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Intestinal carriage of epidemic strains of Vibrio cholerae O1 'hybrid' by bats – A first time report from Chennai, South India.

KEYWORDS: Cholera, Vibrio cholerae, ctxAB, Bats.

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ABSTRACT *Vibrio cholerae, the causative agent of cholera is primarily an inhabitant of the aquatic environment. They have been associated with a variety of aquatic flora and fauna which have been found to be the reservoirs of the organism. Some of the terrestrial animals including bats have also been found to carry the non-epidemic and the environmental strains of V. cholerae. Presence of epidemic strains of V. cholerae in bats have not been reported so far and this study for the first time establishes this association. The bat species studied were found to carry V. cholerae O1 Inaba 'hybrid' strains.*

Introduction

Vibrio cholerae, the causative agent of the devastating disease cholera is primarily a microflora of the aquatic environment found in marine, brackish water and estuarine environment. They have also been isolated from water sediment, plankton, invertebrates and fish.[1-4] *V. cholerae* has two serogroups, the O1 (biotypes classical and El Tor) and the O139 which are capable of causing epidemics and pandemics.[5] Other serogroup strains of *V. cholerae* are known as non-epidemic strains, as there have been no report of epidemics caused by them.[5] The primary virulence factor of *V. cholerae* is the cholera enterotoxin (CT) encoded by *ctxAB* gene and almost all of the epidemic causing strains possess this gene.[5] Majority of the non-epidemic strains of *V. cholerae* do not carry the *ctxAB* gene, and that is the reason they are considered to be incapable of causing an epidemic or pandemic. However a few percentage of non-epidemic strains carry the *ctxAB* gene.[6]

Both the biotypes of the *V. cholerae* O1 can cause severe cholera,[5] but the phenotypic characters and the *ctxAB* gene possessed by the classical and the El Tor biotypes are different.[7,8] Hybrid strains of *V. cholerae* O1 with traits of both the classical and El Tor biotypes have also been reported.[7] Studies have found out that, these hybrid strains have completely replaced the *V. cholerae* O1 El Tor.[9-13]

The reservoirs of *V. cholerae* are primarily the aquatic environments, however studies have indicated that *V. cholerae* O1 can be carried by household animals, including cows, dogs, and chicken.[14] Non epidemic strains of *V. cholerae* have also been isolated from the flying mammals bats.[15] So far, intestinal carriage of epidemic strains of *V. cholerae* by bats has not reported from anywhere. Though transmission of *V. cholerae* by the flying mammal bat has not been recorded so far, it would be interesting to know whether these animals carry the deadly epidemic strains of *V. cholerae*. To investigate this, two colonies formed by two species of coexisting evening bats, *Scotophilus kuhlii* and *Pippistrellus dormeri* inhabiting the crevices of an office building in the city of Chennai, South India were screened for the presence of pathogen.

Materials and methods

Sample from Bats

The study was carried out for a period of one year. Two bat colonies were located on the crevices of an office building, in Taramani, Chennai, South India. The colonies were formed by two species of co-existing insectivorous bats which were identified as *Scotophilus kuhlii* and *Pippistrellus dormeri*. Each of these colonies comprised of about twenty individuals of bats. The bat faecal pellets (Guano) were collected as samples, three times a month, in ten days interval.

Collection and processing of bat faecal pellets

Sterile Petri dishes were kept underneath the bat colonies. Bat faecal pellets which fell on the Petri dishes were collected and transferred to sterile test tubes and were used as samples. One pellet each was inoculated into each test tube containing 2 mL of alkaline peptone water (with 1% NaCl, pH 9.0), and incubated at 37^{0}C for 4 - 6 hours. After enrichment, the samples were streaked onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar and incubated at 37^{0}C for 18-24 hours. Representative colonies were picked up and processed.

