Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2021.1.7

Research Article
Quantitation of *mec*A and *sea* genes on *Staphylococcus aureus* using Quantitative PCR Assay

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Abstract

Background and Objective: Methicillin Resistant *Staphylococcus aureus* (MRSA) is one of the major causes of nosocomial infections and are most profound in community in previously healthy individuals. To detect and quantify antibiotic resistant and virulence genes present in methicillin sensitive *S. aureus* (MSSA) strains from wounds and burns patients. **Materials and Methods:** About 200 clinical samples were obtained for *S. aureus* isolation, identified and characterized by using standard microbiological procedures. Methicillin resistance was determined by using β-lactamase assay and oxacillin disk (Oxoid) susceptibility test. Quantification of the *S. aureus* strains was performed using quantitative Polymerase Chain Reaction (qPCR) assay. Agarose gel electrophoresis was carried out on the qPCR products using 1.5% agarose gel with a standard DNA ladder (100 bp), visualized under UV transilluminator and the image taken using digital camera. **Results:** Almost 44 (22%) *S. aureus* were isolated and characterized with 36 (82%) strains producing β-lactamase and were resistant to oxacillin (MRSA) while, 8 (18%) strains do not produce β-lactamase and were sensitive to oxacillin (MSSA). The β-lactamase and non-β-lactamase isolates were resistant to other antibiotics. The quantification of PCR products indicated that *sea* genes (virulence enterotoxin factor) were detected from the antibiotic resistant staphylococci ranging from 0-13551.84 nmoles while, the quantification of *mec*A genes detected ranged from 0-2601.76 nmoles. The agarose gel electrophoresis of the PCR products of *mec*A and *sea* genes showed amplicon size of 657 bp for *mec*A and 526 bp for *sea* genes after amplification of the antibiotic resistant *S. aureus* strains. **Conclusion:** This study detected the presence of antibiotic resistant and virulence genes associated with MRSA in MSSA, which calls for urgent clinical and pharmaceutical attention.

Key words: Antibiotic resistant gene, virulence gene, quantitative polymerase chain reaction, methicillin resistant *Staphylococcus aureus*, methicillin sensitive *Staphylococcus aureus*, β-lactamase assay, agarose gel electrophoresis

Citation: S.K.S. Ojo, F.I. Esumeh and G.T. Sunmonu, 2021. Quantitation of *mec*A and *sea* genes on *Staphylococcus aureus* using quantitative PCR assay. Res. J. Microbiol., 16: 1-7.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Staphylococcus aureus is a general normal flora of humans and its primary habitat is the moist squamous epithelium of the anterior nares[1]. From the report of Mansour *et al.*[1], MRSA accounted for 55.1% in wound infections with 18 *mec*A genes of 29 MRSA. It has become apparent that these organisms among hospital staff provides a source for infection in hospitalized patients especially in pediatric and intensive care units, thus, making nasal carriage rate to be higher among hospital staff and patients than in the community[2].

Previous studies were critical on Methicillin resistant *S. aureus* (MRSA) and Methicillin sensitive *S. aureus* (MSSA) which has posed a serious therapeutic challenge and multidrug resistance among hospitalized individuals with the detection of *mec*A genes and other virulence genes[3,4]. Out of the 512 MSSA strains, 449 (87.7%) were resistant to penicillin while, 155 (89.6%) of 173 MRSA were resistant to penicillin as reported by Naik and Teclu[2]. Also, various serological enterotoxin types were detected in *S. aureus* causing food poisoning in humans and animals have been isolated from foods, faeces and healthy carriers[5]. Enterotoxin *sea* genes were reported by Saadati *et al.*[5] to be present in *S. aureus* isolated from 95 nasal carriers among other enterotoxins.

However, owing to paucity of information on the methicillin sensitive strains and the detection of both resistant and virulent genes from other studies have not been linked with wound and burn samples, thus the need for this study.

MATERIALS AND METHODS

Study area: Clinical samples were obtained from different tertiary hospitals within Delta state, Nigeria and processed at the Department of Microbiology, Ekpoma, Edo state, Nigeria and Department of Microbiology, Federal University, Oye-Ekiti, Ekiti state, Nigeria, within the period of 2017 and 2019.

