African Journal of
Microbiology Research
Volume 11 Number 44 28 November, 2017
ISSN 1996-0808
Academic Journals

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Vol. 11(44), pp. 1586-1599, 28 November, 2017
DOI: 10.5897/AJMR2017.8725
Article Number: A9A831266842
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

A novel *groel* gene from the endosymbiont of beet leafhopper, *Candidatus Sulcia muelleri*

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Received 4 October, 2017; Accepted 8 November, 2017

Curtoviruses are transmitted by the beet leafhopper *Circulifer tenellus*, in a circulative (non-propagative) manner. Curtoviruses are phloem-limited and are acquired by the vector during feeding. Sap-feeding insects harbor endosymbionts which can help provide essential nutrients required for the insects' survival. *Candidatus Sulcia muelleri* is an endosymbiont present in the beet leafhopper identified during this study. A housekeeping gene, *groel*, was identified from the endosymbiont. The *groel* gene sequence from this strain of *Ca. S. muelleri* differs from all other strains published in NCBI, suggesting the presence of a new strain, which was named *S. muelleri* beet leafhopper (SMBLH). A GroEL-homolog protein produced from *groel* was found in different vectors with circulative transmission. Analysis of nucleotide and translated sequences, using alignment, phylogenetic trees, and predicted secondary and tertiary structures showed that SMBLH GroHp has similarities to *Escherichia coli* GroEL and the GroEL-homolog proteins from *Hamiltonella* and *Buchnera*, endosymbionts of whiteflies and aphids, respectively. GroHp and GroEL were expressed as fusion proteins. Electron microscopy analyses indicate that purified expressed GroHp and GroEL proteins demonstrate correct folding.

Key words: Beet leafhopper (BLH), *Candidatus Sulcia muelleri,* endosymionts, GroEL homolog protein (GroHp).

INTRODUCTION

Plant viruses are economically important and can infect a wide range of host plants (Hogenhout et al., 2008). Insects play important roles in plant virus transmission. The insect vector acquires the virus while feeding on one plant then infects another plant when it takes its next meal (Hogenhout et al., 2008). Viruses must be able to survive, and sometimes replicate, in their insect hosts and still be transmissible and infective.

Phloem feeding insects, such as mealybugs, aphids, and whiteflies, harbor bacterial endosymbionts. These endosymbionts may play roles important for the hosts' survival. *Wolbachia* and *Spiroplasma* (endosymbionts of *Drosophila* flies) and *Buchnera* (aphids), provide protection against microbial pathogens (Shokal et al.,

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2016). Endosymbionts can provide the minimal host diet with essential nutrients (Douglas, 2009). Furthermore, they can also provide better temperature tolerance and parasite and insecticide resistance, as well as, sex determination (Montllor et al., 2002; Oliver et al., 2003; Kontsedalov et al., 2008).

Two types of bacterial endosymbionts can be found in insects, primary (obligate) and secondary (facultative) (Oliver et al., 2010). The location of the endosymbionts differs depending on the insect itself and the type of endosymbionts. Whiteflies that vector cotton leaf curl virus (CLCuV), harbor *Portiera* as their primary endosymbiont, and is found only in bacteriocytes, while the secondary endosymbiont *Arsenophonus* can be found in salivary glands, midgut, and bacteriocytes (Rana et al., 2012). For the aphid, *Myzus persicae*, the *Buchnera aphidicila* endosymbiont is restricted to bacteriocytes or mycetocytes found in the hemolymph (Bouvaine et al., 2011; van den Heuvel et al., 1994). Mealybug (Pseudococcidae, Hemiptera). Vectors of the ampelovirus grapevine leafroll-associated virus 3 (GLRaV-3) (Kono et al., 2008), have primary betaproteobacteria, endosymbiont (P-endosymbiont) that may act as a host for a secondary, gammaproteobacteria, endosymbiont (S-endosymbiont) (von Dohlen et al., 2001). It is very likely that insects acquire their primary endosymbionts through vertical maternal transmission (Baumann, 2005; Hogenhout et al., 1996), while secondary endosymbionts can be transmitted both vertically and horizontally (Oliver et al., 2010).

Leafhoppers belong to the order Hemiptera, suborder Auchenorrhyncha, family Cicadellidae (Moran et al., 2005). Auchenorrhyncha harbor the obligate endosymbiont *Candidatus Sulcia muelleri*. This bacterium is a nutritional endosymbiont belonging to phylum Bacteriodetes. It can be found in strap-shaped bacteriomes which can be found as a pair in the abdomen of adult insects (Moran et al., 2005; Moran, 2007). Although, different endosymbionts can be found in separate bacteriocytes in the insect body, *Ca. S. muelleri* along with *Candidatus Baumannia cicadellinicola*, a gammaproteobacterium, were found together in the same bacteriome of sharpshooters. In spittlebugs, *Ca. S. muelleri* was found together with *Candidatus Zinderia insetticola*, a betaproteobacterium (Wangkeeree et al., 2012; Moran, 2007). In the leafhopper *Matsumuratettix hiroglyphicus*, *Ca. S. muelleri* was associated with bacterium associated with *M. hiroglyphicus* (BAMH) in more than one region of single insect body, such as fat bodies, ovaries, and bacteriocytes (Wangkeeree et al., 2012). More than one type of facultative endosymbiont has been found in some leafhoppers but the different types of endosymbionts and the lack of regularity in finding them has prevented the formation of a clear account of the bacterial fauna in leafhoppers (Ishii et al., 2013).

Van den Heuvel et al. (1994) carried out research on the aphid, *M. persicae,* and a virus it transmits, potato leafroll virus (PLRV, Luteoviridae). The researcher discovered the presence of a protein produced by the aphid endosymbiont, *Buchnera* and named it symbionin. This protein can readily interact with the coat protein (CP) of PLRV. Analysis of symbionin, especially of the N-terminal, showed sequence homology with the *Escherichia coli* heat shock protein GroEL. Symbionin is a GroEL-like protein (GroHp) produced by *Buchnera* and is important for preserving the symbiont-aphid (*Acyrthosiphon pisum*) relationship (Ishikawa, 1982; van den Heuvel et al., 1997). GroHp is thought to interact and protect the virus while circulating in the insect, thus, facilitating its transmission (van den Heuvel et al., 1994).

GroEL is a chaperonin, which reduces the number of aggregated proteins within the small confines of cells by assisting in the folding of proteins into their three dimensional structure (Skjaerven et al., 2015). The GroEL protein, or its homologues, can be found in all bacteria including endosymbiont bacteria. The function of GroHp in a virus/vector system could involve not only protecting the virus from degradation or from detection by the immune system, but also virus trafficking throughout the vector and preventing virus aggregation or disassembly (van den Heuvel et al., 1994; Morin et al., 1999; Gottlieb et al., 2010).

GroEL and GroHps are the most abundant proteins produced by bacteria (Baumann et al., 1996; Kupper et al., 2014). The *groel* gene is highly conserved in primary endosymbionts (Kupper et al., 2014).

The beet leafhopper, *Circulifer* (*Neoaliturus*) *tenellus* (Baker) is a hemipteran insect (Cicadellidae) that transmits curtoviruses (*Geminiviridae*) which cause curly top disease. Curly top disease (CTD) is economically important affecting many plant crops including common bean, pepper, spinach, sugar beet, cucurbits, and tomatoes (Baliji et al., 2004).

The endosymbiont(s) of the beet leafhopper (BLH) have not yet been identified. Furthermore, the GroHp produced by any endosymbiont(s) has not been explored. This report identifies the endosymbiont(s) of the BLH and analyzes a GroHp produced by the endosymbiont(s). The sequence of the *groel* gene responsible for this protein is analyzed. This gene has been amplified, cloned, and sequenced. The relationship between fourteen different GroHps produced by the endosymbionts of insect vectors has also been investigated and of *C. tenellus* was investigated and BLH GroHp predicted tertiary structure validity was determined using TEM imaging.

MATERIALS AND METHODS

Leafhoppers and sugarbeets

The beet leafhoppers (*C.* (*Neoaliturus*) *tenellus*) used in this study were gifts from Carl Strausbaugh, USDA, Kimberly, ID, or collected from Las Cruces, NM, Leyendecker Plant Science Farm from

Table 1. Primers for identifying beet leafhopper endosymbionts.

Primer	Sequence (5-3)”	Annealing temperature (°C)
16SF	AGGTTTATGTATTTTTGGGGA	53
16SR	CTGAATTACAACGTACAAAACCC	
16S/RicF	TGACGGTACCTGACCAAGA	52
16S/RicR	AAGGGATACATCTCTGCTT	
WolbF	TTAAATATGGGAAGTTTACTTTCTGTATTAC	47
WolbR	GTAATACAGTAAACTTCCCATATTTAA	
qSulF	AGGTTTATGTATTTTTGGCGA	51
qSulR	CAATCATCGTCTTGGTAAGCC	

Universal 16S, 16S/Ric, and Wolb primers were from Noda et al. (2012). The qSul primers were designed by the authors.

Kochia scoparia plants.

Beet leafhoppers were reared on sugarbeet plants maintained at 28°C day and 26°C night with a 16 h photoperiod. Adult insects were used for DNA extraction. The sugarbeet plants, *Beta vulgaris*, were grown from seeds of breeding line P1518-6 provided by Kelly Richardson, USDA, Salinas, CA.

DNA extraction from leafhoppers

Total DNA was extracted from whole beet leafhoppers using the “Purification of total DNA from insects” protocol of DNeasy Blood & Tissue Kit (QIAGEN Inc. Valencia, CA). The extracted DNA was diluted using molecular grade water to a final concentration of 30 ng/μl. It was stored at -20°C until needed.

Identification of *C. tenellus* endosymbiont(s)

To identify the symbiont(s) inhabiting *C. tenellus*, 16S rRNA primers (Table 1) were used (Noda et al., 2012). To test for specific endosymbionts known to colonize some phloem-feeding insects, specific PCR primers were used for *Rickettsia* (Noda et al., 2012), *Wolbachia* (Gonella et al., 2011), and *Sulcia* (Table 1). For the negative control, no DNA template was used in the amplification reaction. The PCR reaction contained 2.5 μl of 10X standard Taq reaction buffer (BioLabs, GA), 0.5 μl of 10 mM of dNTPs, 0.5 μl of 10 μM of each primer, 0.125 μl of Taq DNA polymerase (BioLabs, GA), 2 μl of DNA template, and nuclease-free water to a final volume of 25 μl. The PCR cycles were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, annealing cycle with various temperatures (Table 1), for 2 min, and 72°C for 1.5 min and a final extension at 72°C for 10 min, then ending at 4°C forever. PCR amplicons were electrophoresed in a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA), stained by GreenView™ (GeneCopoeia, MD), and viewed under a UV light.

The amplified products were cleaned using QIAquick PCR Purification kit (QIAGEN, MD), according to manufacturer’s instructions. The cleaned products were sequenced by MCLAB (South San Francisco, CA). Obtained sequences were compared with available sequences using NCBI Basic Local Alignment Search Tool (BLAST) algorithm.

Amplifying *Ca. S. muelleri groel* gene from *C. tenellus*

The *Ca. S. muelleri groel* gene was amplified from 2 μl of diluted beet leafhopper DNA using primers designed from identical regions of *Ca. S. muelleri groel* gene sequences of strains ALF, ML, DMIN, GWSS, BGSS, and PUNC (GeneBank accession no. CP006060.1, CP010105.1, CP001981.1, CP000770.2, CP008986.1, and CP013212.1, respectively). The PCR cycles were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1.5 min, 42°C for 2 min, and 72°C for 1.0 min, and a final extension at 72°C for 10 min, then ending at 4°C forever. PCR amplicons were electrophoresed in a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA), stained by GreenView™ (GeneCopoeia, MD). Table 2 lists all primers used for sequencing *groel*.

Cloning of *S. muelleri groel* into pGEM-T Easy

Fresh (less than 24 h) *groel* PCR product was cleaned using QIAquick PCR Purification Kit (QIAGEN, MD) according to manufacturer’s instructions. The purified samples were eluted using molecular grade water and the concentration was measured using NanoPhotometer™ P-class spectrophotometer (IMPLEN, Germany). PCR amplicons of the *groel* gene were then cloned into pGEM-T Easy vector system (Promega, WI) according to instructions with a 5:1 ratio of insert to vector and incubation for 24 to 48 h at 4°C. The cloned plasmid was transformed into JM 109 high Efficiency Competent Cells (Promega, WI), also according to instructions. The culture was plated on LB plates supplemented with 100 μl/ml ampicillin, 1 mM IPTG, and 20 mg/ml X-gal. The plates were incubated at 37°C for 12 h. White cells were tested using PCR colony method with *groel* amplification primers. PCR amplicons were electrophoresed using a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA), stained by GreenView™ (GeneCopoeia, MD). Colonies containing inserts were inoculated on LB broth supplemented with ampicillin and incubated while shaking (200 rpm) at 37°C overnight. The plasmid was extracted using E.Z.N.A. Plasmid DNA Mini Kit I (OMEGA) and eluted using molecular grade water.

