J Vector Borne Dis 52, March 2015, pp. 52–57

Isolation and characterization of *Vagococcus* sp from midgut of *Culex quinquefasciatus* (Say) mosquito

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ABSTRACT

Background & objectives: Mosquito gut is a rich source of microorganisms. These microorganisms exhibit close association and contribute various physiological processes taking place in mosquito gut. The present study is aimed to characterize two bacterial isolates M19 and GB11 recovered from the gut of *Culex quinquefasciatus* mosquito collected from Bhuj and Jamnagar districts of Gujarat, India.

Methods: Both the strains were characterized using polyphasic approach including, phenotypic characterization, whole cell protein profiling and sequencing of 16S rRNA gene and *groESL* region.

Results: Sequences of 16S rRNA gene of M19 and GB11 were 99% similar to *Vagococcus carniphilus* and *Vagococcus fluvialis.* But phenotypic profile, whole cell protein profile and sequence of *groESL* region of both isolates were found to be similar to *V. fluvialis.*

Conclusion: Based on phenotypic, genotypic and protein profiling, both the strains were identified as *V. fluvialis.* So far this species was known from domestic animals and human sources only. This is the first report of *V. fluvialis* inhabiting midgut of *Cx. quinquefasciatus* mosquito collected from Arabian sea coastal of India.

Key words *Culex quinquefasciatus*; midgut bacteria; mosquito; *Vagococcus*

INTRODUCTION

The mosquito *Culex quinquefasciatus* (Diptera: Culicidae) is a cosmopolitan mosquito and transmits lymphatic filariasis and West Nile virus, affecting millions of people every year[1]. In the process of transmission, irrespective of their category all the parasites come in contact with mosquito gut environment which is harboured by a large number of bacteria[2]. These bacteria are involved in various physiological processes taking place inside the mosquito gut. Some of the bacteria are closely associated with mosquito, and their absence could lead to reduced fitness or even death of mosquito vector[3–4]. Midgut microbiota may also contribute in vector competence of mosquito vector by either influencing or inhibiting parasite transmission[5]. *Cx. quinquefasciatus* mosquito has drawn special attention due to its competence to survive in diverse range of environmental conditions. Diverse bacterial population were reported from the midgut of wild caught *Cx. quinquefasciatus* mosquitoes[6].

In the present study, two bacterial isolates were isolated from the midgut of *Cx. quinquefasciatus* mosquitoes collected from coastal region of Arabian sea from Gujarat, India. Detailed characterization of strains was carried out using polyphasic taxonomic approach.

MATERIAL & METHODS

Source of bacterial isolates

Indoor resting adult mosquitoes were collected from Bhuj, Gujarat (23°15' N/69°39' E) and Jamnagar, Gujarat (22°27' N/70°04' E), using mouth aspirator and kept in plastic cage which was sterilized by 70% ethanol prior to use. Mosquitoes were anesthetized with chloroform and species were identified morphologically[7]. Midguts were dissected out only from female adult mosquitoes under sterile conditions. Total 100 female mosquitoes were analysed for the presence of midgut bacteria. The midgut was isolated and kept in eppendorff tubes containing 100 ml of brain heart infusion broth (Himedia). Tubes were incubated for 4 h for enrichment. After incubation equal volume of 40% glycerol was added in these tubes and preserved in liquid nitrogen and transported to laboratory for further processing. Midgut contents were serially diluted in PBS and spread on tryptose soya agar (Himedia) supplemented with 5% sheep blood and incubated at 30°C for 48 h. Thus, obtained colonies were fur-

ther streaked to get single colony. Strains M19 and GB11 were recovered from the midgut of *Cx. quinquefasciatus* mosquito. Both the strains were maintained on tryptone soya agar (TSA) at 30°C.

Phenotypic characterization of strains

Phenotypic analysis of both the isolates (M19 and GB11) was carried out using conventional biochemical tests[8]. Briefly, haemolysis was assessed in columbia agar containing 5% sheep blood. Motility was determined at 25, 30 and 37°C in semi solid medium containing 0.25% noble agar (Difco), 1% Tryptose (Difco) and 0.5% NaCl. Growth at various concentrations of NaCl (0.5 to 8%) and at various temperatures (20, 25, 30, 37, 40 and 45°C) was determined. Other biochemical tests such as gas production, aesculine hydrolysis in the presence of 40% bile, arginine hydrolysis, hippurate hydrolysis, methyl red voges proskauer, pyrrolidonyl arylamidase β-galactosidase activity, arginine utilization, lysine decarboxylase, ornithine decaroxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production, citrate utilization, malonate, and indole were also assessed.