Research procedure: Two hundred clinical wound and burn samples were obtained with sterile cotton swabs from tertiary health care facilities within Delta state, Nigeria, for *S. aureus* isolation. The cotton swabs were applied on freshly prepared nutrient agar and Mannitol Salt Agar (MSA) and were incubated at 37°C for 24 h. Identification and characterization were done by using standard microbiological procedures.

β-lactamase assay: Strips of starch paper (4×7 cm) were cut and disinfected with 70% ethanol before been soaked for 10 min in benzyl penicillin dissolved in phosphate buffer. The method as described by Ojo *et al.*[6] was employed.

Determination of antibiotic resistance profile: Antibiotic susceptibility of *S. aureus* strains by disk diffusion method[7] were performed by using overnight inocula marched with 0.5 McFarland turbidity index. The *S. aureus* strain (ATCC 25923) was used as positive control. Multiple antibiotic resistances of the strains were determined based on the number of resistance pattern of each strain to the exposed antibiotics.

Detection of *mec*A and *sea* genes by quantitative PCR (qPCR)

Chromosomal DNA extraction: Chromosomal DNA was extracted following the procedure of Mansour *et al.*[1] by using an overnight subculture of the *S. aureus* strains. The bacterial genomic DNA isolation kit was obtained from Norgen Biotek Corp., Thorold, ON, Canada and the extraction was based on manufacturer's manual.

The DNA sequence for *mec*A gene used in this study contains 657 bp, which is:

TTTCCTCTATTCGTATTTTTTATTACCGTTCTCATATAGCTCATCATACACTTTACCTG
AGATTTTGGCA while the *sea* gene has 526 bp sequence:
ATCCTAATTACTTTCATAACCTATAATCCTTCTCTATGAAGGTTCCAACAAGTTGTTA
TGATTGCAGTCG

Primer and probe design: The oligonucleotide primers and probes used for duplex qPCR assay in this study were purchased from Inqaba Biotechnical Industries (Pty) Ltd., South Africa (Table 1)[8].

The followings represent the DNA sequences of both *mec*A and *sea* genes as reported and obtained from NCBI entrez GenBank[8].

DNA sequence for *mec*A gene used in this study contains 657 bp, which is shown as[8]:

TTTCCTCTATTCGTATTTTTTATTACCGTTCTCATATAGCTCATCATACACTTTACCTG
AGATTTTGGCA
TTGTAGCTAGCCATTCCTTTATCTTGTACATCTTTAACATTAATAGCCATCATCATGT
TTGGATTATCTT
TATCATATGATATAAACCACCCAATTTGTCTGCCAGTTTCTCCTTGTTTCATTTTGAG
TTCTGCAGTACC
GGATTTGCCAATTAAGTTTGCATAAGATCTATAAATATCTTCTTTATGTGTTTTATTT
ACGACTTGTTGC
ATACCATCAGTTAATAGATTGATATTTTCTTTGGAAATAATATTTTTCTTCCAAACTT
TGTTTTTCGTGT
CTTTTAATAAGTGAGGTGCGTTAATATTGCCATTATTTTCTAATGCGCTATAGATTG
AAAGGATCTGTAC
TGGGTTAATCAGTATTTCACCTTGTCCGTAACCTGAATCAGCTAATAATATTTCATT
ATCTAAATTTTTG
TTTGAAATTTGAGCATTATAAAATGGATAATCACTTGGTATATCTTCACCAACACCT
AGTTTTTTCATGC
CTTTTTCAAATTTCTTACTGCCTAATTCGAGTGCTACTCTAGCAAAGAAAATGTTAT
CTGATGATTCTAT
TGCTTGTTTTAAGTCGATATTACCAAT

Table 1: List and characteristics of oligonucleotide primers and probes used in the duplex qPCR

