia, India), and plates were incubated at 37°C for 24 hours. Presumptive colonies based on the morphology and hemolytic pattern on BA were Gram-stained. Then, a sub-culture was made on mannitol salt agar (MSA) (HiMedia, India) and incubated at 37°C for 24 hours.

Golden-yellowish colonies were picked for biochemical tests (catalase and slide/tube coagulase). Coagulase-positive isolates were stored on nutrient agar slants at -20°C until further analysis.

Antimicrobial Susceptibility Testing (AST)

The AST was carried out on confirmed isolates using the agar disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2019). The following antimicrobials that are commonly used for the treatment of mastitis and growth promotion in livestock production and as primary class of antibiotic for the treatment of bacterial infections in humans (Global Antibiotic Resistance Partnership-Tanzania Working Group, 2015) were used: oxacillin (1 µg), cefoxitin (30 µg), amox- icillin/clavulanic acid (30 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg), tetracycline (30 µg), chloramphenicol (50 µg) and penicillin G (10 IU) (Liofilchem, Italy). Briefly, two to three colonies were picked and suspended in sterile normal saline. Turbidity of bacterial suspension was adjusted to 0.5 McFarland standard, then streaked onto the surface of sterile Muller Hinton agar (Oxoid, UK) using sterile cotton swabs and allowed to dry. Antimicrobial disks were placed on the inoculated agar plate surfaces using sterile forceps and incubated at 37°C for 24 hours. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as quality control strains. The diameter of inhibition zones was measured and interpreted as sensitive (S), intermediate (I), or resistant (R) according to the Clinical and Laboratory Standard Institute table (CLSI, 2019).

DNA extraction and molecular characterization

Genomic DNA of *S. aureus* was extracted using Quick DNA fungal/bacterial Miniprep Kit (ZymoResearch Corp, USA) according to the manufacturer's instructions. Screening for virulence and MRSA encoding genes was performed by multiplex polymerase chain reaction (PCR) as previously described by Stegger et al. (2012). PCR mixture contained 12.5 µL of the master mix, 5.5 µL of nuclease-free water, 0.5 µL of each of the sets of primers, and 3 µL of bacterial genomic DNA. Thermal cycling conditions were initial denaturation at 94°C for 5 minutes, then 35 cycles of 94°C for 30 seconds, 59°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 2 minutes. Agarose gel (2%) electrophoresis was used to separate PCR products in 1×TAE buffer with 10 µL EZ Vision staining dye (VWR, USA) at 120 volts for 60 minutes using Midi plus 15 electrophoresis system (VWR, USA). A 100bp DNA ladder (ZymoResearch Corp, USA) was used as a molecular marker to estimate the amplified PCR products' size. The number of tandem repeats (N) in *spa* gene was calculated using the formula reported by Fr´enay et al. (1996).

Data analysis

Collected data were analyzed using a Microsoft Excel spreadsheet and Epi info (Version 7). All results at $p<0.05$ were considered statistically significant.