Sequencing of *Ca. S. muelleri groel* gene

The amplified and cloned *Ca. S. muelleri groel* gene-plasmid were cleaned using QIAquick PCR Purification Kit (QIAGEN, MD) and E.Z.N.A. Plasmid DNA Mini Kit I (OMEGA), respectively, according to manufacturers’ instructions. The concentrations of the cleaned products were measured using NanoPhotometer™-P-Class. The concentration of amplified *groel* was adjusted to 20 ng/μl. The concentration of the purified *groel* clone plasmid was adjusted to

Table 2. Primers used for amplifying and sequencing beet leafhopper *groel*.

Primer	Sequence (5-3)"	Corresponding position (bp)	Use
Sul_Alf_F'	ATGGCAAAAAATATTCA	1	*Groel* amplification
Sul_general_R'	GAAGATTTTCCTTTTT	1,644	
PilotSulGrR1	TCCATAGGATTAGCTCCAGCA	320	-
PilotSulGrF2	TCTGAAGAAGTTGAAGGAGAAGCA	750	
PilotSulGrF3	GCTGGAGGAGTTGCTGTTCTA	1,116	*Groel* sequencing
4_SGr_pGemR	TAGAACAGCAACTCCTCCAGC	1,116	
PilotSulGrR5	TTTCCAGATTGAGCAACGGGT	713	-

Table 3. Sequence analysis for the 16S and qSul primers and the endosymbionts they detected.

Primer	Endosymbiont	GeneBank no.	E-value	% Identity	Host
16Sr	Proteobacterium	FJ774959.1	0.0	99	Brown planthopper (*Nilaparvata lugens*)
	Acetobacteraceae	JQ726821.1	0.0	98	*Nysius expressus*
qSul	*Asaia sp*	Several	0.0	97	Hemiptera and Diptera
	Ca. S. muelleri	Several	1e-99	99	Sub order Auchenorrhyncha
	Mycoplasma	DQ679965.1	1e-93	99	*Macrosteles* spp.

The universal 16S primers amplified three types of endosymbionts with an E-value of 0.0. The insect hosts of these endosymbionts is listed. The qSul primers identified two endosymbionts.

100 ng/µl. Both samples were sequenced by MCLAB. Seven sequencing primers were used to sequence the complete *groel* gene (Table 2).

Analysis of sequences

The sequence of the complete *groel* gene was assembled using Geneious (Biomatters Inc, Newark, NJ). This sequence was analyzed using NCBI BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3). The *groel* gene sequences of all *Ca. S. muelleri* published in NCBI were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and multiple alignments were created. The amino acid sequence of this *groel* was obtained by translating the gene using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Multiple alignments of eight nucleotide and twelve amino acid sequences were generated using Clustal Omega. The sequences analyzed included all *groel* genes of *Ca. S. muelleri* strains published in NCBI, as well as, *E. coli, Hamiltonella*, and *Buchnera groel*s. Phylogenetic trees of maximum likelihood were constructed using the program Phylogeny.fr (Dereeper et al., 2008, 2010), using "One Click" settings.

Beet leafhopper (BLH) endosymbiont, *Ca. S. muelleri* GroEL-homolog protein (SMBLH GroHp) and the GroHps sequences of *E. coli, Hamiltonella*, and *Buchnera*, were used for structure prediction. Secondary structure was predicted using the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) (data not shown). The tertiary structure was predicted using SWISS-MODEL https://swissmodel.expasy.org/interactive).

Cloning the genes

E. coli (MG6155) *groel* gene was cloned in the HindIII site (Table 5) of the expression plasmid pRsetC (Invitrogen, Carlsbad, CA). The resulting construct plasmid pRset C/EcoGroEL was propagated in *E. coli* BL21(DE3)lysS cells, on media supplemented with 100 µl/ml Amp and 30 µg/ml chloromphenicol. The SMBLH *groel* gene (without the stop codon), was cloned into NotI and XhoI sites (Table 5) of the expression vector pTXB1 (NEB, Ipswich, MA), upstream of the Intein site (IMPACT kit^{TM}). The resulting construct plasmid pTXB1/SuGroHp was propagated in *E. coli* ER2566 cells, supplemented with 100 µl/ml Amp. The integrity of the genes was confirmed by sequencing.

Purification of over-expressed protein

Induction of pRset C and pTXB1 constructs with IPTG was performed according to instructions. Twenty five milliliters of LB supplemented with 100 µg/ml ampicillin was inoculated with a single colony of pLysS or *E. coli* ER2566 cells containing the construct plasmid, and incubated for 18 h at 37°C, while shaking at 220 rpm. Ten milliliters of culture was diluted in 1 L of LB supplemented with 100 µg/ml ampicillin, and incubated at 37°C while shaking until O.D600 = 0.4-0.6. Isopropylthio-β-Dgalactoside (IPTG) was added to a final concentration of 1 mM for pRset C clones and 0.4 mM for pTXB1 clones and the cells were incubated at 22°C for another 18 h. The cells were collected by centrifuging at 5,000 ×*g* for 15 min at 4°C. The supernatant was discarded and the pellet was kept at -80°C. The induction was assessed on a 10% SDS-PAGE and stained with Coomassie Brilliant Blue (CBB).

Purification of pRset C constructs

The induced protein pellet was resuspended on ice in 80 ml of 1X Native buffer (50 mM NaH_2PO_4, pH 8.0, and 500 mM NaCl, 10 mM imidazole, pH to 8.0). Then, sonicated five times, on ice at 60%

amplitude for 30 s at 1 min intervals. The lysate was cleared by centrifugation at 15,000 ×*g* for 30 min at 4°C. The lysate was filtered through a 0.22 um filter then incubated with 10 ml of Ni-NTA beads (ThermoScientific, Waltham, MA) overnight on ice at 4°C, while rocking gently. The mixture was transported to a column and the flow through collected. The beads were washed four time with 100 ml of native buffer supplemented with 20 mM imidazole. The recombinant protein was eluted in 1 ml fractions (30 ml), with elution buffer (native buffer with 300 mM imidazole). The presence of protein in the fractions was tested on a 10% SDS-PAGE. The fractions with the protein were pooled, then concentrated using 30 kDa centricon.

Purification of pTXB1 constructs

The pellet was re-suspended on ice in cold 100 ml of column buffer (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 1 mM EDTA, 0.5% Triton-100, and 0.2% Tween 20), at 4°C. The re-suspended pellet was sonicated five times, on ice at 60% amplitude for 30 s at 1 min intervals. The lysate was cleared by centrifugation at 15,000 ×*g* for 30 min at 4°C. The lysate was filtered through a 0.22 µm filter and loaded to a 10 ml calibrated chitin column (NEB, Ipswich, MA). The chitin slurry was calibrated with 100 ml of column buffer. The lysate was allowed to flow through at a 0.5 to 1 ml/min. The chitin bed was washed with 200 ml of column buffer at a flow rate of 2 ml/min. On-column cleavage to release the protein was induced by a thiol reagent. 30 ml of cleavage buffer (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, 1 mM EDTA, 50 mM DTT), was used to quickly flush the column. After the quick flush, the column was stopped leaving around 1.5 cm of cleavage buffer on top of the chitin bed. Incubate at 4°C for 24 to 30 h. The target protein was eluted from the column using 30 ml column buffer in a 1 ml fraction size. The presence of protein in the fractions was tested on a 10% SDS PAGE. The fractions with the protein were pooled then concentrated using 30 kDa centricon. The proteins were stored at 80°C.

Transmission electron microscopy (TEM)

Partially purified GroEL and GroHp were dialyzed in cold glycine/sodium hydroxide buffer, pH 8.1 (25 ml of 0.5 M glycine, titrated with 0.5 M NaOH). The samples were mounted on Carbon Film 200 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA), then stained with 2.5% uranyl acetate. The samples were visualized in the bright field-imaging mode with a model H-7650 electron microscope (Hitachi High Technologies, Pleasanton, California, USA), at 50,000 ×magnification.

RESULTS

Detection and characterization of *C. tenellus* endosymbionts

The 16S rRNA gene is commonly used for identification of bacteria, because it is both conserved and ubiquitous, and targets a wide variety of bacteria. This gene was used to identify the endosymbionts of the beet leafhopper, *C. tenellus,* which vectors curtoviruses (Geminiviridae). The PCR amplification of the 16SrRNA gene gave a band of around 900 bp. The sequenced 16SrRNA genes were analyzed for similarity to sequences in the NCBI databank using the BLASTN algorithm and the top hits showed the endosymbionts to be mostly similar to proteobacterium (99% identity), acetobacteraceae (98% identity), and *Asaia* species (97% identity) (Table 3).

Primers specific for the endosymbionts *Ricketsia* and *Wolbachia* were also tested but neither showed PCR products. Primers for *Ca. S. muelleri* (Table 1) amplified a product of size of around 210 bp. Sequence comparison using BLASTN algorithm when set for ‘Somewhat similar sequences’ identified the top hits of a 209 bp alignment with different strains of *Ca. S. muelleri* (99% identity), and a 199 bp alignment with *Mycoplasma* spp. (Table 3).

Ca. S. muelleri groel gene from *C. tenellus*

BLASTN and BLASTX were used to compare the *groel* from *C. tenellus* and 15 other *groel* sequences found in NBCI. Twelve of the 15 sequences belonged to different *Ca. S. muelleri* strains, while three were from *Hamiltonella* (whitefly endosymbiont), *Buchnera* (aphid endosymbiont), and *E. coli* (free living). Table 4 shows both nucleotide and amino acid sequences percent identities with the newly identified *groel*; the NCBI GeneBank accession numbers for the nucleotide and protein, and the host of the endosymbionts. Because this *groel* gene differs from all other *groels* from the strains of *Ca. S. muelleri,* it was deduced that it belongs to a new strain of *Ca. S. muelleri,* which can be found in BLHs. The strain SMBLH (*Sulcia muelleri* beet leafhopper) was denoted. The sequence was submitted to GeneBank under the accession no. KY569409.

The *Ca. S. muelleri groel* nucleotide sequence percent identities ranged from 90 to 99% compared to the beet leafhopper endosymbiont sequenced gene, while *Hamiltonella*, *E. coli*, and *Buchnera*, had 67, 64, and 71% identity, respectively. All *Ca. S. muelleri groel* amino acid sequences percent identities (and percent similarities), ranged from 92 (97) to 99 (99), compared to the beet leafhopper endosymbiont *groel* translated amino acid sequence, while *Hamiltonella*, *E. coli*, and *Buchnera*, had 64 (78), 64 (79), and 63% (79), identities, respectively.

Aligned *Ca. S. muelleri groel* sequences

All twelve *Ca. S. muelleri groel* sequences published in NCBI, and the newly sequenced (and translated) SMBLH were aligned using Clustal Omega. Figure 1 shows the first 60 nucleotides (the 5’-end) of *groel* gene of eight strains of *Ca. S. muelleri* (seven were published in NCBI and SMBLH). The strains TETUND, SMDSEM, SMMAGTRE, and PSPU, did not show enough identity at this region so they were omitted from comparison. At nucleotide 39, SMBLH was identical to the ML, ALF, PUNC, and NC strains. At the 3’-end, SMBLH lacked six nucleotides (GGTATG), when compared with DMIN, GWSS, BGSS, and (GGAATG) compared to ML, ALF, PUNC, and NC. But when comparing SMBLH to CARI,

Table 4. Nucleotide and amino acid sequences percent identities of *groel*s; the NCBI GeneBank numbers for the nucleotide and *groel* protein; and the host of the endosymbionts.