Gene and Primer or probe name	Sequence (5' 3')	L (bp)	Amplicon size (bp)	GC (%) min/max	Tm min/ max	Conc. (nmole)	Optical Density (OD)	GenBank accession No.	Reference
***mec*A**			657						
F *mec*A	CCCAATTTGTCTGCCAGTTT	20		45/45	58.35/58.35	36.35	7.1060	NC002745.2	Jang *et al.*[8]
R *mec*A	TCAGGTTACGGACAAGGTGA	20		50/50	60.4/60.4	26.99	6.2453		
P *mec*A	CAGTACCGGATTTGCCAATT	20		45/45	58.35/58.35	7.39	1.5751		
sea			526						
F *sea*	GTCGTGTGACGTGCATCAAT	20		50/50	60.4/60.4	32.60	6.9692	NC002745.2	Jang *et al.*[8]
R *sea*	AATAAAAAGCCATGCCGATG	20		40/40	56.3/56.3	31.14	7.5050		
P *sea*	ATGAGTTGGGCAAGATGGTT	20		45/45	58.35/58.35	36.35	0.7923		

F: Forward, R: Reverse, P: Probe (Taqman), L: Length of primer in Base Pair (bp)

The *sea* gene has 526 bp sequence and it is as shown as[8]:

ATCCTAATTACTTTCATAACCTATAATCCTTCTCTATGAAGGTTCCAACAAGTTGTTA
TGATTGCAGTCG
TGTGACGTGCATCAATTACTGCGGATTATTAGGTTAAGGGAGGTGGATATAATGA
GTTGGGCAAGATGGT
TATCATGTTGATTTGTATGGTCGTAAACTGTAAATAATGTTTTTGGTCAGTGCATCG
GCATGGCTTTTTA
TTTTGATTGAAAACGAGGTACGTACATGGTATTACACAGCTCAAAAGACAGGAAG
CATACTGCAAGTGAA
GTTGGGAAGTGTTGTTAATACCAAGTAAGTAAGATATCTGAAATGTATAATAGAGT
AAAAATGAAATCTT
TTTATTATAGACAAATATAAAAAGTGTATAGTAATATATGTATGTATAATTAAATGA
TAATCATTTCATA
ATTATTGTATATAACTAAATAACTACTTAACAAAAATAATTATGCTTTAGAGGTGAG
CAAAATGAAAAAA
ACAGCATTTACATTACTTTTATTCATTGCCCTAACG

Amplification of *mec*A and *sea* genes using quantitative PCR (qPCR) assay: Modified protocol of Grisold *et al.*[9] was employed for the amplification of *mec*A and *sea* genes in methicillin resistant and sensitive *S. aureus* strains. Duplex quantitative PCR analysis working solution were prepared by dispensing 16 µL of each reconstituted primers and probes into separate eppendorf tubes and made up with 184 µL of PCR grade water, vortexed for 10 sec followed by the addition of 1 µL each of the forward and reverse primer and 1 µL of the probe. A 2 µL of *sea* DNA template was added to the content of the PCR tube followed by the addition of 25 µL of 2×PCR Master Mix and the reaction mixture was brought to a total volume of 50 µL using Nuclease-free water. The PCR mixture was vortexed and spinned down briefly. The *mec*A DNA template was also added as in *sea* DNA template. The PCR tubes were placed into the Hybaid OmniGene thermocycler PCR instrument (Model no: TR3SM2). The PCR cycle conditions used in this study were an initial denaturation process at 95°C for 2 min at 1 cycle, another denaturation process at 95°C for 30 sec, followed by annealing at 65°C for 30 sec and an extension at 72°C for 1 min in 40 cycles. The final extension temperature of 72°C ran for 5 min in 1 cycle while, the hold temperature of 10°C was held for about 120 min in 1 cycle.

The PCR products were then subjected to quantification in a Thermomax Microplate reader (Molecular Devices) at a wavelength of 405 and 450 nm. To determine the absolute quantity of the resistant and virulence genes (*mec*A and *sea*), 50 µL of PCR grade water was dispensed in to seven microtiter well with 5µL of the working solution of primer (for *mec*A and *sea* genes using separate microtiter plates) in the 1st well.

Serial dilution was carried out by dispensing 5 µL of the mixture in well 1 to well 2 and continuously to the 7th well. A 30 µL of the mixture was discarded from well (1-7), while 20 µL of the primers and PCR grade water were dispensed into 8th and 9th well, respectively, the 10th well as blank, all serving as standards for the quantification. A 20 µL of the PCR products from the 44 multiple antibiotic resistant *S. aureus* strains were introduced into the other wells. The reading of the samples for quantification was done within 10 sec and the data was analyzed by using MYASSAYS software.