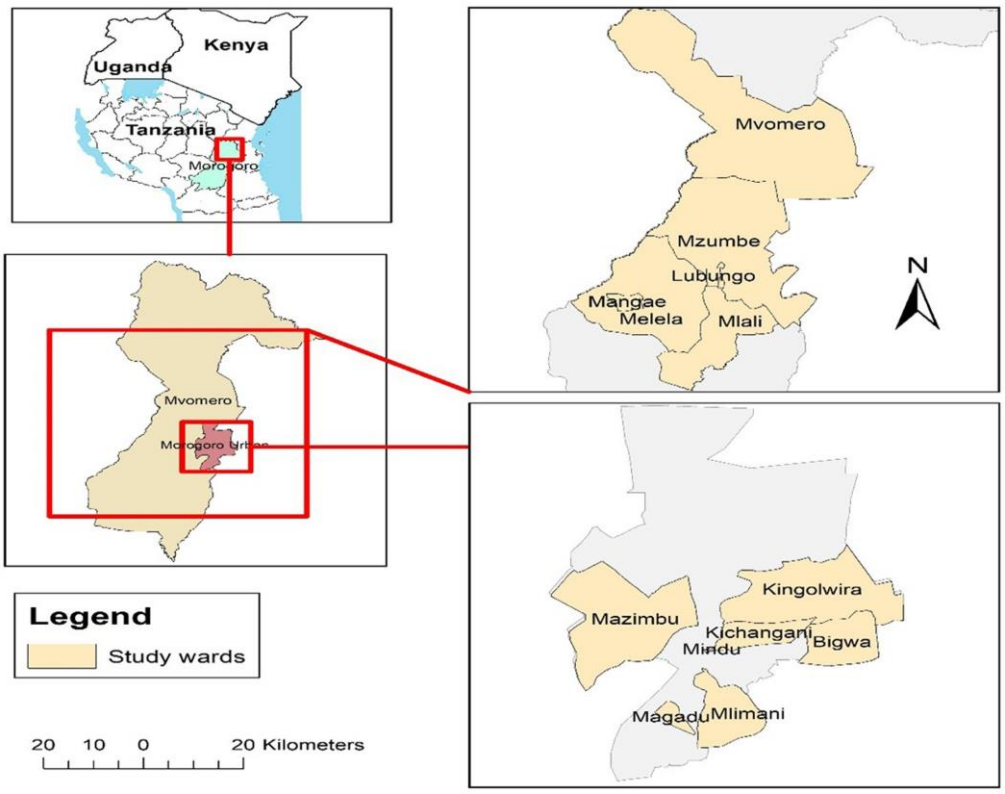


Figure 1: A map showing the study wards in Morogoro Urban and Mvomero districts, Tanzania.

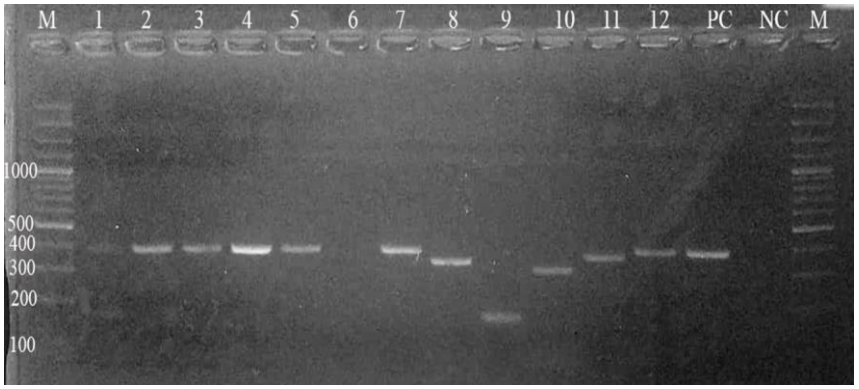


Figure 2: PCR amplification of *spa* gene in *S. aureus* isolates; *spa* gene positive isolates (Lane 1, 2, 3, 4, 5,7, 8, 9, 10, 11 and 12), *spa* gene negative isolate (Lane 6), PC; positive control, NC; negative control, M; DNA ladder marking from 100bp to 1kb.

Results and discussion

Out of 397 raw milk samples, *S. aureus* was isolated from 124 samples with a prevalence of 31.2%. Notably, dairy farms had a higher prevalence of 78/190 (41.1%) than pastoral farms 46/207 (22.2%). The observed prevalence of *S. aureus* is slightly lower than the previous prevalence reported in dairy farms of the Sokoine University of Agriculture, Tanzania, at 49% (Kashoma et al., 2015) and in local markets of Morogoro Municipality at 41% (Mohammed et al., 2018). Other studies in Ethiopia (Elemo et al., 2017) and Pakistan (Maalik et al., 2019) reported similar prevalence at 39.2% and 34.2%, respectively. However, it is also slightly higher than reports of 16.7% in smallholder dairy farms in Mbeya, Tanzania (Massawe et al., 2019),20.3% in Uganda (Asiimwe et al., 2017) and 22.5% in India (Hamid et al., 2017).

Milk contamination by *S. aureus* can occur through the shedding of the organism into milk from cows with clinical or subclinical mastitis (McMillan et al., 2016) or unhygienic milking practices during pre-milking udder preparation and cleaning of milking utensils/equipment (Gwandu et al., 2018). The hands of milkers can also be a primary source of microbial transmission during milking (Orwa et al., 2017). This study found that most of the dairy cows were kept and milked on dirty or muddy floors, utensils were not properly washed, and most milk handlers did not use detergents to clean their hands before and after milking each cow (Table 1). Similar unhygienic activities are responsible for the contamin-

Table 1: Association between dairy farming practices and prevalence of *S. aureus* isolates from raw bovine milk in dairy and pastoral farms of Morogoro Urban and Mvomero districts, Tanzania.