NCBI GeneBank no. Nucleotide/Amino acid	Host	% Nucleotide identity	% Amino acid identity (similarity)
PUNC CP013212.1 /ALP70160.1	Pestiferous leafhopper (*Macrosteles quadripunctulatus*)	99	99 (99)
ALF CP006060.1 /AGS33420.1	Aster leafhopper (*Macrosteles quadrilineatus*)	99	99 (99)
ML CP010105.1 /AIZ48895.1	Maize leafhopper (*Dalbulus maidis)*	99	99 (99)
BGSS CP008986.1 / AIN47733.1	Blue-green sharpshooter *Graphocephala atropunctata*	99	99 (99)
DMIN CP001981.1 /ADE35468.1	Green sharpshooter (*Draeculacephala minerva*)	99	98 (99)
GWSS CP000770.2 /ABS30591.1	Glassy-winged sharpshooter (*Homalodisca vitripennis*)	99	98 (99)
NC CP016223.1 /ANO35772.1	Rice leafhopper (*Nephotettix cincticeps*)	99	99 (99)
CARI CP002163.1 /ADM90008.1	Arizona spittlebug (*Clastoptera arizonana*)	95	96 (98)
PSPU AP013293.1 /BAO66356.1	Meadow spittlebug (*Philaenus spumarius*)	95	96 (98)
SMDSEM CP001605.1 / ACU52899.1	Cicada (*Diceroprocta semicincta*)	90	94 (96)
SMMAGTRE CP010828.1 /ALA22796.1	Cicada (*Magicicada tredecim)*	89	92 (97)
TETUND CP007234.1 / AHL31279.1	Cicada (*Tettigades undata*)	90	93 (97)
Hamiltonella AF130421.1/ AAD26368.1	Whitefly (*Bemisia tabaci*)	67	64 (78)
Buchnera CP002701.1 / AHG61490.1	Aphids	71	63 (79)
E. coli NZ_KE702337.1 /WP_021570575.1	Free living	64	64 (79)

Table 5. Primers used for cloning into expression vectors.

Gene/primer	Sequence	Annealing temperature (°C)	Expression vector/Ab^r
E. coli groel			
EcoliGrHind_F	AAGCTTATGGCAGCTAAAGACG	59	pRsetC/Amp^r
EcoliGrHind_R	AAGCTTTTACATCATGCCGCCCATG		
SMBLH *groel*			
ImpSulNot_F	GCGGCCGCATGGCAAAAAATA	62	pTXB1/Amp^r
ImpSulXho_R	CTCGAGCATCATTCCTCCACTATTAGGC		

Underlined nucleotides indicate the restriction enzyme. Both the gene and the restriction enzyme are found in the first column.

TETUND, SMDSEM, SMMAGTRE, and PSPU, lacked 15 nucleotides (not shown). DMIN, GWSS, BGSS, ML, ALF, PUNC, and NC have a 99% identity with SMBLH.

At the N-terminus SMBLH GroEL-homolog protein (GroHp) is identical to the first 50 amino acids of GroHps from *Ca. S. muelleri* BGSS, DMIN, GWSS, PUNC, ALF, and NC (Figure 2). At the C-terminus SMBLH GroHp lacks two amino acids (MG) at positions 536 and 537 when compared with CARI, BGSS, DMIN, GWSS, PUNC, ALF, and NC, while it lacks four amino acids, GG-MG (Figure 2). SMBLH has the same conserved amino acid residues as *E. coli* GroEL, with similarities to *Buchnera* GroHp, except for 474,

```
The 5'-end
DMIN     ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGATAAATTAAAAAAAGGAGTAGAT 60
GWSS     ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGATAAATTAAAAAAAGGAGTAGAT 60
BGSS     ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGATAAATTAAAAAAAGGAGTAGAT 60
SMBLH    ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGACAAATTAAAAAAAGGAGTAGAT 60
ML       ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGACAAATTAAAAAAAGGAGTAGAT 60
ALF      ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGACAAATTAAAAAAAGGAGTAGAT 60
PUNC     ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGACAAATTAAAAAAAGGAGTAGAT 60
NC       ATGGCAAAAAAATATTCAATTTGATATTGAAGCTAGAGACAAATTAAAAAAAGGAGTAGAT 60
         ************************************** *********************

The 3'-end
DMIN     CTATGCCACAAATGCCTAATAGTGGTATGGGAGGAATGATGTAA----------    1632
GWSS     CTATGCCACAAATGCCTAATAGTGGTATGGGAGGAATGATGTAA----------    1632
BGSS     CTATGCCACAAATGCCTAATAGTGGTATGGGAGGAATGATGTAA----------    1632
SMBLH    CTATGCCACAAATGCCTAATAGT------GGAGGAATGATGTAA----------    1626
ML       CTATGCCACAAATGCCTAATAGTGGAATGGGAGGAATGATGTAA ---------    1633
ALF      CTATGCCACAAATGCCTAATAGTGGAATGGGAGGAATGATGTAA----------    1632
PUNC     CTATGCCACAAATGCCTAATAGTGGAATGGGAGGAATGATGTAA----------    1632
NC       CTATGCCACAAATGCCTAATAGTGGAATGGGAGGAATGATGTAA              1642
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Figure 1. Aligned 5'- and 3'- ends of seven *Ca. S. muelleri groel* and of SMBLH nucleotide sequences, using Clustal Omega. The first 60 nucleotides of SMBLH *groel* is identical to ML, ALF, PUNC, and NC strains, while it lacks six nucleotides at the 3'-end of the gene. Asterix (*) indicates identical nucleotides. The SMBLH sequence is boxed.

where Asp is replaced with Gly, similar to *Hamiltonella* GroHp at this position. This substitution is at a conserved region. Furthermore, SMBLH shares the same polypeptide, and ATP binding sites as *Buchnera*, *Hamiltonella*, and *E. coli*, except for SMBLH and *E. coli* residues 479, where Asp is replaced with Asn. This change is at a less conserved region. Figure 3 shows the alignments and the polypeptide, and ATP binding sites, as well as the substituted residues.

Phylogenetic analyses of *groe*l and GroHp

Using maximum likelihood and neighbor joining, phylogenetic analyses were performed on 13 *groels* from different strains of *Ca. S. muelleri*, including SMBLH, for nucleotide and protein sequences, providing very similar results. The outgroups used were *groels* from *Hamiltonella*, *Buchnera*, and *E. coli*. The trees for nucleotide and amino acid sequences were highly concordant (Figures 4 and 5, respectively). All strains of *Ca. S. muelleri* were divided into two major clades with a strong support of 1 (Figures 4 and 5). The clade that SMBLH occupies has a support of 0.94 and greater. The phylogeny matches the grouping of endosymbionts and their host insect. The clades separate into cidada (TETUND, SMMAGTRE, and SMDSEM) and cicadellids (all others) (Figures 4 and 5 and Table 4). Furthermore, the clade of cicadellids divides into three branches, also in agreement with host, giving spittlebugs (CARI and PSPU), sharpshooters (DMIN, GWSS, and BGSS), and leafhoppers (SMBLH, NC, ML, ALF, and PUNC), the clade that has the SMBLH.

Prediction of the secondary and tertiary structures

The predicted secondary structures of SMBLH, *E. coli*, *Hamiltonella*, *Buchnera*, and GroHps show very similar motifs throughout the structures, except for three regions (data not shown). The residues between regions 184-191, 313-316, and 463-473, show similar and differences in motifs between the predicted secondary structures among the four GroHps (Table 6). In region 1 (residues 184-191), SMBLH GroHp has a β-strand motif, while the GroHps of *E. coli*, *Buchnera* and *Hamiltonella,* have a coil. In region 2 (residues 313-316), SMBLH GroHp had a coil motif, similar to that found in the GroHps of *Buchnera* and *Hamiltonella*, but unlike *E. coli,* which had an α-helix. At region 3 (residues 463-473), SMBLH GroHp had both β-strand and α-helix, while the GroHps from both *E. coli* and *Buchnera* had a β-strand and *Hamiltonella* had an α-helix.

Table 6 shows the predicted homo-heptemer structure

```
The N-terminus
SMMAGTRE    ----------MAKNIKFDIEARDKLKKGVDALANAVKVTLGPKGRNVVFQKSFGGPQVTK    50
SMDSEM      ----------MAKNIKFDIEARDKLKKGVDALANAVKVTLGPKGRNVVFQKSFGGPQVTK    50
TETUND      MVVNLMSDARMAKNIKFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    60
CARI        ----------MSKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
PSPU        ----------MSKNIKFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
BGSS        ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
DMIN        ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
GWSS        ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
SMBLH       ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
ML          ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
PUNC        ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
ALF         ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
NC          ----------MAKNIQFDIEARDKLKKGVDALANAVKVTLGPKGRNVVLQKSFGGPQVTK    50
                      *.***.********************************.***********

The C-terminus
SMMAGTRE    GEYKNMISEGIIDPTKVTRVALENAASVAGMLLTTDCVITEIKKEEPA IP---AN-SGMGGMV    541
SMDSEM      GEYKNMISEGIIDPTKVTRVALENAASVAGMLLTTDCVITEIKKEEPA MPAMPGN-SGMGGMV    542
TETUND      GEYKNMISEGIIDPTKVTRVALENAASVAGMLLTTDCVITEIKKEEPA -PAMPGN-SGMGGMV    553
CARI        GEYKNMIYEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEQT MPQMPSGGGGMGGMM    545
PSPU        GEYKNMIYEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEQT MPQMPSGGGGMGGMM    545
BGSS        GEYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
DMIN        GEYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
GWSS        GEYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
SMBLH       GDYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--G--GMM    541
ML          GDYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
PUNC        GDYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
ALF         GDYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
NC          GDYKNMISEGIIDPTKVTRVALENAASVSGMLLTTECVITEIKKEEPA MPQMPNS--GMGGMM    543
            *:***** ********************:******:********** :  *     . *  **:
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Figure 2. Aligned N- and C- termini of *Ca. S. muelleri* amino acid sequences and SMBLH, using clustal Omega. The sequence of SMBLH lacks two amino acids when compared to those of BGSS, DMIN, GWSS, ML, PUNC, ALF, and NC strains, and four amino acids when compared to CARI, and PSPU strains. Asterix (*) indicates identical amino acids, while the dots (:) indicate similar amino acids and (.) some similarities between amino acids. The SMBLH sequence is boxed.

without a ligand. *E. coli*, *Buchnera*, and *Hamiltonella* GroHp proteins show similar tertiary structures. The GroHps from *Ca. S. muelleri* strains were represented by NC, TETUND, and SMBLH. The predicted structures of GroHps from *Ca. S. muelleri* strains NC, TETUND, and SMBLH were also similar to each other and to that of *E. coli*, *Buchnera*, and *Hamiltonella* predicted homo-heptemer structures, with mean model identities of 65. The predicted model for a 14-homomers for the SMBLH GroHp, showed it formed 2 rings, each with seven identical units, with a cavity in the middle. Table 6 shows the top view of the predicted SMBLH GroHp.

SMBLH GroHp TEM visualizing

Both partially purified GroEL and GroHp were visualized by TEM. GroEL structure appears to be folding as predicted (Figure 6). A ring of seven units was seen in the top view, while the side view displayed the staked rings. The SMBLH GroHp appeared to form similar structures, albeit the resolution was not as sharp as that of GroEL (Figure 6).

DISCUSSION

This study identified the endosymbiont *Ca. S. muelleri* from the beet leafhopper (BLH), *C. tenellus*. This was an expected result since *Ca. S. muelleri* is the primary endosymbiont of Auchenorrhyncha which includes the Cicadellidae family (leafhoppers).

This endosymbiont has many strains, of which twelve complete genomes have been deposited into NCBI. This