Analysis of whole cell protein profile by one dimensional SDS PAGE

Strains M19, GB11 and type strain of *V. fluvialis* (DSMZ 21402^T) were grown on TSA plates for 24 h at 37°C. Preparation of protein extracts and analysis of whole cell protein profiles by one dimensional SDS PAGE were performed as described by Merquior *et al*[9]. Type strain of *V. fluvialis* (DSMZ 21402^T) was procured from DSMZ, Germany.

Amplification and sequencing of 16S rRNA gene

Chromosomal DNA was isolated by Gentra Puregene Yeast/Bact kit (Qiagen) following manufacturer's instructions. Complete 1.5 kb sequence of 16S rRNA gene was amplified from the chromosomal DNA by PCR employing the method using universal eubacteria specific primer[6]. Amplified PCR products were then purified using MiniElute PCR purification kit (Qiagen) following manufacturer's instructions. Purified PCR amplicons were sequenced by ABI 3730 automated DNA sequencer. Sequences were submitted to GenBank under the accession number JF690756 and JF690757.

Sequence analysis

Sequences of 16S rRNA gene from isolates were analyzed by BLASTn analysis (*www.ncbi.nlm.nih.gov/BLAST*). For phylogenetic analysis 16S rRNA sequences of related taxa were obtained from the GenBank database. The multiple sequence alignment was carried out using ClustalW program available at EMBL server (*www.ebi.ac.uk/clustalw*). Unaligned sequences at the beginning and at the end of the alignment file were trimmed by software DAMBE (DNA analysis for molecular biology and evolution). The phylogenetic tree was constructed using the neighbor-joining method using Kimura 2 parameter distance in MEGA 4.0 software with bootstrap values based on 1000 replicates[10]. 16S rRNA sequence of *Catellicoccus phocoenae* (AJ854484.1) was taken as out group.

Amplification and sequencing of groESL gene

Whole *groESL* region (1881 bp) was amplified. PCR primers were designed based on *groESL* sequence of *V. fluvialis* available at GenBank (Accession No. AY328534). The PCR conditions used were, initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min. After PCR reaction amplicons were purified and sequenced as described earlier, to get complete sequence of *groESL* region 4 internal primers along with PCR primers were used (Table 1). Obtained partial sequences were assembled to get complete sequence. Complete sequences from both isolates were aligned with GenBank sequence of *V. fluvialis* (Accession No. AY328534). Sequences were submitted to GenBank under the accession number JF732882 and JF732883.

Antibiotic susceptibility assay

The disc diffusion method was adopted to check antibiotic susceptibility status of the isolates[11]. The test was conducted in triplicate for each isolate and the results were included in this study only for those which exhibited the same resistant pattern in all the plates. Antibiotic suscep-

Table 1. List of primers designed for amplification and sequencing of *groESL* region

Primer name	Sequence	Position
groF1	5'–TTG TTA AAA CCA TTA GGA GAT CGT G –3'	1 – 25
groR2	5'–ATA TCA GCT AAT GTA TCA ACA C–3'	1499 – 1521
groR3	5'–TTG TTC TAA TAA AGG TAA AAT G–3'	861 – 883
groF2	5'–GTG GTT ACT TAT CTC AAT AC–3'	892 – 911
groF3	5'–TGA TCG TGA AAA ACT ACA GG–3'	1382 – 1401
groR1	5'–CAT TAC TTG GTT TGT CAG CAA TAA C–3'	1856 – 1881

tibility assay was carried out on Mueller Hinton Agar plates (Himedia). About 100 µl of culture specimen having turbidity of 0.5 MacFarland standard was spread uniformly. Antibiotic discs (Himedia) were placed on the surface of medium and were allowed to settle then inverted and kept at 37°C for 18–24 h. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimetre. Antibiotic susceptible, intermediate resistant, and resistant patterns were determined according to CLSI standard[12]. Zone diameter was determined after 18 h for all the disks except oxacillin and vancomycin that was determined after 24 h. Antibiotic susceptibility status of both the isolates have been determined against following antibiotics: gentamycin, kanamycin, neomycin, streptomycin, tobramycin, methicillin, nafcillin, imipenem, cefaclor, ciprofloxacin, gatifloxacin, norfloxacin, vancomycin, clindamycin, erythromycin, oxacillin, piperacillin, penicillin G and nalidixic acid.