Variables	Category	Frequency (%)	x^2 value	p-value	CI-95%
Hygiene status of the farm	Good	3 (9.38)	6.790	0.009	1.313-14.687
	Poor	29 (90.62)			
Floor-type	Concrete	5 (15.63)	3.431	0.064	0.923-6.519
	Soil	27 (84.37)			
Cleaning quality of milking utensils	Good	7 (21.87)	1.223	0.269	0.683-3.851
	Poor	25 (78.13)			
Milking method	Hands	30 (93.75)	50.295	<0.001	0.007-0.129
	Machine	2 (6.25)			
Hand pre-washing	With detergent	1 (3.12)	11.332	<0.001	1.901-104.316
	Without detergent	31 (96.88)			
Handwashing after milking every cow	Yes	1 (3.12)	11.332	<0.001	1.901-104.316
	No	31 (96.88)			
Udder/teat washing before milking	Yes	32 (100)	60.514	n/a	0
	No	0			
Antibiotics usage	Yes	32 (100)	60.514	n/a	0
	No	0			
Observing withdrawal periods	Yes	29 (90.62)	45.524	n/a	0.014-0.157
	No	3 (9.38)			
AMR awareness	Yes	10 (31.25)	0	n/a	0.459-2.174
	No	22 (68.75)			

Table 2: Antimicrobial susceptibility profiles of *S. aureus* isolates (n=124) from raw bovine milk in dairy and pastoral farms of Morogoro Urban and Mvomero districts

Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Penicillin G	8 (6.5)	0	116 (93.5)
Tetracycline	75 (60.5)	14 (11.3)	35 (28.2)
Oxacillin	70 (56.5)	26 (21.0)	28 (22.6)
Amoxicillin-clavulanic acid	93 (75.0)	0	31 (25.0)
Gentamicin	111(89.5)	3 (2.4)	10 (8.1)
Trimethoprim-sulfamethoxazole	122 (98.4)	2 (1.6)	2 (1.6)
Chloramphenicol	124 (100)	0	0
Cefoxitin	124 (100)	0	0

tion of milk by *S. aureus* has also been documented in prior studies (Reta et al., 2016; Elemo et al., 2017). Therefore, cleanliness in dairy farms is essential for hygienic milk production and animal welfare (Hauge et al., 2012).

Antimicrobial-resistant *S. aureus* negatively affects the management of the associated infections in both animals and humans (WHO, 2017). The antimicrobial susceptibility profiles of 124 *S. aureus* isolates from dairy and pastoral farms are shown in Table 2. *S. aureus* isolates showed high susceptibility to chloramphenicol cefoxitin, followed by trimethoprim-sulfamethoxazole, gentamicin, and amoxicillin-clavulanic acid. A study in Ethiopia (Abunna et al., 2016) also reported high susceptibility to chloramphenicol (81.8%), trimethoprim-sulfamethoxazole (81.8%), and gentamicin (94.5%). On the other hand, *S. aureus* isolates exhibited high resistance to penicillin G, which concurs with studies carried out in South Korea (Hamid et al., 2017) and Ethiopia (Marama et al., 2016). Lower resistance to penicillin G (15.8%) has been reported in India from bovine mastitis milk samples (Patel et al., 2017). Higher resistance to penicillin in this study area could be associated with its widespread use in Tanzania as a primary choice antibiotic for treating livestock infections (Katakweba et al., 2012; Kashoma et al., 2015).

The multidrug resistance pattern was observed in 5/124 (4.0%) *S. aureus* isolates (penicillin, tetracycline, gentamicin, trimethoprim-sulfamethoxazole), which is substantially lower than the previous report of (26.1%) in Morogoro Municipal

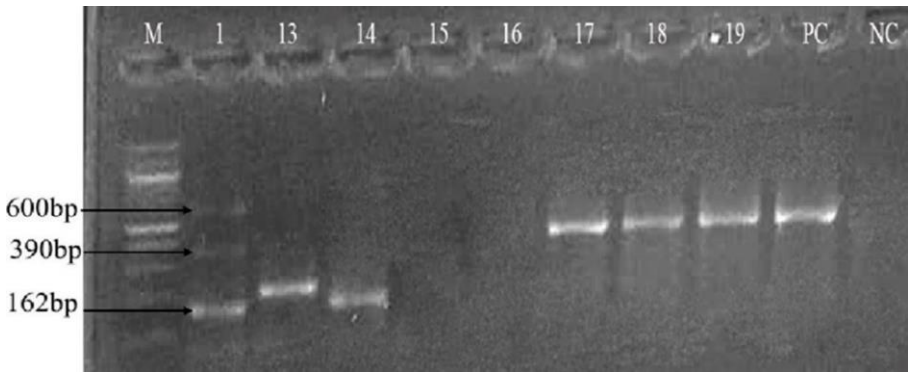


Figure 3: Multiplex PCR detection of *mecA* and *mecC* gene; *S. aureus mecA* (162bp) positive isolate (Lane 1), *S. aureus spa* gene positive isolates (Lane 1, 13, 14, 17, 18, 19); *S. aureus spa* negative isolates (Lane 15, 16); PC, positive control; NC, negative control; M, DNA ladder marking from 100bp to 1kb.