Agarose gel electrophoresis of PCR products: The method of Sambrook and Russell[10] with some modification was employed for the electrophoresis of the PCR products. Agarose powder of 1.5 g was dissolved in 100 mL of diluted 1×TBE and heated to dissolve in temperature controlled water bath at 70°C and allowed to cool to 50°C. A 20 µL ethidium bromide was added to the cooled agarose solution, the gel was allowed to set for 30 min. The PCR products of 0.2 µL were mixed with 0.2 µL of 6×TBE sample buffer (270 mM, Tris, 270 mM Boric acid, 0.025% Bromophenol blue, 0.025% Xylene cyanol and 6 mM EDTA) on a microtiter plate while, 0.2 µL of the mixture was pipetted into the agarose gel well. A standard DNA molecular weight ladder (50 bp-1 kb) of PCR ranger 100 bp was loaded on one of the wells as standard marker. The gel was thereafter, electrophoresed in a horizontal tank at a constant voltage of 100 volts for 30 min. The DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light trans illuminator and the photograph taken with a digital camera.

Statistical analysis: The SPSS version 20 software was used for the statistical analysis of the data. A p-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$).

RESULTS AND DISCUSSION

Quantitative expression of *mecA* and *sea* genes from multiple antibiotic resistant *Staphylococcus aureus*: Forty four *S. aureus* strains from our study yielded 36 MRSA and 8 MSSA (Table 2). Out of the 8 MSSA, 7 (87.5%) had *mec*A antibiotic resistant genes while, 5 (62.5%) had virulence *sea* genes. All the 36 MRSA strains had *mec*A antibiotic resistant genes while, 33 MRSA strains had virulence *sea* genes (Table 2).

The amount of *sea* genes (virulence factor) expressed by the multiple antibiotic resistant *S. aureus* ranges from 0-13551.84, while *mec*A expressed genes ranges from 0-2601.76 (Fig. 1). The MSSA *mec*A genes ranged from 0-1037.46 and MSSA *sea* genes ranged between 0 and 1278.12 (Fig. 1). The amplification of the PCR products of MRSA and MSSA revealed the presence of *mec*A and *sea* genes on the agarose gel electrophoresis plate (Fig. 2).

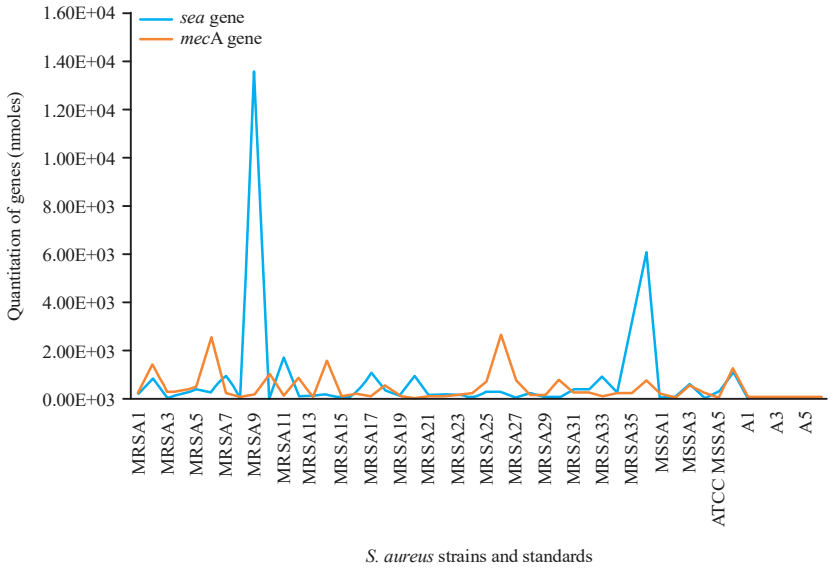

Fig. 1: Quantitative expression of *mec*A and *sea* genes from methicillin resistant and sensitive multiple antibiotic resistant *Staphylococcus aureus* strains
A1-A4: Standards as primers and PCR grade water