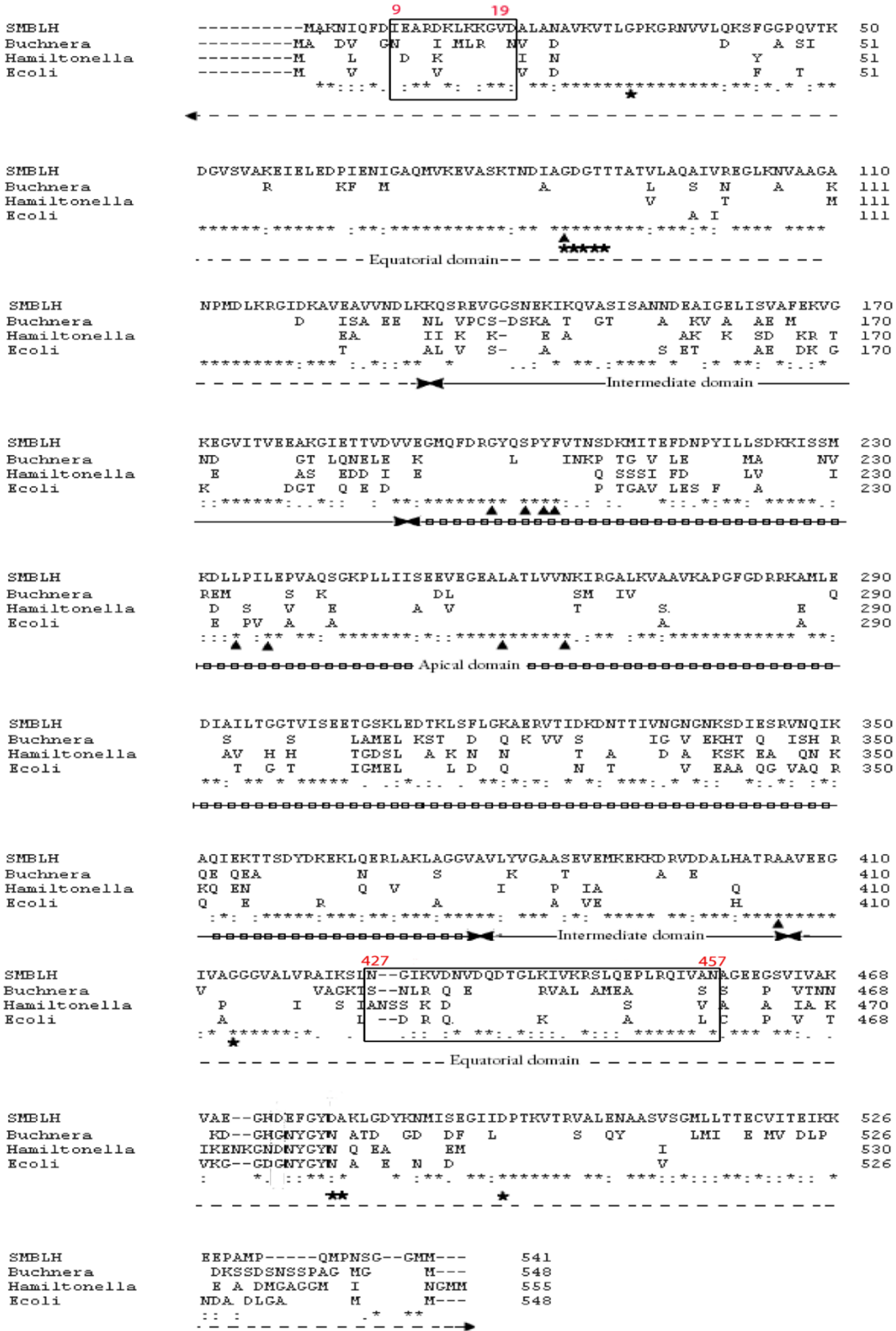


Figure 3. Amino acid sequence alignment of GroHps of *E. coli* and of SMBLH, *Buchnera*, and *Hamiltonella*. The polypeptide binding amino acids are indicated by arrow heads, the ones implicated in ATP binding are indicated by stars. The red stars above the residues indicate where SMBLH GroHp differs from the others.

gene differs from all other *groels* produced by the *Ca. S. muelleri* published strains. It has the greatest identity to *Ca. S. muelleri* DMIN, GWSS, ML, ALF, PUNC, and NC. Because this *groel* was different to all other *groels*

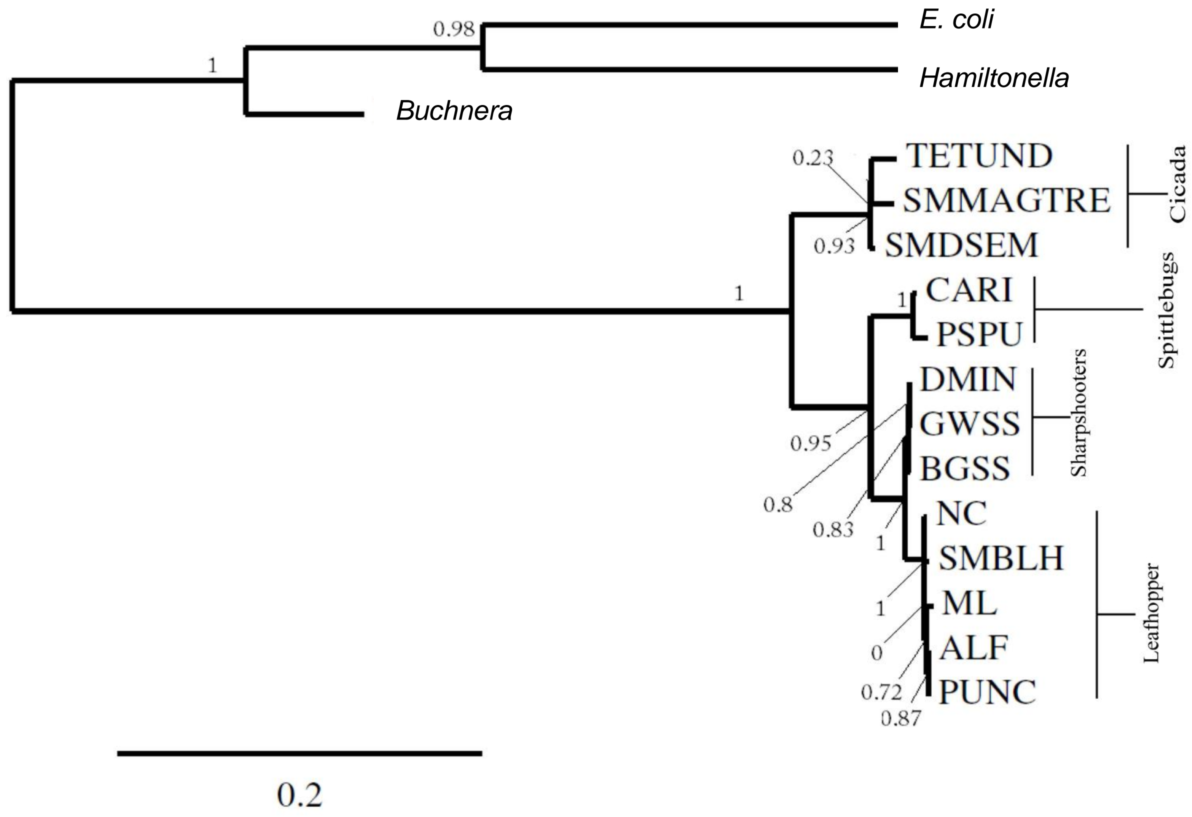


Figure 4. Maximum likelihood (ML) phylogenetic tree of *groel* nucleotides indicating the relationship among 13 strains of *Ca. S. muelleri.* The outgroups chosen are endosymbionts of whiteflies (*Hamiltonella*), aphids (*Buchnera*), and free living *E. coli.* The ML tree is almost identical to the Neighbor Joining tree. The clades with *Sulcia* strains show the host insect at the right of the tree. Nodes show >70% confidence for the clade for leafhopper endosymbionts.

produced by any strain of *Ca. S. muelleri* published in NCBI, It was deduced that it belongs to a new strain not published in NCBI, which was named *S. muelleri* beet leafhopper (SMBLH).

The SMBLH *groel* length is shorter than the other strains *groels* by at least six bases at the 3'-end. The SMBLH GroEL-homolog protein (GroHp) sequence shows that it shares the same ATP binding sites and conserved regions as those of *Buchnera*, *Hamiltonella*, and *E. coli* GroHps. By aligning SMBLH GroHp with *E. coli* GroEL, it was found that both proteins retained the same conserved residues amongst these regions.

The presence of different binding sites on the GroHp of endosymbiont *Buchnera* was explored by Hogenhout et al. (1998, 2000), by studying the binding of PLRV to GroHp. They found that the virus bound to the GroHp in the equatorial domain, mainly two regions. One region contains residues 1 to 121 of the N-terminal and more specifically residues between 9 and 19. The other region contains residues 409 to 474 of the C-terminal, mainly residues between 427 and 457. In *E. coli* GroEL, the same domain exhibits polypeptide binding (Fenton et al., 1994). These regions in SMBLH GroHp still differ somewhat from *Buchnera, Hamiltonella*, and *E. coli* GroHp even in areas where they generally agree. The SMBLH GroHp differs at four residues between amino acids 9 to 19 and eight residues between 427 and 457. It is unknown if these differences play a role in binding to viruses, but the conservation of these regions is maintained.

Phylogenetic analyses using maximum likelihood and neighbor joining gave similar results. Both showed that SMBLH *groel/*GroHp had high identity with strains ML, ALF, PUNC, and NC of *Ca. S. muelleri* and all had leafhoppers as their host, such as *Dalbulus*, *Macrosteles*, and *Nephotettix* species. This is in agreement with Noda et al. (2012). The strains TESUND, SMMAGTRE, and SMDSEM with cicada as their host had the least homology with SMBLH *groel/*GroHp. The phylogeny was in agreement with their insect host suggesting co-evolution/co-speciation. *Ca. S. muelleri* is an endosymbiont of Auchenorrhyncha insects including leafhoppers, sharpshooters, spittlebugs, and cicada. This suggests that the strain SMBLH endosymbiont has the same evolutionary path and outcome as that of other *Ca. S. muelleri* strains.

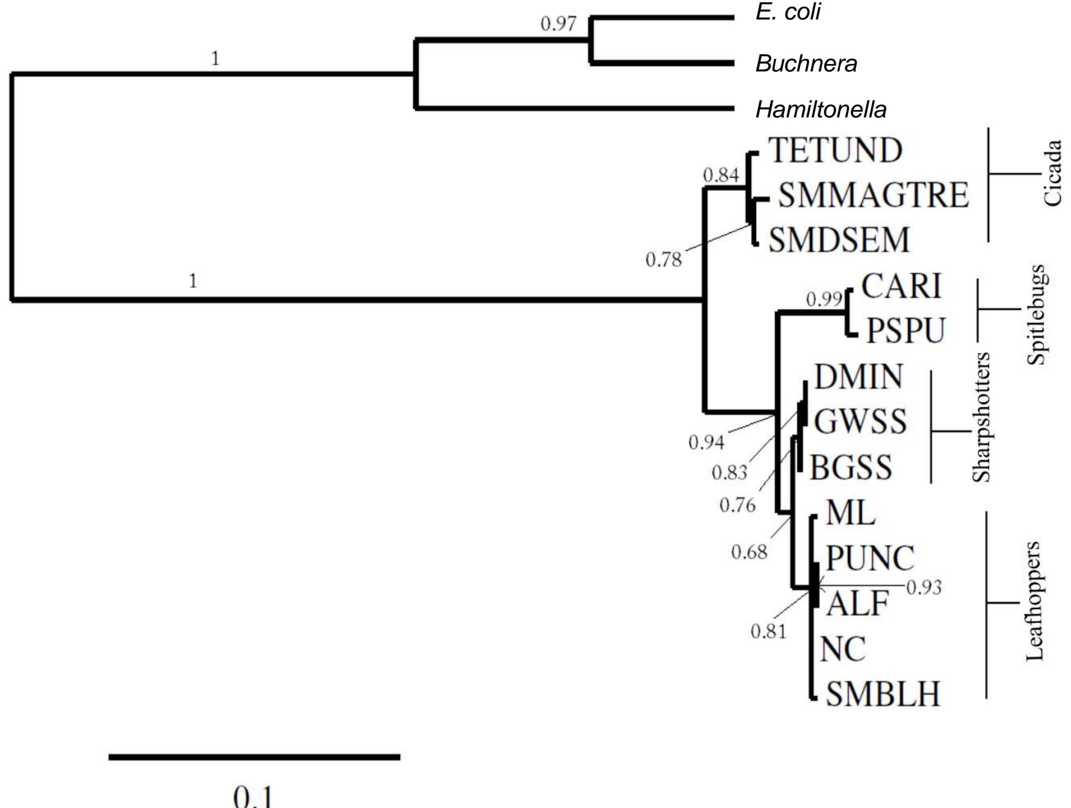


Figure 5. Maximum likelihood (ML) Phylogenetic tree of GroHp proteins indicating the relationship between 13 strains of *Ca. S. muelleri*. The outgroups chosen are endosymbionts of whiteflies (*Hamiltonella*), aphids (*Buchnera*), and free living *E. coli*. The ML tree is almost identical to the Neighbor Joining tree. The clades with *Sulcia* strains show the host insect on the right of the tree. Nodes show >70% confidence for the clade for leafhopper endosymbionts.

Secondary structure was predicted for SMBLH GroHp, *E. coli* GroEL, *Buchnera*, and *Hamiltonella* GroHps. The structures show three regions with differences in the presence or absence of β-stand, α-helix, or coil motifs. SMBLH GroHp was different from the others in region 1 (at the intermediate domain) and region 3 (at the equatorial domain). Comparing these differences with residues at active sites, these differences do not appear to affect the binding sites or conserved regions.

Tertiary structure prediction was employed to see if differences and similarities in the motifs of the predicted secondary structures would affect the folding. This folding might be more important for the protein function than the amino acid sequence itself. *E. coli* GroEL is made of two stacked rings. Each ring is made of seven identical subunits arranged together in a circle, forming 14 to homomer structure. The predicted homo-heptemer structures for *E. coli*, *Hamiltonella*, *Buchnera*, and SMBLH, appeared very similar to each other. Furthermore, the predicted 14-homomer structure of SMBLH GroHp, is similar to the solved structure of *E. coli* GroEL (Braig et al., 1994). Thus, the differences in the secondary structure did not affect the tertiary folding. To validate the predicted tertiary structure of SMBLH GroHp, it was compared to that of *E. coli* GroEL. Both visualized *E. coli* GroEL and SMBLH GroHp, appeared to fold correctly. This further strengthens the conclusion that the differences in the motifs in the predicted secondary structure between the proteins did not have a role in folding.