Identification of the isolates

V. cholerae isolates were confirmed biochemically, by performing tests like Oxidase, Indole, Lysine decarboxylase, Arginine dihydrolase, Citrate utilization, Methyl Red test and Voges Proskauer test.[16] Organisms which were biochemically identified as *V. cholerae*, were serogrouped and serotyped with O1- Inaba, Ogawa and O139 type specific anti-serum. Bio-typing of the *V. cholerae* O1 isolates was done by performing, test for haemolysis, Polymyxin B (50 U) sensitivity, Voges Proskauer test and chick cell agglutination (CCA) test.[16] *V. cholerae* isolates, which did not agglutinate with the O1- Inaba, Ogawa and O139 type specific

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anti-serum were serogrouped at the National Institute of Infectious Diseases (NIID), Tokyo, Japan.

Polymerase Chain Reaction (PCR)

All the *V. cholerae* isolates were subjected to PCR assay to amplify the species specific gene *ompW* (588 bp, to genotypically confirm the isolates as *V. cholerae*) and the cholera toxin gene *ctxA2B* (566 bp, to prove the enterotoxigenicity of the isolates). PCR was carried out with target specific primers by previously described methods, using bacterial cell lysate as the source of template DNA.[17, 18] *V. cholerae* grown over night on Luria Bertani broth incubated at 37^{0}C, were centrifuged at 10, 000 rpm for 5 minutes and the cell pellet was collected. The pellet was re-suspended in 100 µL of sterile nuclease free water, boiled for 10 minutes and cell debris were removed by centrifugation at 12,000 rpm for 10 minutes. The supernatant containing the template DNA was used for PCR assay.

PCR was carried out in a thermal cycler (Eppendorf Mastercycler Gradient, Germany); with 200 µL PCR tubes with a reaction mixture volume of 50 µL. Primers and master mix (2X) were obtained from Sigma-Aldrich, U.S.A. Amplification of the target genes was carried out using *ompW* – F primer (5'- CACCAAGAAGGTGACTTTATTGTG-3'), *ompW* – R primer (5'- GAACTTATAACCACCCGCG-3'), for amplifying the species specific gene *ompW* and *ctxA2B* – F primer (5'- TAGAGCTTGGAGGGAAGAGCCGT-3') and *ctxA2B* – R primer (5'- ATTGCGGCAATCGCATGAGGCGT-3') for amplifying the cholera toxin gene *ctxA2B*. The amplification consisted of Initial denaturation at 94^{0}C for 2 minutes, followed by 30 cycles consisting of 94^{0}C for 1 minute, 62^{0}C for 1 minute and 72^{0}C for 1 minute and a final extension at 72^{0}C for 10 minutes. PCR products obtained, were electrophoresed through 1.5% (wt/vol) agarose gel with ethidium bromide (final concentration 0.5 µg/mL), and visualized. The results were recorded on a BioRad UV gel documentation system (BIO-RAD, Hercules, CA, USA). The *ctxA2B* amplicons were purified using the Qiagen's Qiaquick spin columns (U.S.A.) and were sequenced in an ABI PRISM 3730XL Analyzer (96 capillary type) at Ms/Macrogen Inc., Seoul, Korea. Each sequence was given a unique identification number. All the *ctxA2B* sequences were submitted to the GenBank. The accession numbers are KT002407, KT002408, KT002409 (3 sequences).

Sequence alignment

The *ctxA2B* nucleotide sequences from the *V. cholerae* strains were subjected to an initial BLASTn and tBLASTx analysis to confirm the source of origin and to estimate the sequence identity before further analysis. The sequences were translated into their respective amino acid sequences, and all the nucleotide and translated amino acid sequences were aligned along with sequences of *V. cholerae* O1 El Tor (N16961), classical (O395) and O139 reference strains obtained from GenBank, edited using the software application BioEdit, version 7.2.5, to remove regions of ambiguity and were analyzed.

Results

Vibrio isolates from bats

V. cholerae belonging to serogroups, O1 Inaba, O6, O18, O45, O94, O123, O140, O201 and other vibrios namely *V. mimicus* and *V. fluvialis* were isolated from bat faecal pellets obtained from both the colonies. A total number of 46 *V. cholerae* were isolated during all sampling and the epidemic strain, *V. cholerae* O1 Inaba was isolated once from each colony. Based on the phenotypic characterization, both the *V. cholerae* O1 Inaba isolates were found to belong to the biotype El Tor. Some serogroup strains were repeatedly isolated during subsequent isolation process.