RESULTS

Two non-motile gram positive bacterial isolates, M19 and GB11 were isolated from the midgut of *Cx. quinquefasciatus* collected from Bhuj and Jamnagar (Gujarat) respectively. For identification of these isolates, 16S rRNA genes have been amplified, sequenced and found to be identical to each other. On BLASTn analysis both the sequences showed maximum sequence similarity (99.9%) with *V. fluvialis* (GQ337040) and *V. carniphilus* (AY669387). Figure 1 shows the phylogenetic relationship of isolated species with other members of genus *Vagococcus*. Both the isolates are showing close association with *V. fluvialis.*

The key differentiating phenotypic test of the strains studied are summarised in Table 2. Both the strains have produced acid from adonitol, arbutin, cyclodextrin, cellobiose, D-fructose, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, D-Saccharose, D-sorbitol, D-trehalose, glycerol, L-arabinose, salicin, α-methyl-D-glucoside. Whereas acid is not produced from amygdalin, arbutin, D-arabinose, D-arabitol, D-galactose, D-tagatose, D-turanose, dulcitol, D-xylose, esculine, gentibiose, gluconate, inositol, inuline, L-arabitol, L-fucose, melibiose, methyl β-D-xylose, mehtyl α-D-glucopyranoside, N-acetyl glucosamine, rahmnose, reffinose, and sucrose. In case of D-lactose, M19 does not produce acid but GB11 has produced acid. Both isolates, M19 and GB11 are positive for arginine dihydrolase, leucine arylamidase, pyrolidonyl arylamidase, β-galactosidase and β-glucosidase. Whereas, both the strains are negative for alkaline phosphatase, VP, α-galactocidase and α-glucocidase. Esculine is hydrolysed but Hippurate was not hydrolyzed.

Growth at various concentrations of NaCl (0.5 to 8%) has been studied. Both isolates were able to grow up to the NaCl concentration of 3.5%. Growth at different temperatures has also been observed and isolates were found to be able to grow at 20, 25, 30, 37 and 40°C but could not grow at 45°C.

Whole cell protein profiling by SDS PAGE is a very

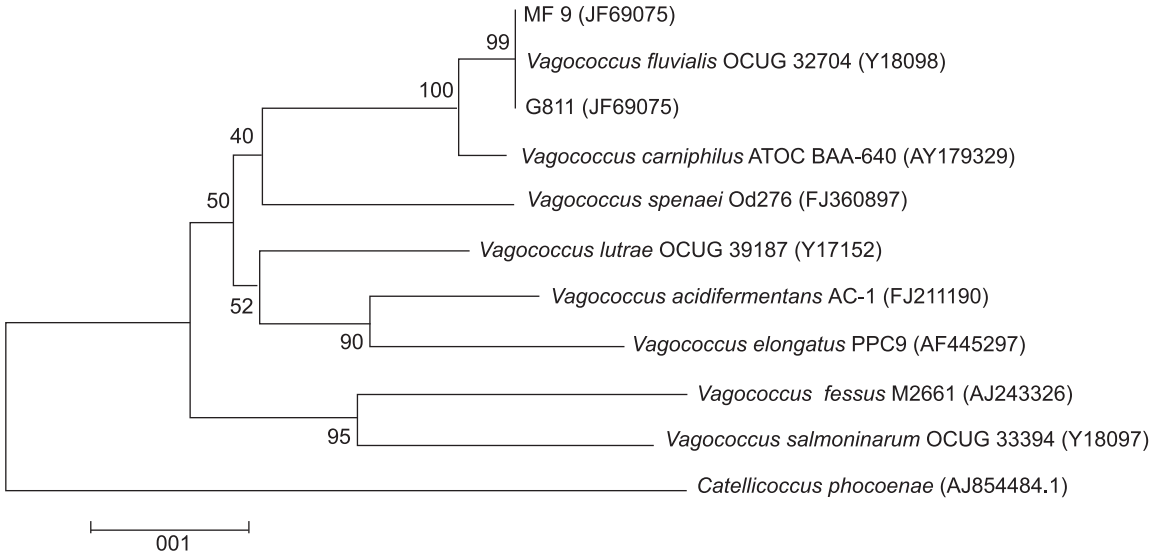

Fig. 1: Rooted tree showing the phylogenetic relationship of strain M19 and GB11. Neighbour-joining phylogenetic tree constructed from a comparative analysis of 16S rRNA gene of M19 and GB11 with *V. fluvialis* and other related species of Vagococcus genus. Tree was generated using the neighbour-joining method with Kimura 2 Parameter distance in MEGA 4.0 software. Number at the nodes indicates percent bootstrap value. The bar indicates the Jukes-Center evolutionary distance.