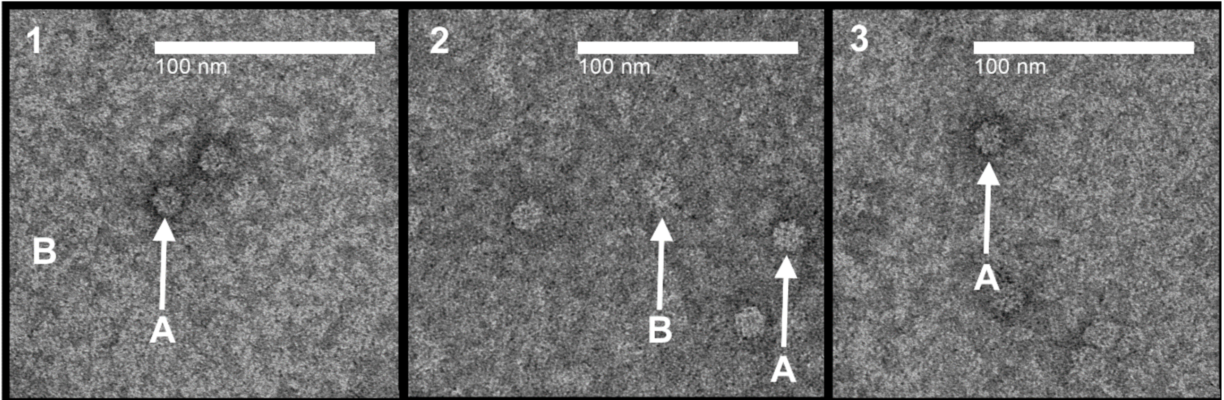
Curly top disease (CTD) is economically important affecting many plant crops including common bean, pepper, spinach, sugar beet, cucurbits, and tomatoes (Baliji et al., 2004). Beet leafhopper (BLH) harbors a new strain of *Ca. S. muelleri* endosymbiont. It produces a GroHp that had not been previously identified.

The role of aphids' and whiteflies' endosymbionts' GroHp in plant virus transmission and plant resistance had been investigated. Interrupting the interaction between the virus's coat protein (CP) and GroHp can reduce virus transmission capacity. This was done by feeding whiteflies, vector of begomoviruses (Geminiviridae), anti-GroHp antibodies derived from *Buchnera* GroHp, providing more than 80% reduction in

Table 6. Predicted secondary and tertiary protein structures of GroHp.

Organism	Region in Secondary structure			Tertiary structure of a single Homo-heptemer SWISS-MODEL modeling
	184-191	313-316	463-473	
E. coli	Coil	α-helix	β-strand	
Buchnera	Coil	Coil	β-strand	
Hamiltonella	Coil	Coil	α-helix	
SMBLH	β-strand	Coil	β-strand and α-helix	
SMBLH GroHp predicted tertiary structure (top view of the seven identical units)				

The three regions of the secondary structures with the position/number of the amino acid residues are listed along with the type of motifs they form. Models of the predicted tertiary structures of GroHp homo-heptemer show that there are no differences between SMBLH GroHp structure and those of *E. coli*, *Buchnera*, and *Hamiltonella*. A predicted ring of SMBLH ring made of seven identical units can be seen at the bottom of the right column.



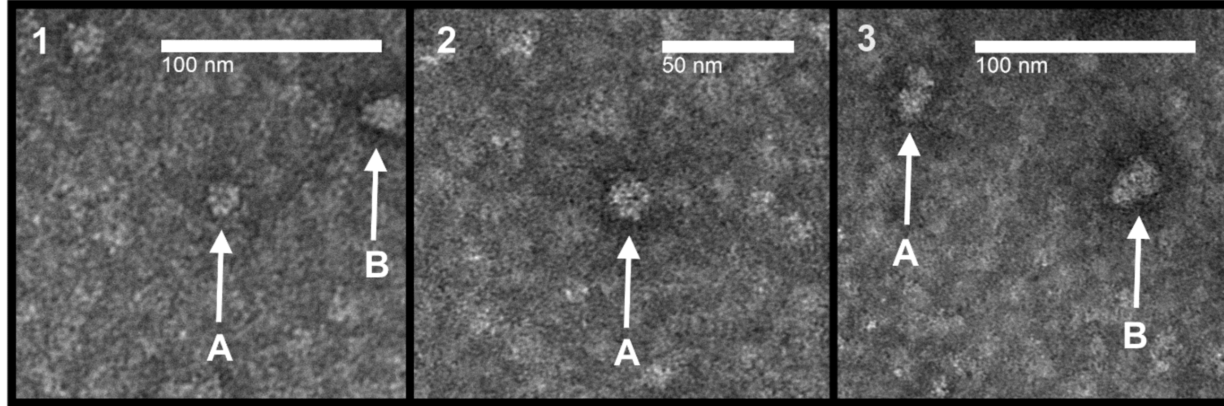


Figure 6. Expressed and purified SMBLH GroHp and *E. coli* GroEL visualized by TEM. (A) Side view showing stacked rings of GroHp/GroEL. (B) Top view of the seven units making up a GroHp/GroEL ring.

virus transmission (Morin et al., 2000). Even if the insect vector harbors more than one endosymbiont, only one GroHp derived from one of the endosymbionts has been implicated in virus transmission (Morin et al., 1999; Gottlieb et al., 2010; Rana et al., 2012; Su et al., 2013).

GroHp could offer some resistance in transgenic plants which carry the gene for the whitefly endosymbiont GroHp protein. These plants were able to tolerate tomato yellow leaf virus (TYLCV), as well as *Cucumber mosaic virus* (CMV) infections. This was because both TYLCV and CMV were able to interact with GroHp, possibly trapping them in the plant and preventing movement of the virus (Edelbaum et al., 2009). Furthermore, GroHp from *Xenorhabdus nematophila* was used to bestow protection against the herbivorous insect *Helicoverpa armigera*, when ectopically produced by transgenic plants (Kumari et al., 2015).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 11(44), pp. 1600-1605, 28 November, 2017
DOI: 10.5897/AJMR2017.8716
Article Number: B9B012766847
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Extended-spectrum beta-lactamase- and carbapenemase-producing Enterobacteriaceae clinical isolates in a Senegalese teaching hospital: A cross sectional study

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Received 23 September 2017; Accepted 13 November, 2017

Extended spectrum β-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae (CPE) have been increasingly reported worldwide. The objective of this study is to determine the prevalence of these multidrug-resistant strains in a major university teaching hospital in Dakar, Senegal. A total of 1205 Enterobacteriaceae stains were tested for ESBL and carbapenemase production. Antibiotics susceptibility test was performed with disk diffusion method. ESBL was detected using a double-disk synergy method. Carbapenemase production was detected with ertapenem 10 µg disk charge. The overall prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae was 26.2 (316/1205) and 5.1% (62/1205), respectively. Interestingly, 3.8% of these pathogens were both ESBL-carbapenemase producers. Among the Enterobacteriaceae ESBL positive, *Escherichia coli* (45.2%, 143/316), *Klebsiella pneumoniae* (26.3%, 83/316), *Enterobacter cloacae* (12.7%, 40/316), and *Proteus vulgaris* (9.2%, 29/316) were the most prevalent. These strains were mainly isolated from urine (56.6%) and pus (22.7%) specimen. The most prevalent CPEs were *E. coli* (45.2%, 28/62), *K. pneumoniae* (27.4%, 17/62), and *E. cloacae* (16.1%, 10/62), particularly isolated from urine (58%) and pus (19.3%). The majority of these MDR strains were isolated from patients hospitalized in urology (32.4%), surgery (27.7%), internal medicine (18.5%), and intensive care units (10%). ESBL-producing Enterobacteriaceae remain highly susceptible to fosfomycin (94.1%), amikacin (92.5%) and ertapenem (88.6%), while carbapenemase producers were fully susceptible to amikacin (100%), and to a lesser extent, fosfomycin (66.7%) and colistin (60%). This study revealed increasing prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae with limited therapeutic options, suggesting a need for continuous multi-drug resistant (MDR) surveillance patterns particularly in hospital settings.

Key words: Extended spectrum β-lactamase, carbapenemase, Enterobacteriaceae.

INTRODUCTION

Multi-drug resistant (MDR) Gram-negative bacilli have been increasingly associated with life-threatening infections worldwide (Girish et al., 2012; Poulou et al., 2014). In the last two decades, bacterial resistance to antibiotics, particularly by extended-spectrum β-lactamase (ESBL) production has become a major public health concern, particularly in resource limited settings (Vasoo et al., 2015). ESBLs have the ability to hydrolyze oxyimino-β-lactam antibiotics (e.g., cefotaxime, ceftriaxone, and ceftazidime) and monobactams (aztreonam), but not cephamycins (e.g., cefoxitin and cefotetan) and carbapenems (imipenem, meropenem, doripenem, and ertapenem) (Paterson et al., 2005).

Although ESBLs have been described in a range of Enterobacteriaceae, these enzymes are predominantly found in the bacterial species of *Klebsiella pneumoniae* and *Escherichia coli* (Pitout and Laupland 2008).

ESBL-producing strains of *E. coli* and *K. pneumoniae* are increasingly reported all over the world, and are important pathogens in community- and hospital-onset infections (Paterson and Bonomo, 2005). ESBL-producing Enterobacteriaceae are associated with life-threatening infections, increased morbidity and mortality and healthcare-associated costs (Pitout, 2010).

Extensive use of expanded-spectrum antibiotics including β-lactams is one of the most important risk factors associated with high prevalence of ESBLs (Chopra et al., 2015; Shukla et al., 2004; Oteo et al., 2010).

Carbapenems are generally stable against ESBLs and still mainly used as treatment of last resort in infections caused by MDR Gram-negative bacilli (Morosini et al., 2006). The emergence of carbapenemase-producing Enterobacteriaceae (CPE) is causing an unprecedented public health threat leaving few treatment options, and consequently leads to high clinical mortality rates (Tzouvelekis et al., 2012).

Carbapenemases are the most versatile family of β-lactamases and are able to hydrolyze carbapenems and other β-lactams (Queenan and Bush 2007). The most important mechanism of carbapenem resistance in Enterobacteriaceae is the production of carbapenemases, although resistance can also result from the synergistic activity between AmpC-type or ESBL combined with decreased outer membrane permeability (Pitout et al., 2015; Ruppé et al., 2015).

The recognition of MDR isolates is a major laboratory challenge and their inappropriate or delayed detection may have negative impacts on patients' management and on the implementation of infection control measures (Nordmann and Poirel, 2014). To our knowledge, the prevalence of MDR Enterobacteriaceae is not well documented in Senegal. Therefore, this study was designed to determine the prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae and its antibiotic susceptibility profiles at Aristide Le Dantec University Teaching Hospital in Dakar, Senegal.

MATERIALS AND METHODS

Bacterial isolates

A total of 1205 non-duplicate clinical isolates were collected from January to December 2016. Clinical specimens were cultured on Eosin Methylene Blue (EMB) agar (Merck, Germany), and incubated at 37°C for 24 h. Clinical isolates were identified using standard biochemical galleria (for *E. coli*, *K. pneumoniae*, *Salmonella* species, *Shigella* species) or Api 20E (for *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Providencia stuartii*) for Enterobacteriaceae.

Antibiotic susceptibility testing

Antimicrobial susceptibility test was performed using the disk diffusion method (Bio-Rad, France) as recommended by the Antibiogram Committee of the French Society for Microbiology (CA-SFM, 2016). Briefly, bacterial suspensions of 10^7 CFU/ml, adjusted with a McFarland densitometer, were inoculated on Mueller-Hinton agar and incubated for 16 to 24 h at 37°C. The following antibiotics were tested: amoxicillin (AMX, 25 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), cefalotin (CF, 30 µg), cefamandole (MA, 30 µg), cefoxitin (FOX, 30 µg), cefotaxim (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), aztreonam (ATM, 30 µg), ertapenem (ERT, 10 µg), gentamicin (GM, 10 µg), amikacin (AN, 30 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), sulphamethoxazole-trimethoprim (SXT, 1.25/23.75), ciprofloxacin (CIP, 5 µg), and fosfomycin (FOS, 50 µg). *E. coli* ATCC 25922 was used for quality control.

ESBL production was detected by double-disk synergy test with disks of amoxicillin-clavulanic acid surrounded at a radius of 30 mm by cefotaxime, ceftriaxone, ceftazidime and aztreonam. Bacterial suspensions at a concentration of 10^7 CFU/ml were inoculated on Mueller-Hinton agar.