PCR and sequence analysis

All the forty six *V. cholerae* isolates were positive for *ompW* gene. Among them, three strains, two *V. cholerae* O1 Inaba (VCO1BATTSRPVJR1; GenBank accession no. KT002407, VCO1BATTSRPVJR2; GenBank accession no. KT002408) and one O201 (VCO201BATTSRPVJR2; GenBank accession no. KT002409) were positive for *ctxA2B* gene. The BLASTn and tBLASTx analysis of the *ctxA2B* sequence data of these three isolates confirmed the source of origin. They showed that the *ctxA2B* sequences from the three isolates were 98 – 99% identical (maximum identity) with that of the sequences from the database. Multiple sequence alignment and comparative analysis of the nucleotide and the translated amino acid sequence data from the *ctxB* region of the three *V. cholerae* isolates with that of the *V. cholerae* reference strains *V. cholerae* O1 El Tor (N16961), classical (O395) and O139 revealed the replacement of amino acid Tyrosine by Histidine at position 39 and Isoleucine by Threonine at position 68. This is the characteristic feature of the *V. cholerae* classical strains, indicating that all the three epidemic strains of *V. cholerae* isolated from bats were phenotypically El Tor strains, but carried a classical CTX prophage or the classical *ctxB* gene. Figure 1 shows the multiple sequence alignment (partial) of the amino acid sequences of the *ctxB* gene with the amino acid replacements.

Figure 1 - Multiple sequence alignment of the amino acid sequences of the *ctxB* gene with the amino acid replacements

Figure 1 legend - Amino acid abbreviation - G – Glycine, **P** – Proline, **A** – Alanine, **V** – Valine, **L** – Leucine, **I** – Isoleucine, **M** – Methionine, **C** – Cysteine, **F** – Phenylalanine, **Y** – Tyrosine, **W** – Tryptophan, **H** – Histidine, **K** – Lysine, **R** – Arginine, **Q** – Glutamine, **N** – Asparagine, **E** - Glutamic Acid, **D** - Aspartic Acid, **S** – Serine, **T** – Threonine.

Discussion

Presence of epidemic causing hybrid strains of *V. cholerae* in bats has not been reported so far. In the present study, various serogroups of *V. cholerae,* including the epidemic causing *V. cholerae* O1 Inaba hybrid strain, *V. mimicus and V. fluvialis* were found to be shed by bats in their faeces. All these organisms can cause acute diarrhoeal disease in human beings, and the *V. cholerae* O1 Inaba hybrid strain are known to cause very severely dehydrating cholera.[19]

V. cholerae was isolated during each sampling, proving their continuous presence in the bats. The source, where the bats acquire the *V. cholerae* is not well known. The presence of hybrid strains of *V. cholerae* in this region has been established by these authors (unpublished data), and there is every possibility that the bats acquired the organism either from the water sources or the surrounding environment.

Presence of enterotoxigenic *V. cholerae,* especially the epidemic causing strain in bats is alarming. Bats are found

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commonly in places where people live. Presence of *V. cholerae* was detected in bat colonies that were present in the corridors of an office campus, where a lot of people pass by daily. Bats are flying mammals and there is always the risk of them bringing the *V. cholerae* into the houses. Bats can be spotted in space above the water tanks in houses and other buildings. When *V. cholerae* carrying bats reside in such places, the risk of transmission of the organism increases, as it can directly reach the water. This type of continuous contamination may be a great risk to the people drinking or using such water. If such a thing happens in public water distribution systems, the possibilities of a sudden outbreak cannot be ruled out. Even though the involvement of the animal carriage in cholera cases is not clear, and transmission of *V. cholerae* by bats has not been recorded anywhere, these findings cannot be neglected. More emphasis has to be laid on the carriage of *V. cholerae* by bats.

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