Table 2. Differential phenotypic characteristics of isolate M19, GB11, *V. fluvialis* and *V. carniphilus*—1: M19; 2: GB11; 3: *V. fluvialis;* 4: *V. carniphilus*[*]

Characteristics	Species			
	1	2	3	4
Acid production from substrates				
Adonitol	+	+	–	–
Amygdalin	+	+	+	
Arbutin	+	+	+	–
D-arabinose	–	–	–	+
D-cellobiose	+	+	+	–
D-lactose	–	+	–	–
D-mannitol	+	+	+	–
D-mannose	+	+	+	–
D-saccharose	+	+	+	–
D-sorbitol	+	+	+	–
Esculine	–	–	–	+
L-arabinose	+	+	–	–
L-fucose	–	–	–	+
Methy-α-D-glucopyranoside	–	–	–	+
N-acetylglucosamine	–	–	–	+
Salicin	+	+	–	+
Sucrose	–	–	+	V
Enzyme activity and others tests				
Arginine dihydrolase	+	+	+	–
α-glucosidase	–	–	–	+
β-galactosidase	+	+	+	–
β-glucosidase	+	+	+	–

*Biochemical test for *V. carniphilus* was taken from Shewmaker *et al*[13]; + Positive reaction, – Negative reaction; V–Variable reaction.

useful tool and routinely used method for differentiation of bacterial species belonging to *Enterococci*[13]. Whole cell protein profiling of both isolates was carried out by one dimensional SDS PAGE. The profiles were compared with typed strain of *V. fluvialis.* M19 and GB11 had virtually indistinguishable protein profile when compared with typed strain of *V. fluvialis.*

Identity was further confirmed by amplification and sequencing of *groESL* regions. Complete *groESL* regions were sequenced and were identical to each other and found to be similar to the sequence of *V. fluvialis* available at DNA database. Only one base change has been observed in the spacer region at the position 289 of complete amplified region (Fig. 2). There A is substituted with C in both the isolates but the length of the spacer region remained unaltered.

Susceptibility status of both isolates was determined against the different classes of antibiotics using disc diffusion method and susceptibility status was determined based on CLSI guidelines and summarized in Table 3. Both the isolates were found to be susceptible for gentamycin, streptomycin, imipenem, cefaclor, ciprofloxacin, gatifloxacin, erythromycin, oxacillin and penicillin G whereas, strains were resistant to norfloxacin, vancomycin, clindamycin and nalidixic acid.

DISCUSSION

The genus *Vagococcus* was erected by Collins *et al*[14] to accommodate *Lactococcus* like but motile bacteria which are reactive with Lancefield anti serum N. The type species of genus Vagococcus was *Vagococcus fluvialis* which was isolated from the excreta of chicken[15]. So far

Table 3. Susceptibility pattern of strains M19 and GB11 to different classes of antibiotics

S. No.	Antibiotic	Antimicrobial group	Size of zone of inhibition	
			M19	GB11
1.	Gentamycin (30 mcg)	Aminoglycoside	S	S
2.	Kanamycin (30 mcg)	Aminoglycoside	R	R
3.	Neomycin (30 mcg)	Aminoglycoside	I	R
4.	Streptomycin (25 mcg)	Aminoglycoside	S	S
5.	Tobramycin (10 mcg)	Aminoglycoside	I	I
6.	Methicillin (30 mcg)	β-lactam	I	I
7.	Nafcillin (30 mcg)	β-lactam	R	S
8.	Imipenem (10 mcg)	Carbapenem	S	S
9.	Cefaclor (30 mcg)	Cephalosporine	S	S
10.	Ciprofloxacin (10 mcg)	Fluoroquinolone	S	S
11.	Gatifloxacin (30 mcg)	Fluoroquinolone	S	S
12.	Norfloxacin (30 mcg)	Fluoroquinolone	R	R
13.	Vancomycin (10 mcg)	Glycopeptide	I	I
14.	Clindamycin (10 mcg)	Lincosamide	R	R
15.	Erythromycin (15 mcg)	Macrolide	S	S
16.	Oxacillin (5 mcg)	Panicillin	S	S
17.	Piperacillin (100 mcg)	Panicillin	I	S
18.	Penicillin G (10 units)	Panicillin	S	S
19.	Nalidixic acid (30 mcg)	Synthetic quinolone	R	R