Carbapenemase producing strains were detected with an inhibition zone diameter of <25 mm with ERT antibiotic disk as in CASFM (2016).

Statistical analysis

Differences in continuous and categorical variables between groups were analyzed with non-parametric Mann-Whitney U and chi-squared tests, respectively. The level of significance for all statistical tests was set at $p < 0.05$. Statistical analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software.

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Table 1. Demographics and specimen type characteristics from patients infected with ESBL-and carbapenemase-producer and non-producer isolates.

Parameter	Value	% of patients					
		ESBL producer (n=316)	Non ESBL producer (n=889)	p	CPE (n=62)	Non CPE (n=1143)	p
Sex	M/F	191/125	569/320	0.367	38/24	722/421	0.905
Age, year, median (IQR)	-	46 (29-65)	50 (29-64)	0.774	51.5 (32-65)	48 (29-65)	0.753
Hospital unit, n (%)	Internal medicine	50 (18.6)	141 (17.5)	0.634	7 (13)	184 (18)	0.476
	Pediatrics	9 (3.3)	34 (4.2)		4 (7.4)	39 (3.8)	
	ICU	27 (10)	64 (7.9)		7 (13)	84 (8.2)	
	Surgery	74 (27.5)	210 (26)		16 (29.6)	268 (26.2)	
	Urology	92 (32.3)	370 (37.7)		17 (31.5)	369 (36.1)	
	Other	21 (7.8)	59 (7.3)		3 (5.5)	77 (7.5)	
Specimen type, n (%)	Wound swab	76 (22.7)	281 (26)	0.482	12 (19.3)	322 (29.8)	0.711
	Urine	189 (56.6)	583 (54)		36 (58)	711 (65,8)	
	Liquid of effusion	5 (1.5)	17 (1.6)		1 (1.6)	20 (1.8)	
	Genital	9 (2.7)	50 (4.6)		3 (4.8)	50 (4.6)	
	Blood	12 (3.6)	28 (2.6)		3 (4.8)	36 (3.3)	
	Catheter	7 (2.1)	27 (2.5)		0	30 (2.8)	
	Respiratory	32 (9.6)	87 (8)		7 (11.3)	102 (9.4)	
	Fecal	4 (1.2)	7 (0.6)		0	10 (0.9)	

ESBL: Extended-spectrum beta-lactamase; CPE: carbapenemase producing *Enterobacteriaceae*; M: male; F: female; IQR: inter quartile range; ICU: intensive care unit.

RESULTS

Prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae (CPE)

During the study period, a total of 1205 Enterobacteriaceae stains were tested for ESBL and carbapenemase production. The overall prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae was 26.2 and 5.1%, respectively. 3.8% of these pathogens were, however, both ESBL and carbapenemase producers.

The demographic characteristics of the study population are shown in Table 1. Male patients predominated either in ESBL-producer and CPE or in non ESBL-producer and non-CPE isolates. There was no significant difference regarding age between patients infected by these two groups of pathogens.

The majority of these MDR (ESBL and CPE) strains were isolated from patients hospitalized in urology (32.4%), surgery (27.7%), internal medicine (18.5%), and ICUs units (10%).

The prevalence of strains is depicted in Table 2. Among the Enterobacteriaceae ESBL positive, *E. coli* (45.2%), *K. pneumoniae* (26.2%), *Enterobacter cloacae* (12.6%), and *Proteus vulgaris* (9.2%) were the most prevalent. These strains were mainly isolated from urine (56.6%) and pus (22.7%) specimens. The most prevalent CPEs were *E. coli* (46.8%), *K. pneumoniae* (27.4%), and *E. cloacae* (16.1%) particularly isolated from urine (58%) and pus (19.3%).

Susceptibility of ESBL- and carbapenemase-producing Enterobacteriaceae to antimicrobial agents

Table 3 shows the results of the susceptibility test of 316 ESBL- and 62 carbapenemase-producing clinical isolates against several antibiotics. All ESBL producing isolates were resistant to amoxicillin, cefalotin, cefamandole, and cefotaxim or ceftriaxone. The majority of these ESBL strains had associated high resistance rates to non-β-lactam antibiotics, including chloramphenicol (76.4%), sulfamethoxazole/trimethoprim complex (76.2%), tetracycline (61.3%), and ciprofloxacin (50.2%). Interestingly, fosfomycin, amikacin, and imipenem, remain very effective against the majority of the ESBL strains, with 94.1, 92.5 and 88.6% activities, respectively, while colistin activity is declining (66.7%).

Regarding CPE, all isolates were resistant to β-lactam antibiotics, except aztreonam (17.8%). High rates of

Table 2. Distribution of *Enterobacteriaceae* strains ESBL-and CARB-producer and non-producer isolates.

Parameter	Value	% of patients					
		ESBL producer (n=316)	Non ESBL producer (n=889)	p	CPE (n=62)	Non CPE (n=1143)	p
Isolated micro-organisms, n (%)	*Escherichia coli*	143 (45.2)	408 (45.9)	0.773	28 (45.2)	523 (45.7)	0.876
	Klebsiella pneumoniae	83 (26.3)	233 (26.2)		17 (27.4)	299 (26.2)	
	Enterobacter cloacae	40 (12.6)	104 (11.7)		10 (16.1)	134 (11.7)	
	Citrobacter freundii	13 (4.1)	45 (5)		1 (1.6)	57 (5)	
	Proteus vulgaris	29 (9.2)	71 (8)		4 (6.5)	96 (8.4)	
	Salmonella spp.	1 (0.3)	5 (0.6)		-	6 (0.5)	
	Shigella spp.	-	1 (0.1)		-	1 (0.1)	
	Serratia marcescens	-	2 (0.2)		-	2 (0.2)	
	Morganella morganii	7 (1.4)	1 (0.1)		2 (3.2)	18 (1.6)	
	Providencia stuarti	-	7 (0.8)		-	7 (0.6)	

ESBL: Extended-spectrum beta-lactamase; CPE: carbapenemase producing Enterobacteriaceae.

Table 3. Susceptibility rate for ESBL- and carbapenemase-producing isolates to different antibiotics.

Anti-biotics	MDR Enterobacteriaceae	
	% ESBL producer	CPE (%)
Amoxicillin	0	0
Amoxicillin-clavulanic acid	44.2	40.7
Cefalotin	0	0
Cefamandole	0	0
Cefotaxim	0	0
Ceftriaxone	0	0
Aztreonam	33.3	17.8
Ertapenem	88.6	0
Gentamicin	61.5	57.4
Amikacin	92.5	100
Chloramphenicol	23.6	17.7
Tetracycline	38.7	9.7
Sulphamethoxazole-trimethoprim	23.8	14.3
Ciprofloxacin	49.8	28
Fosfomycin	94.1	66.7
Colistin	66.7	60

ESBL: Extended-spectrum beta-lactamase; CPE: carbapenemase producing Enterobacteriaceae.

resistance patterns were detected with tetracycline (90.3%), sulfamethoxazole/trimethoprim complex (85.7%), chloramphenicol (82.3%), and ciprofloxacin (72%). Interestingly, amikacin remained fully active to all isolates.

DISCUSSION

ESBL- and carbapenemase-producing Enterobacteriaceae (CPE) strains are increasingly reported worldwide pathogens in community- and hospital-onset infections (Paterson and Bonomo 2005; Tzouvelekis et al., 2012), suggesting the need for continuous surveillance of antimicrobial resistance (AMR) patterns. In this study, the prevalence of ESBL- and CPE and its antibiotic susceptibility patterns in Aristide Le Dantec Teaching Hospital in Dakar, the major university hospital of Senegal was investigated. The rate of ESBL- and carbapenemase-producing strains was found to be 23.6 and 5.1%, respectively.

High rate of Enterobacteriaceae producing ESBLs have also been reported across Africa, namely Ghana (49.3%) (Obeng-Nkrumah et al., 2013), Gabon (45%) (Schaumburg

et al., 2013), Egypt (38.5%) (Bouchillon et al., 2004), and Tanzania (21%) (Blomberg et al., 2005).

In this study, ESBL production was mainly detected towards *E. coli* (45.2%) and *K. pneumoniae* (26.3%). Similar results have been reported from USA (Ajao et al., 2013) and India (Taneja et al., 2010), showing ESBL prevalence rates from 60 to 71% for *K. pneumoniae* and 35.0 to 42% for *E. coli*. The majority of ESBL producers were recovered from urine specimens (56.6%) and pus (22.7%). This is consistent with data reported in other studies (Obeng-Nkrumah et al., 2013; Severin et al., 2010). Our findings confirm the reports of Pitout et al. (Pitout and Laupland 2008) showing higher frequencies of ESBL-producing Enterobacteriaceae among patients with severe infections including UTIs, suppurative infections, bacteremia, and intra-abdominal. Indeed, ESBL-producing Enterobacteriaceae have been associated with serious nosocomial infection outbreaks that lead to prolonged hospital stay, increased morbidity and mortality, and consequently increased healthcare associated costs with limited therapeutic options (Pitout, 2010). Increasing rate of community-acquired infections caused by ESBL-producing Enterobacteriaceae has, however, been recently reported (Lonchel et al., 2012), representing a potential reservoir for ESBL producers.

ESBL-producing strains were more dominant among patients admitted in urology, surgery, internal medicine, and intensive care units, as reported elsewhere (Obeng-Nkrumah et al., 2013; Shu et al., 2010).

In this study, all strains were resistant to cefotaxim or ceftriaxone (99.9%). These ESBLs are plasmid mediated β-lactamases resistance and are associated with co-resistance to other classes of antibiotics (Paterson and Bonomo 2005). This would explain the high rates of resistance to non-β-lactam antibiotics observed in our study, including tetracycline, sulfamethoxazole/ trimethoprim complex, chloramphenicol, and ciprofloxacin, which are comparable to rates found in other studies (Lin et al., 2012; Simner et al., 2011). Low rates of amikacin and fosfomycin resistance were detected, which is in agreement with findings from Korea (Lee et al., 2012), Taiwan (Liu et al., 2015), and Japan (Wachino et al., 2010). Carbapenems are generally stable against ESBLs and still mainly used as treatment of last resort in infections caused by multi-drug resistant bacteria. As reported elsewhere (Liu et al., 2015), the result of the present study data showed that imipenem remains very active against ESBL-producing *E. coli* and *K. pneumoniae* clinical isolates. However, ESBL-producing Enterobacteriaceae carbapenem resistant are emerging worldwide. This might be due to acquisition of cabapenem-hydrolyzing β-lactamases (Nordmann et al., 2011) or a combination of plasmid-mediated AmpC β-lactamase and loss of an outer membrane protein (Dahmen et al., 2012), limiting thus the drug treatment choices. The most prevalent CPEs detected in this study were *E. coli* (45.2%), *K. pneumonia* (27.4%), and *E. cloacae* (16.1%), particularly isolated from urines (58%), and pus (19.3%). All these isolates were resistant to β-lactam antibiotics. In addition, high resistance rates were observed with tetracycline (90.3%), sulfamethoxazole/trimethoprim complex (85.7%), chloramphenicol (82.3%), and ciprofloxacin (72%). Interestingly, amikacin remained fully active, while fosfomycin and colistin were respectively effective only in 66.7 and 60% of these MDR strains. In fact, only few remaining antibiotics are currently in use to treat infections caused by carbapenemase-producing Gram-negative bacilli including, colistin, tigecycline, amikacin, fosfomycin, and temocillin. Appropriate combination therapy with 2 or more drugs is superior to monotherapy and associated with better survival rate (Tumbarello et al., 2015; Daikos et al., 2014; Zarkotou et al., 2011).

Conclusion

ESBL- and carbapenemase-producing Enterobacteriaceae strains are important pathogens in community- and hospital-onset infections. Emergence of carbapenem-resistant clinical isolates underscores the need for continuous surveillance of antimicrobial resistance patterns. This study shows high prevalence rates of ESBL-producing isolates and emerging prevalence of CPE with limited therapeutic options, suggesting a need for continuous MDR surveillance patterns and antibiotic combination recommendation, particularly in hospital settings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Mame Yacine Fall, Bineta Laly Ba, Mamadou Moustapha Cisse, Khady Gueye, and Maguette Diop Diop for their technical assistance.