(Mohammed et al., 2018) and comparable with the prior report of Elemo et al. (2017). The occurrence of AMR *S. aureus* in milk is majorly attributed to the uncontrolled use of antimicrobials in the treatment of livestock infections and as feed supplements to promote the growth of animals and improve productivity. Misuse of antimicrobials has been previously reported in the Morogoro region and other regions of Tanzania, where farmers treat their animals without proper diagnosis, use expired antimicrobials, arbitrarily combine drugs, fail to observe the recommended therapeutic doses and withdrawal periods (Mohammed et al., 2018; Massawe et al., 2019).

Out of 124 *S. aureus* isolates, 80 (64.5%) produced amplicons of *spa* gene (Figure 2) with eight different product sizes amplified at 180, 200, 300, 340, 370, 390, 400, and 600 bp with 6, 6, 11, 12, 14, 14, 15 and 23 number of tandem repeats, respectively. This finding is in agreement with other studies in India (Yadav et al., 2015; Bhati et al., 2016), which reported seven to nine diverse-sized *spa* gene bands ranging from 120bp to 330bp.

Out of 80 *S. aureus* isolates possessing *spa* gene, 76/80 (95.0%) were harboring more than seven tandem repeats. The most frequent tandem repeat observed was 14 in 70/80 (87.5%) of the *S. aureus* isolates. The observed wide degree of polymorphism confirmed the pathogenicity potential of *S. aureus* isolates in dairy cows. This study did not detect any *S. aureus* isolate carrying the Panton-Valentine leukocidin (PVL)-encoding gene at 85bp. PVL-encoding genes are rare in the bovine population, and their presence could indicate transmission from humans during milking (Shrivastava et al., 2018).

None of the isolates was phenotypically identified as MRSA positive in the present study, but PCR revealed 1/124 (0.8%) isolate as *mecA* positive amplified at 162bp. There were no positive isolates in the detection of *mecC* to confirm the *S. aureus* resistance gene at 138 bp. Failure to detect the *mecA* gene phenotypically could be linked to inconsistencies in antimicrobial susceptibility tests efficiency or lack of *mecA* gene expression by the specific isolate (Cabrera-Contreras et al., 2019).

Similar to this study, discrepancies have been reported between cefoxitin disk diffusion and *mecA*-based PCR results on MRSA in Uganda (Asiimwe et al., 2017) and Italy (Corrente et al., 2007). Furthermore, a study conducted in Ethiopia did not find any *mecA* gene among *S. aureus* isolates of milk origin (Kalayu et al., 2020).

Sequencing techniques are higher in terms of sensitivity in detecting low-frequency variants. This study did not use sequencing techniques to confirm the presence of resistant genes in *S. aureus* due to the limitation of funds. Nevertheless, multiplex PCR was able to detect the low prevalence of MRSA in raw milk, providing a baseline for future research to consider confirming resistance genes using sequencing techniques and including nasal and hand swab samples of milk handlers to monitor *S. aureus* transmissions between humans and cows. It is worth mentioning that pastoralists did not consent to use hand gloves during the collection of milk samples, but we used hand sanitizer to reduce the risk of contamination.

Conclusions

S. aureus isolates were prevalent in raw bovine milk from both dairy and pastoral farms of the Morogoro region, Tanzania, and were associated with poor hygienic milking practices. The isolated *S. aureus* showed varying degrees of resistance to the tested antimicrobials with higher resistance to penicillin. This calls for concerted efforts for a better implementation of the National Action Plan for AMR in Tanzania. We also recommend campaigns on the awareness of the AMR issue and regular training on proper farm hygienic practices at the community level.

Article Information

Funding. This research was funded through a Master's scholarship offered to Nancy E. Kalee by the Inter-University Council of East Africa/World Bank Women scholarship program.

Conflict of Interest. The author declares no conflict of interest.

Acknowledgments. We sincerely thank Mrs. Enesa Mlay and the livestock extension officers for providing the needed support during data collection.

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