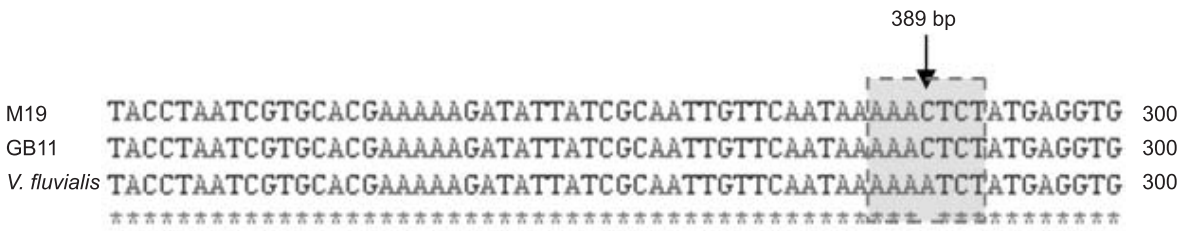

Fig. 2: Multiple sequence alignment showing variation in intergenic spacer of *groESL* region.

eight bacterial species have been identified from diverse environmental sources. These include *V. salmoninarum* from fish[16], *V. lutrae* from Otter *(Lutra lutra)*[17], *V. fessus* from seal and harbour porpoise[18], *V. carniphilus* from ground beef[13], *V. elongatus* from swine manure storage pit[19], *V. penaei* from cooked shrimp[20] and *V. acidifermentans* from acidogenic fermentation bioreactor[21].

V. fluvialis is a motile gram positive coccus shaped bacterium and has been isolated from chicken faces, lesions and tonsils of domestic animals and also from clinical samples of peritoneal fluid and wound from human sources[22–23]. Recently, it has been isolated from periradicular lesions of root filled tooth[24].

When the genus *Vagococcus* formed, motility was the key character to differentiate from non-motile *Lactococcus* bacteria[14]. But later on some non-motile *V. fluvialis* isolates were also retrieved from domestic animals[22]. Similarly, the present isolates of *Vagococcus* from the midgut of *Cx. quinquefasciatus* are also non-motile. Among other variations in the present strain, we observed acid production from sugars. Both M19 and GB11 produced acid from Adonitol, L-Arabinose and Salicine whereas typed *V. fluvialis* has not produced acid from these sugars.

The *groESL* genes are also known as cpn10/60 or hsp10/60 and encodes for 10 kDa (*groES*) and 60 kDa (*groEL*) heat shock proteins and; *hsp10* and *hsp60* are ubiquitous and highly conserved among bacteria[25]. This region includes complete *groES* (hsp10) and *groEL* (hsp60) genes and intergenic spacer region. The *groESL* is extensively used for the identification of bacteria such as *Staphylococcus*[26], *Enterococcus*[27–28], *Vagococcus*[29], *Streptococcus*[30]. In the present study, *groESL* sequences of both the isolates were identical to each other and to the sequence of *V. fluvialis* except on base pair change at position 289 of spacer region. Such intraspecies variation in *groESL* region is already been reported in other bacterial species[31–32]. Such variation may exist in other strain of *V. fluvialis* but their sequence information of *groESL* is not available.

Based on polyphasic analysis, both the isolates were identified as *V. fluvialis.* Present study is the first report of isolation of this bacterial species from mosquito gut. Midgut bacteria play an important role in biology of the mosquito. In the absence of midgut bacteria, the survival and fecundity of mosquito is reduced[3,32]. Bacterial community also may influence the developmental stages of pathogens, for example, in the presence of gram negative bacteria, the oocysts formation of malaria parasites is reduced, thus affecting the transmission cycle[33]. Many species of the vectors are refractory, i.e. unable to transmit the pathogens, besides innate immunity of vectors, difference in midgut microbiota may be one of the reason. Midgut bacteria may also be used to interrupt disease transmission through paratransgenesis[34]. Paratransgenesis is the technique where, midgut bacteria is genetically transformed to expressed antiparasitic molecules, and again reintroduced in insect gut. When parasite comes in midgut environment which is already harboured by these genetically modified bacteria. Antiparasitic molecules produced by bacteria kills parasite within midgut and block further transmission[34]. Significant reduction (99%) in *Trypanosoma cruzi* infection in *Rhodinus prolixus* has been observed using genetically modified symbiotic bacteria *Rhodococcus rhodnii*[35]. Same strategy may be tested in mosquito and isolates recovered in present study could be further evaluated for its suitability for paratransgenesis.

ACKNOWLEDGEMENTS

Authors are thankful to Prof. (Dr) M.P. Kaushik, Director, Defence Research and Development Establishment, Gwalior for providing all facilities and financial support required for this work.

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Received: 24 July 2013 *Accepted in revised form:* 25 July 2014