ORIGINAL ARTICLE

Predominance of Genomovar I among Burkholderia cepacia Complex bacteremia in the Neonatal Population – a study from Karachi, Pakistan

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Abstract

Background
Burkholderia cepacia complex (BCC) has been reported as an emerging cause of healthcare associated neonatal bacteremia and sepsis. Genomic speciation (genomovar determination) by *recA* PCR has been employed to study genetic variability among different patient populations, but no previous reports have determined genomovars within the neonatal population. The aim of the study was to determine most prevalent genomovar of BCC causing bacteremia among neonates in Karachi, Pakistan, through species-specific PCR for *recA* gene.

Material and Methods
Of 589 BCC neonatal bacteremic episodes detected from May 2015 - September 2016 at the Clinical Microbiology Laboratory at The Aga Khan University Hospital, 138 isolates were randomly selected for genomovar determination. *recA* PCR was performed on 138 isolates.

Results
All isolates were identified as *Burkholderia cepacia* complex by detection of BCR1 and BCR2 gene. BCC was differentiated further into lineages and *B. cepacia* genomovar I was found to be the most prevalent species, being isolated from 129 of 138 patients (93.4%). *B. cenocepacia* which is the predominant species in cystic fibrosis patients was found in only 2.9% cases (n=4).

Conclusions
We confirm the dominance of *B. cepacia* genomovarI in neonatal population in Karachi, Pakistan. Further studies are required to elucidate transmissibility and genetic similarity of *B.cepacia* genomovar I isolated from neonatal bacteremia cases.

Introduction
Burkholderia cepacia complex (BCC) is a group of Gram-negative bacteria, widely distributed in natural and hospital environments.[1] These gram-negative rods which were previously classified as *Pseudomonas* species are non-lactose fermenters, motile, aerobic and often resistant to multiple antibiotics. Although nonpathogenic for healthy individuals, BCC are recognized pathogens in patients with cystic fibrosis (CF) and chronic granulomatous diseases, where heavy lung colonization and consequent respiratory infections in these patients have been associated with poor outcomes.[2] Moreover, it has emerged as an important opportunistic pathogen in hospitalized and immunocompromised patients, with crude mortality rates reported as high as 53.8 %.[2] BCC have also been reported as a cause of neonatal sepsis.[3] Prematurity, very low birth weight, frequent use of broad spectrum antibiotics, and peripheral and central intravenous catheters predispose these neonates to BCC sepsis. In neonates, a case fatality rate of 17% has been reported.[4]

BCC has undergone dramatic taxonomic changes in last few decades and now exhibits an extensive diversity of genotypes composed of at least 17 genetically distinct genomic species associated with different levels of severity and transmissibility, including *B. cepacia* (genomovarI), *B. multivorans* (genomovarII), *B. cenocepacia* (genomovarIII), *B.stabilis* (genomovarIV), *B. vietnamiensis* (genomovarV),*B. dolosa* (genomovarVI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII), and *Burkholderia pyrrocinia* (genomovar IX).[5] *B. cenocepacia*has been the most prevalent genomovar in patients with CF in the past and has been replaced by *B. multivorans*with passing years, whereas, *B. cepacia* genomovar I is the least common.[6,7,8] *B.cenocepacia* genomovar III is also the most common genomovar causing bacteremia in non-CF patients in critical care settings.[7,10] No previous studies have reported the genomovar distribution among neonatal sepsis cases.

BCC outbreaks have been reported from a neonatal intensive care unit (NICU) in Karachi.[11] Subsequent to these outbreaks, BCC has become a frequent isolate from bacteremic episodes among neonates admitted to NICUs in Karachi. However, given the variation in genomovar distribution in non-CF populations and geographical locations, genomovar III cannot be assumed to be the prevalent genomic species in this population. We report on neonatal bacteremic episodes cultured at a laboratory

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which serves several hospitals in Karachi, and the most prevalent genomovar of BCC causing neonatal bacteremia in Karachi, Pakistan.

Subjects and Methods

Study setting, study period, sample size, and bacterial isolates: The Aga Khan University Clinical microbiology laboratory receives culture samples from several hospitals across Karachi. On average, the laboratory processes 150 blood cultures per month obtained from neonates. The laboratory receives neonatal blood cultures from hospitals across the Karachi city, which are collected in BACTEC Peds Plus/F bottles and analyzed with the BACTEC 9240 instrument (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). Transport to the laboratory is within 6-18 hours at room temperature and cultures are incubated immediately on receipt, till 5 days or until positive. In this study, we obtained isolates from positive neonatal blood cultures or bacteremic episodes identified as *Burkolderia cepacia* complex to study the genomic species distribution.