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Vol. 11(44), pp. 1606-1613, 28 November, 2017
DOI: 10.5897/AJMR2017.7088
Article Number: 1F5868E66854
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and identification of methicillin-resistant *Staphlococcus aureus* from mastitic dairy cows in Bishoftu town, Ethiopia

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Received 27 August, 2014; Accepted 11 August, 2017

This study determines prevalence of methicillin-resistant *S. aureus* from bovine mastitis in dairy cows from October 2012 to June 2013 in Bishoftu town, Ethiopia. In this cross-sectional study, 16 dairy farms were included and 384 lactating cows sampled. Clinical examination and California mastitis test (CMT) were performed to diagnose clinical and subclinical mastitis, respectively. Milk samples were obtained from the quarters that reacted positively to California mastitis test and cultured to isolate *S. aureus*. Antimicrobial sensitivity test was conducted on the isolates using antibiotics including penicillin G (10 IU), amoxicillin (25 µg), streptomycin (10 µg), erythromycin (15 µg), oxacillin (1 µg), chloroamphenicol (30 µg), vancomycin (30 µg) and ampicillin (10 µg), all from Oxoid and tetracycline (30 µg) and gentamicin (10 µg) with agar diffusion technique. Data were analyzed using Statistical Package for Social Science. Results reveal that of the 384 milk samples collected and subjected to CMT examination, 177(46.09%) were found to be mastitis positive, of which 23(12.99%) and 154(87%) showed clinical and subclinical mastitis, respectively. Of the 177(46.09%) mastitis positive cases, *S. aureus* was isolated in 110(28.65%) samples. The antibiotic susceptibility test indicated that the highest resistance was observed for penicillin (100%) followed by oxacillin (65.45%), erythromycin (61.82%) and amoxacillin (59.09%). There was a statistically significant difference (P<0.05) between *S. aureus* prevalence and risk factors (age and lactation stage). This study indicates that there is high existence of methicillin-resistant *S. aureus* in dairy cows. Therefore, public awareness on transmission, prevention and control of methicillin-resistant *S. aureus* is suggested.

Key words: Bishoftu, dairy cows, methicillin-resistant *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus is a versatile and dangerous pathogen in both humans and animals. It causes skin infections, fatal septicaemia, pneumonia and food poisoning as well as life threatening postsurgical infections. In animals, *S. aureus* is the most notorious pathogen that causes mastitis in dairy cows (Salyers and Whitt, 2002; Butaye et al., 2007). It is highly resistant to adverse environmental conditions and it resists drying as

well as high sodium chloride concentrations. This enables a probably temporary and even permanent colonization of skin and nasal mucosa (Ivanka and Vladimir, 2008).

More than 80% of *S. aureus* strains produce penicillinases and thus beta-lactam antibiotics such as methicillin, which are resistant to penicillinases are widely used to treat *S. aureus* infections (Armand-Lefevre et al., 2005). One basic reason for the continuing important role of *S. aureus* in disease is its propensity to become resistant to antimicrobials. The introduction of penicillin had a profound effect on staphylococcal infections, but penicillin resistance soon followed. Similarly, after the introduction of new antimicrobials such as methicillin, it was not long before methicillin-resistant *S. aureus* (MRSA) developed (Abraham et al., 2007). MRSA, initially described in the 1960s, is now endemic in many hospitals settings, veterinary hospitals and clinics and may account for up to 30% of the staphylococcal infections. MRSA is *S. aureus* strain that is resistant to all classes of beta-lactam antimicrobials, including penicillin, cephalosporin, oxacillin, ampicillin and amoxicillin (Bjorland et al., 2001).

Staphylococcal antibiotic resistance has been associated with resistant plasmids that have the ability to mediate the production of drug inactivating enzymes such as beta-lactamases (Hiramatsu et al., 2014) and other functions (King et al., 2006; Diep et al., 2008). MRSA is not only resistant to methicillin but also different antibiotics. This resistance is mediated by the acquisition of Staphylococcal cassette chromosome mec (SCCmec) encoding for meticillin-resistance gene, mecA (Diep et al., 2008).

S. aureus isolates of human and animals have been extensively analyzed with respect to their virulence patterns and clonal relatedness in developed countries but there is only sparse information on the molecular diversity of *S. aureus* isolates of human from Africa (Schaumburg et al., 2011). In particular, little is known about *S. aureus* infection and asymptomatic carriers in human and animal in Ethiopia except pronounced reports on the effect of *S. aureus* infection as a major cause of bovine mastitis which resulted in great economic loses (Mekonnen et al., 2005).

S. aureus remains one of the most significant organisms associated with clinical and subclinical bovine mastitis worldwide. It is evidenced that these infections respond poorly to therapy with antimicrobial agents (Vintov et al., 2003; Rene et al., 2008). Moreover, the public health of this issue is of great importance because antibiotic therapy of infectious diseases in animals poses risk of selection of resistant strains and introduction of these strains into the food chain (Lee, 2003).

Determination of susceptibility or resistance of strains to antibiotics is very important from a clinical and economic point of view. Moreover, it is crucial to conduct a surveillance of *S. aureus* in neglected sub-Saharan Africa in order to pave way for the control of development and spread of antibiotic resistance strains with special emphasis on MRSA.

Therefore, this study was carried out to estimate the prevalence of MRSA from bovine mastitis, assess antibiogram profile of *S. aureus* to methicillin group of antibiotics and identify associated potential risk factors.

MATERIALS AND METHODS

Study area

The study was conducted in Bishoftu town, East Shoa Zone of Oromia Regional State, Ethiopia, which is located at 47 km south east of the capital, Addis Ababa. The area ranges from 9°N latitude and 4°E longitude at an altitude of 1850 m above sea level in the central highlands of Ethiopia. The mean annual rainfall of the area is 866 mm, with mean relative humidity of 61.3% and daily mean maximum and minimum temperatures of 26 and 14°C, respectively of which 84% is in the long rain season (June to September). The dry season extends from October to February (NMSA, 2012).

Study animals

The study animals included cross bred lactating dairy cows found in purposively selected dairy farms in Bishoftu town, comprising age groups of 3 to 9 years and above.

Sample size determination

Sample size was calculated according to the formula of Thrusfield (2005). Accordingly, the total numbers of sample required for this study were 384 (dairy cows screened for mastitis, both clinical and subclinical), taking into account expected prevalence of 50% as no study has been conducted on isolation and identification of MRSA in the study area with 95% confidence interval and 5% level of precision.

$$n = \frac{1.96^2 \text{ Pexp } (1\text{-exp})}{d^2}$$

Where, n = required sample size; Pexp= expected prevalence; d= precision of the sample estimate.

For eventual selection of the individual study animals, 16 study dairy farms were identified with purposive type of sampling method and on the basis of the ease of accessibility, convenience and

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willingness of the farm owners. The selected dairy farms were clustered into large scale (farms with herd size of 50 and above dairy cows) and small scale or small holders (farms with herd size less than 50 dairy cows). Accordingly, only 3 large scale and 13 small scale dairy farms were identified.

Thus, the calculated 384 lactating cross breed dairy cows were sampled using simple random sampling methods from the 16 dairy farms. For the sake of confidentiality, all the 16 selected dairy farms were designated with alphabetic letters A to P. The alphabetic letters B, G and L represent the large scale groups while the rest 13 represent small scale farms. Moreover, lactating dairy cows were grouped into three categories on the basis of their lactation stages as early, mid and late.

Study design

A cross-sectional study design was employed to investigate the prevalence of methicillin-resistant *S. aureus* from both clinical and sub-clinical mastitic dairy cows in Bishoftu town.

Clinical examination of the udder

Following clinical examination, clinical mastitis was diagnosed based on visible and palpable signs (hard and swollen quarter, kicking on touching the udder and heat) as previously described by Kivaria et al. (2007). In addition, milk from each quarter was withdrawn and examined for any change (watery secretions, clots in milk and blood-tinged secretions). The size and consistency of mammary quarters were inspected for the presence of any anatomical malformation, such as disproportional symmetry, swelling, firmness and blindness (Quinn et al., 1999).

California mastitis test

The California mastitis test (CMT) was conducted to diagnose the presence of subclinical mastitis. This screening test was performed according to the procedure given by Quinn et al. (2002). Briefly, a squirt of milk, about 2 ml from each quarter was placed in each of the four shallow cups in the CMT paddle. An equal amount of the commercial CMT reagent was added to each cup. A gentle circular motion was applied to the mixtures, in a horizontal plane for 15 s. The result was scored as 0, +1, +2 or +3 depending on the intensity of reaction. Samples with CMT result score of 0 and +1 were considered as negative, while those with a score of +2 or +3 were taken as positive and sampled for bacteriological analysis (Quinn et al., 2002).

Milk sample collection

Milk samples were collected aseptically from quarters diagnosed with CMT ≥2 and clinical cases were submitted for bacteriological examination. Briefly, the udder of the cow was thoroughly cleaned with water and dried with a clean towel. After disinfecting the teats with swabs with 70% ethyl alcohol, milk was collected. The first 3 to 4 streams of milk were discarded, and then, about 10 ml of milk was collected from each teat aseptically in separate universal bottles held at slightly horizontal position in order to avoid contamination from the udder (Singh et al., 2007). Tubes were sealed properly and transported on ice to Microbiology Laboratory of College of Veterinary Medicine and Agriculture, Addis Ababa University, where samples were immediately cultured or kept in a refrigerator at 4°C for a maximum of 24 h until cultured on standard bacteriological media (Singh et al., 2007).

Bacterial isolation and identification

Samples collected were cultured following the standard procedure given by Quinn et al. (2002). Milk samples that had been collected from CMT positive and clinical cases were refrigerated, and dispersion of bacteria and fat, were accomplished by warming the samples at room temperature (25°C) for about an hour and then mixed by shaking. The samples were allowed to stand for a while for the foam to disperse and just before inoculation, the tube was inverted gently. One standard loop (0.01 ml) of milk sample was streaked on 7% sheep blood agar. The plates were examined for gross colony morphology, pigmentation and haemolytic characteristics after 24 to 48 h incubation at 37°C. Presumptive colonies of *S. aureus* were selected and sub-cultured on nutrient agar (Oxoid, UK) and incubated aerobically at 37°C for 24 to 48 h. After this incubation on nutrient agar, *Staphylococcus* suspected colonies (based on hemolysis character and pigment production/golden yellow) were taken and subjected to Gram's stain reaction and catalase test. Furthermore, following primary identification, biochemical tests such as tube coagulase test, mannitol and maltose fermentation were carried out to identify the bacterium to the species level (Quinn et al., 2002).

Antimicrobial sensitivity test

A total of 110 *S. aureus* isolated from milk sample of clinical and subclinical mastitis cases of dairy cows were subjected to antimicrobial susceptibility test. Antimicrobials used in this study were penicillin G (10 IU), amoxicillin (25 µg), streptomycin (10 µg), erythromycin (15 µg), oxacillin (1 µg), chloroamphenicol (30 µg), vancomycin (30 µg) and ampicillin (10 µg), all from Oxoid and tetracycline (30 µg) and gentamicin (10 µg) from BBL microbiology systems. The selection of the types of antimicrobial agents used was made purposely to investigate sensitivity pattern of *S. aureus* to methicillin group of antibiotics and antibiotics commonly used in the study of dairy farms. Oxacillin was used in the place of methicillin and the strains of *S. aureus* which are referred to as MRSA are usually oxacillin resistant *S. aureus* (ORSA). Though, methicillin and oxacillin are similar antibiotics, MRSA is the usually accepted designation and this approach was preferred in this study (Gustavo et al., 2001; Cheesbrough, 2002).

Agar disc diffusion (Kirby-Bauer method) was used as described by Quinn et al. (2002). The antibiotic disks were applied on the surface of the inoculated Mueller-Hinton agar plates using aseptic techniques. The disks were deposited with center at least 24 mm apart from each other. Each disk was pressed down with sterile forceps tip to ensure complete contact with the agar surface (Quinn et al., 2002). After measuring the zone of inhibition, isolates were classified sensitive, intermediate and resistant. National Committee for Clinical Laboratory Standard (NCCLS) breakpoints was used to interpret the inhibition zone as adapted from Quinn et al. (1994).

Data management and analysis

All obtained data were stored in Microsoft Excel spreadsheet and analyzed using appropriate statistics. While sampling, animal data related to age, lactation stage, names of the dairy farms and type of antibiotics commonly used in the dairy farms were recorded with proper data collection format. Depending on the findings of clinical inspection and CMT results, cases were categorized as either positive or negative and then positive cases were taken to the

Table 1. Prevalence of *S. aureus* in dairy farms of Bishoftu town.

S/N	Farms designation	Number of animals sampled	Number positive for *S. aureus*	Prevalence (%)
1	A	22	6	27.27
2	B	51	12	23.53
3	C	26	8	30.77
4	D	33	8	24.24
5	E	28	7	25.00
6	F	12	6	50.00
7	G	46	5	10.87
8	H	18	6	33.33
9	I	21	7	33.33
10	J	14	3	21.42
11	K	6	3	50.00
12	L	39	10	25.64
13	M	24	8	33.33
14	N	17	9	52.94
15	O	22	8	36.36
16	P	5	4	80.00
Total	16	384	110	28.65

laboratory for investigations. The age of the study animals was determined from birth records and categorized as young adults (≥ 3 to 5 years), adults (> 6 to ≥ 9 years) and old (> 9 years). The lactation period of lactating dairy cows were obtained from birth records and owners witnessed and categorized them as early lactation (≤3 months), middle lactation (4 to ≤6) and late lactation periods (> 6 months). Logistic regression was used to show the association of the potential risk factors with occurrence of *S. aureus* using SPSS version 20 statistical software. The final model was fit using step wise logistic regression. Pearson's Chi-square test was used to analyze the proportions of categorical data. In all the analysis, the level of significance was set at 5%.