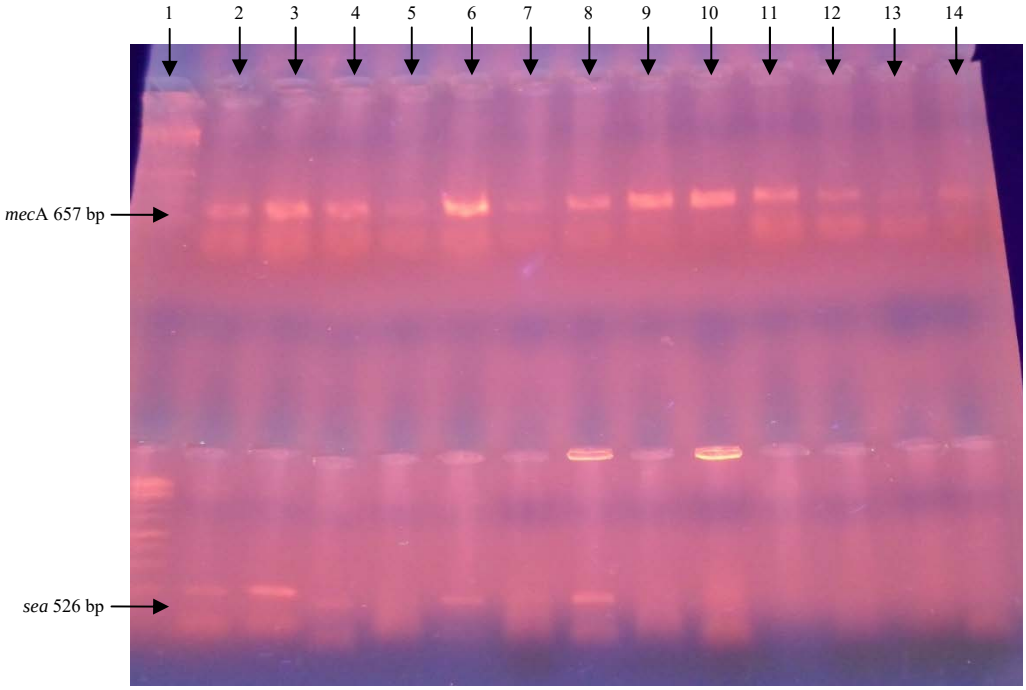

Fig. 2: Agarose gel electrophoresis on the PCR products after amplification of *mec*A (above) and *sea* (below) genes on the multiple antibiotic resistant *Staphylococcus aureus* strains (lanes 2-14). Lane 1: Molecular weight marker (PCR ranger 100 bp DNA ladder: 50 bp-1 kb)

Table 2: Quantitative detection of *mec*A and *sea* genes from methicillin resistant and sensitive multiple antibiotic resistant *Staphylococcus aureus* strains

	Resistant and virulent genes	
Staphylococci strain	*mec*A	*sea*
MSSA (n = 8)	7 (87.5%)	5 (62.5%)
MRSA (n = 36)	36 (100%)	33 (91.7%)
Total (n = 44)	43 (97.7%)	38 (86.4%)

MSSA: Methicillin sensitive *S. aureus*, MRSA: Methicillin resistant *S. aureus*

The absolute quantification value of *mec*A (methicillin-resistant) and *sea* (enterotoxin) genes present in each strain of MRSA and MSSA from this study was not in tandem with most studies on the quantitative determination of various genes encoding resistance in MRSA, which were reported as the frequency of threshold cycles (C_T) and melting point (Tm) curves[9,11].

The *sea* gene detected from this study revealed its presence in 38 (86.4%) of 44 multiple antibiotic resistant methicillin resistant and sensitive *S. aureus*, which was higher in comparison to the reports of previous authors. Saadati *et al.*[5] reported isolating 24 (25.3%) of 56 strains of *S. aureus* associated with the *sea* gene (at 552 bp) while Lovseth *et al.*[12] reported detection of *sea* genes from staphylococci. Sauer *et al.*[13] revealed an incidence of 7 (12.1%) *sea* genes from the 58 skin and wound swab isolates as well as other enterotoxin genes, Klotz *et al.*[14] detected 12 positive *sea* gene isolate out of 44 isolates, while Omoe *et al.*[15] detected 4 (5.6%) of *sea* gene and 18 (25.4%) combined *sea, seb* and *seh* genes from human isolates with food poisoning. Mehrotra *et al.*[16] with 19.6% *sea* genes from nasal swabs isolates and Becker *et al.*[17] with 15.9% *sea* genes from blood and nasal swabs isolates. Thus, the prevalence rate of the virulence enterotoxin genes among in-patients and out-patients in developing countries posed great threat to health of the populace since they are frequently associated with staphylococcal food poisoning.