RESULTS

Prevalence of *S. aureus* from dairy cows

Out of the 384 milk samples collected from cross breed lactating cows and subjected to CMT test, 177 (46.09%) were found to be mastitis positive, of which 23 (12.99%) and 154 (87.00%) showed clinical and subclinical mastitis, respectively. Similarly, out of the 177 (46.09%) CMT positive mastitis quarters, *S. aureus* was isolated in 110 samples with rate of 62.15% positivity. However, the prevalence of *S. aureus* among the examined 384 cross bred lactating cows is only 110 (28.65%) (Table 1). The prevalence of *S. aureus* for the age and lactation period of ≤ 5 years and ≤ 3 months, >6 to ≤ 9 years and ≤ 3 months, and > 9 years and ≤ 3 months were 12.5, 28.6 and 20%, respectively.

Antimicrobial susceptibility test result

A total of 110 isolates of *S. aureus* originating from milk samples were tested for susceptibility to methicillin group of antibiotics and other commonly used antimicrobials in the study area. Accordingly, the antimicrobial susceptibility test result showed that *S. aureus* was highly resistant only to four of the ten antibiotics, specifically, penicillin (100%), oxacillin (65.45%), erythromycin (61.45%) and amoxicillin (59.09%) (Table 2).

Associated risk factors

The prevalence of *S. aureus* as opposed to specific risk factors was investigated using Chi-square test. There was a statistically significant difference (P<0.05) between the prevalence of *S. aureus* and risk factors, namely, age and lactation stage. For instance, older cows more than 9 years of age were noticed to be more affected (48%) than adult cows between 6 and 9 years (29.8%).

The prevalence of *S. aureus* on the basis of different lactation stages showed that highest figure was recorded during late stage of lactation (38.1%) followed by mid lactation (21.4%) and early lactation (20%). The above variation in the prevalence of *S. aureus* on the basis of

Table 2. Drug sensitivity patterns of *S. aureus* isolated from milk samples (n=110).

Antimicrobials	*Staphylococcus aureus*			
	Resistance		Sensitive	
	Number	Percentage	Number	Percentage
Penicillin	110	100.0	-	-
Amoxicillin	65	59.09	45	41.90
Tetracycline	54	49.09	56	51.90
Erythromycin	68	61.82	42	38.18
Gentamycin	7	6.36	15	93.64
Chloroamphenicol	14	12.72	96	87.28
Oxacillin	72	65.45	20	57.40
Ampicillin	57	51.82	53	48.18
Stereptomycin	27	24.55	83	75.45
Vancomycin	22	20.00	88	80.00

Table 3. Prevalence and risk factors for *S.aureus* in dairy cows of Bishoftu town.

Risk factors	Status of *S. aureus*		Number	Prevalence (%)	Chi-square	df	P-value
Age	≤ 5 years	Negative	87	79.8	9.02	2	0.01
		Positive	22	20.2			
		Total	109	100			
	> 6 to ≤ 9 years	Negative	165	70.2			
		Positive	70	29.8			
		Total	235	100			
	> 9 years	Negative	22	55			
		Positive	18	45			
		Total	40	100			
Lactation stage	≤ 3 months	Negative	88	80	14.17	2	0.001
		Positive	22	20			
		Total	110	100			
	4 to ≤ 6 months	Negative	77	78.6			
		Positive	21	21.4			
		Total	98	100			
	> 6 months	Negative	109	61.9			
		Positive	67	38.1			
		Total	176	100			
Overall		Negative	274	71.4			
		Positive	110	28.6			
		Total	384	100			

Df is degree of freedom.

different lactation stages was statistically significant (P<0.05) (Table 3 and Figure 1).

DISCUSSION

The present study addressed the status of MRSA from bovine mastitis in Bishoftu town. In line with this, *S. aureus* isolates were obtained and tested for antimicrobial susceptibility test by using methicillin group of antibiotics. Moreover, potential risk factors associated with mastitis caused by *S. aureus* were assessed. The rate of isolation of *S. aureus* from mastitic milk was found to be 28.65%. The isolation rate of *S. aureus* in this work

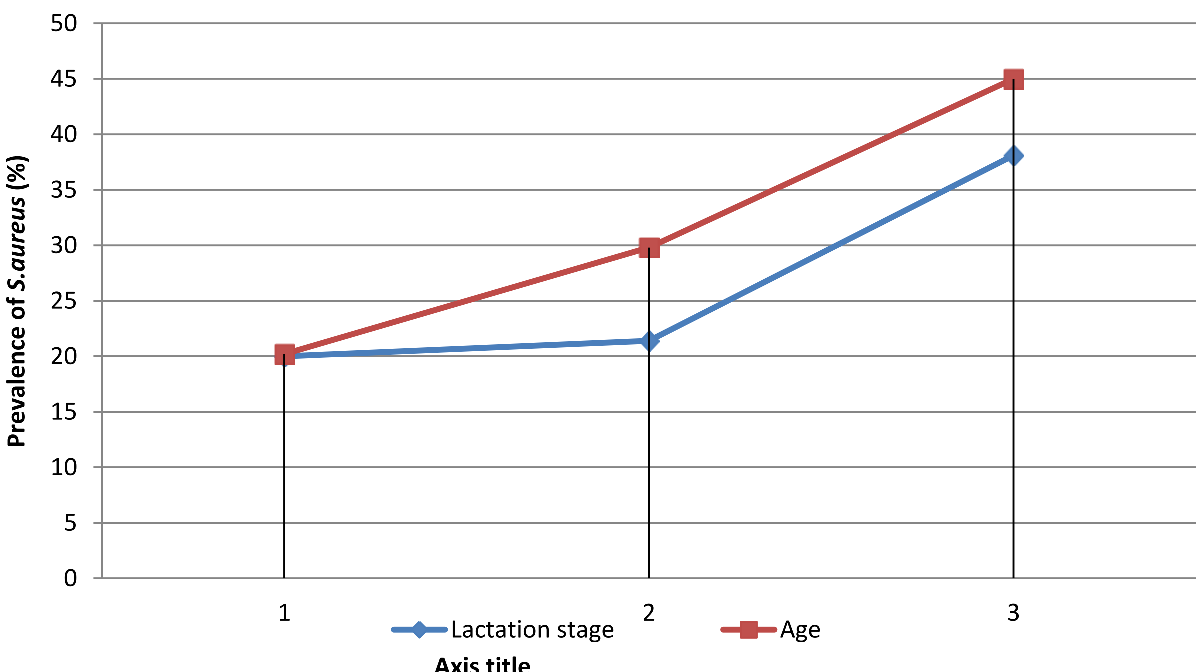

Figure 1. Prevalence and risk factors for *S. aureus* in dairy cows of Bishoftu town. Lactation stage: 1= ≤ 5 years; 2= > 6 to ≤ 9 years, 3= > 9 years; Age: 1=≤ 3 months; 2= 4 to ≤ 6 months; 3= > 6 months.

is slightly similar to that of Hundera et al. (2005) who isolated *S. aureus* at the prevalence rate of 29.20%.

However, the prevalence rate of *S. aureus* in the current study was lower than that of the findings of Lakew et al. (2009), Abera et al. (2010), Mekibib et al. (2010) and Sori et al. (2011) in which the isolation rate of *S. aureus* was 41.4, 42.10, 47.50 and 39.44%, respectively. The relatively high prevalence of *S. aureus* in previous studies can most likely be attributed to the wide distribution of the organism inside the mammary glands and on the skin of teats and udder (Jones et al., 1998). It is well known that *S. aureus* has already adapted survival in the udder and can establish chronic and subclinical infections. From there, it is shed into the milk, which serves as source of infection for healthy cows during the milking process (Takele et al., 2017). *S. aureus* is one of the contagious pathogens that can be easily transmitted from one cow to another during unhygienic milking process (Rowe, 1999).

The findings of the present study (28.65%) was found to be much different from that of Mekonnen et al. (2005) who reported 8% prevalence of *S. aureus*. It was also greater than the reports of Bitew et al. (2010), Salihu et al. (2011), Daka et al. (2012) and Fufa et al. (2013) who identified 20.3, 22.8, 17.9 and 21.13%, respectively. The difference in the prevalence of *S. aureus* between the present study and the previous reports could be due to the variation in the hygienic and management systems of the dairy farms or increase in the spread and abundance of *S. aureus* with time.

The antibiotic susceptibility assay performed for *S. aureus* isolates from clinical and subclinical mastitis cases in dairy farms from Bishoftu town, revealed higher degree of resistance for penicillin (100%), oxacillin (65.45%), erythromycin (61.45%), amoxicillin (59.09%) and ampicillin (51.82%). The present study also showed that gentamycin, vancomycin and streptomycin were found to be effective antibiotics against *S. aureus*. This present report is similar to the findings of Addisalem and Mersha (2012) and Sori et al. (2011). In fact, these antibiotics are effective against Gram negative bacteria. However, the findings of this study showed gentamycin, vancomycin and streptomycin to be effective against *S. auerus*. Probably, these drugs are less circulated in dairy farms of the study area with relatively less chance of resistance development.

Among others, the resistance pattern of penicillin was also in agreement with the reports of Abera et al. (2010) and Shiferaw et al. (2009). The 51.82% resistance recorded for ampicillin in the present study was quite lower than that of Corrales et al. (1995) and Mekonnen et al. (2005) who found 75 and 83% resistance, respectively. This difference in the resistance pattern of *S. aureus* isolates to ampicillin between the current and previous reports may be associated with the infrequent and or frequent use of the drug in the study area.

It has also been documented that MRSA isolates that are resistant to beta-lactam antibiotics may induce cross resistance to vancomycin as stated by Gundogan et al. (2005). In the present study, all the *S. aureus* isolates originating from milk were resistant to beta-lactam group of antibiotics.

Regarding the risk factors, age of the animals and stage of lactation were found to be statistically significant (P<0.05) with prevalence of *S. aureus*. The prevalence of *S. aureus* in the current study showed an increasing pattern of 20, 21.4 and 38.1%, in early, mid and late lactation stages, respectively. Similar increasing prevalence pattern were also reported by Moges et al. (2012) and Mungube et al. (2005) where higher prevalence of *S. aureus* was observed in mid and late as compared to early stages of lactation. This could be attributed to prolonged risk of exposure to pathogens and contact of the dairy cows with other animals though in different lactation stages. Moreover, it is possible for the dairy cows to acquire *S. aureus* infection from different dairy personnel working in the farms. Similarly, the prevalence of *S. aureus* was noted to increase while age advances. For instance, the prevalence of *S. aureus* was 20.2, 29.8 and 45% for age groups ≤ 5, > 6 to ≤9 and > 9 years of animals, respectively. Obviously, similar justifications specifically: prolonged risk of exposure to pathogens and contact of the dairy cows with other animals could be cited for increasing prevalence of *S. aureus* with age. The present study on *S. aureus* infection in dairy cows is significantly important with a higher prevalence of 28.6%. Accordingly, *S. aureus* infection was pointed out to be one of the major dairy animals' health problems in Bishoftu town in particular, and Central Ethiopia at large. The wide spread distribution of *S. aureus* imposed greater resistance to methicillin group of antibiotics in dairy cows. The resistance of *S. aureus* to multiple drugs belonging to methicillin group, especially penicillin, ampicillin, erythromycin and oxacillin calls for serious and immediate public attention in raw milk consumption. On the contrary, few antibiotics namely, vancomycin, gentamycin, chloroampenicol and streptomycin were observed to be effective against *S. aureus*, where proper and ethical use of these drugs might help in the control and limitation of resistance to *S. aureus*. The current work findings also disclosed that the prevalence of *S. aureus* increased as age and lactation period of the dairy cows increased.

Therefore, there is an urgent need to create public awareness at different levels, on transmission, prevention and control of MRSA. It is very important to implement a systematic application of an *in vitro* antibiotic susceptibility test prior to the actual use of antibiotics both in therapeutic as well as prophylactic schemes. Farm level hygienic and improved management practices should be introduced to small and mid-scale dairy farms. Impact and dynamics of genetic antibiotic resistance determinants should also be investigated. Specific strain(s) of MRSA should be identified and characterized, and thus, the responsible zoonotic strain(s) could be known.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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