Previous real-time PCR assays have demonstrated the capability of rapidly detecting MRSA from culture. Report on duplex assay for *mec*A and *S. aureus* specific gene has demonstrated 100% sensitivity and specificity in detecting and differentiating *Staphylococcus* spp. from pure strain isolates[18], which was also observed in this study.

The presence of *mec*A gene in the 36 multiple antibiotic resistant MRSA and 7 of the 8 multiple antibiotic resistant MSSA is very disturbing and alarming especially with high number of quantified genes present. The report of Al-Ruaily and Khalil[19] and Khan *et al.*[20] corroborated with the result of this study with 13 *mec*A gene expression out of 15 isolates

and 33 *mec*A genes of 35 MRSA isolates, respectively. An earlier study[1] observed that out of 29 MRSA selected randomly, 18 were *mecA* positive strains and 11 *mecA* negative strains even though the 29 strains were confirmed to be methicillin resistant by methicillin disk diffusion susceptibility method. In another study[4], a 100% *mec*A gene were reported on all the 35 isolates studied, a study by Grisold *et al.*[9], who showed that 108 of 109 MRSA gave positive results for *mec*A gene, while Jonas *et al.*[21] reported the detection of 64 *mec*A products out of 147 swabs with no *fem*B. Conversely, 6 *mec*A genes out of 93 *S. aureus* strains tested were detected and reported[14]. Out of 29 MRSA that was selected randomly, 18 *mecA* positive strains and 11 *mecA* negative strains were identified[1].

It is noteworthy that 5 (62.5%) strains showed presence of *sea* enterotoxin gene and 7 (87.5%) strains showed *mec*A gene of the 8 multiple antibiotic resistant MSSA strains. It is suggested that the gene encoding for methicillin resistance in these strains could be mediated by other process different from PBP2a, which include: hyper-production of β-lactamase, modified PBP genes or horizontal gene transfer of PBP2a and could be transferred to new cells after some generation becoming resistant to available antibiotics. This was similar to previous studies[18], but contrary to the findings of Fosheim *et al.*[22], who detected 141 *mec*A gene out of 142 MRSA isolates and none in 6 MSSA or in any methicillin susceptible coagulase negative staphylococci. Jonas *et al.*[21] negated the result of this study with no detection of *mec*A in 47 oxacillin susceptible *S. aureus*. However, to the best of our knowledge, detection of *mec*A and *sea* genes in multiple antibiotic resistant MSSA strains has poorly been reported.

CONCLUSION

The emerging and re-emerging development in the detection of *mec*A and *sea* genes among multiple antibiotic resistant *S. aureus* strains could be traced to the presence of extra-chromosomal property (Plasmid) and the various mechanisms of transferring resistant and virulent genes, which are becoming alarming especially among the methicillin sensitive *S. aureus* strains. This, therefore, calls for prompt sensitive diagnostic methods.

SIGNIFICANCE STATEMENT

This study discovered the presence of antibiotic resistant *mec*A genes and virulence enterotoxin *sea* genes from methicillin sensitive *S. aureus* strains, which could imply horizontal gene transfer from resistant to sensitive strains. This study will help the researchers to uncover the critical areas of mechanism(s) of gene transfer from resistant *S. aureus* strains to sensitive *S. aureus* strains.

ACKNOWLEDGMENTS

The authors wish to appreciate the management of General Hospitals Obiaruku, Warri and Ughelli, Delta state, Nigeria, for consent to sample collection and the nursing staff for assisting in the collection of the samples from the patients. We appreciate the technical staff of the Department of Microbiology, Ambrose Alli University, Ekpoma, Edo State and Federal University Oye-Ekiti, Ekiti state, Nigeria, for their technical support.

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