Positive bottles with gram negative bacteria and oxidase positive growth of non-fermenters at 18 hours were further identified by API 20 NE (bioMérieux®) as BCC. Susceptibility testing was performed with Kirby Bauer disc diffusion method for ceftazidime, meropenem, and cotrimoxazole and with the Vitek 2 Compaq system (bioMérieux®) for levofloxacin as per Clinical Laboratory Standards Institute (CLSI) guidelines.[12]

All bacterial isolates identified as BCC were labeled with an exclusive lab identification number and archived at -80 °C till further use. Culture samples with mixed organisms and repeat isolate of *B. cepacia* from same patient were excluded and only the initial blood culture was included. Considering the prevalence of BCC genomovar I bacteremia among the hospitalized population to be 21%.[13] We estimated that 130 episodes of BCC neonatal bacteremia per year will need to be classified into genomovars to determine the prevalence of BCC genomovar I with 95% confidence and with 7% error rate. Assumptions and sample size calculation are provided in Supplementary file 1.

Revival of strains and identification by recA species-specific PCR analysis:

Bacterial isolates of *Burkholderia cepacia complex* were further identified to species level by polymerase chain reaction (PCR) method, exploiting sequence differences in the single locus of the *recA* gene (*recA* is a protein essential for repair and recombination of DNA). This was achieved by DNA extraction from pure isolates followed by PCR using primers specific for the BCC.

Preparation of bacterial DNA:

Archived isolates were thawed, subcultured on MacConkey agar plates and incubated aerobically at 35-37ºC for 18-24 hours to yield isolated bacterial colonies. Bacterial DNA was extracted using QIA amp DNA mini kit (QIAGEN GmbH, Germany) as per manufacturer's protocol for fresh bacterial colonies (spin protocol).

Polymerase Chain Reaction:

Identification of *B. cepacia complex* was carried out using PCR with primers which amplify the entire recA gene of bacteria within the *B. cepacia complex*. PCR assays were performed as described by Mahenthiralingam *et al*.[10] The PCR reaction mixture with a final volume of 25ul constituted of, 0.5µl of 25mM dNTPs (MOLEQULE-ON/New Zealand, cat# PR-M-002-1000), 5µl of BUFFER 5x, 1.5µl of MgCl2 (25mM), 0.5µl of DNA Taq polymerase (Promega), 1µl of each specific primer at a concentration of 10pmol, 2.5µl. DNA template amplification was carried out with following conditions: initial denaturation at 94°C for 60 seconds, followed by 30 cycles of 30 seconds at 94°C at appropriate annealing temperature and at 72°C for 60sec and a final elongation at 72°C for 10 minutes. All primers used in the PCR assays are listed in Table1.[14] *B.cepacia* ATCC 25416 was used as a control organism.

All isolates were first identified using *B. cepacia complex*-specific primers BCR1 and BCR2. Genomovar status was then determined sequentially by using PCR primer specific for *B.cepacia*genomovar I(BCRG11, BCRG12a). Isolates testing negative for *B.cepaciagenomovar I* were further tested with primer specific for *B.cenocepacia* genomovar IIIA (BCRG3A1 BCRG3A2a) and IIIB (BCRG3B1 BCRG3B2a) and those testing negative were further tested with primers specific for *B.stabilis* genomovar IV(BCRG41, BCRG42a).

PCR amplicons were electrophoresed on 1 % agarose gel in 1 X TAE (Tris base, acetic acid and EDTA) containing 2µl of ethidium bromide for 60min at 100mV constant voltage. DNA bands were visualized using Gel Doc (Bio-Rad).

The study protocol was reviewed by the ethical review committee at the Aga Khan University Hospital and permitted to be exempted from patient consent.

Results

Incidence of BCC among culture-positive episodes of neonatal bacteremia:

From May 2015 to September 2016, the laboratory reported 2269 episodes of bacteremia in neonates (positive blood cultures), of which 589 were due to BCC, bringing the estimated incidence to 250 per 1000 episodes of culture-confirmed bacteremia.

Selection of isolates for genomovar determination: Of the 589 isolates, 138 bacterial isolates were randomly selected for genomovar determination. Five of the 138 isolates were selected from cities other than Karachi. The 133 isolates from Karachi were collected from 13 various hospitals in the metropolis and covered approximately 1600 km^2. Figure 1 shows location of hospitals and the catchment area covered in the study.

Table 1: PCR primers used for BCC identification and genomovar determination

Genomovar specificity	Primer name	Sequence 5' to 3'	PCR annealing temperature
B.cepacia complex *recA*	BCR 1 BCR 2	TGACCGCCGAGAAGAGCAA CTCTTCTTCGTCCATCGCCTC	60^{O}C
B.cepacia genomovar I	BCRG11 BCRG12a	CAGGTCGTCTCCACGGGT CACGCCGATCTTCATACGA	62^{O}C
B.cenocepacia genomovar III-A	BCRG3A1 BCRG3A2	GCTCGACGTTCAATATGCC TCGAGACGCACCGACGAG	62^{O}C
B.cenocepacia genomovar III, RG-B	BCRG3B1 BCRG3B2a	GCTGCAAGTCATCGCTGAA TACGCCATCGGGCATGCT	61^{O}C
B.stabilis genomovar IV, RG-4	BCRG41 BCRG42a	ACCGGCGAGCAGGCGCTT ACGCCATCGGGCATGGCA	65^{O}C

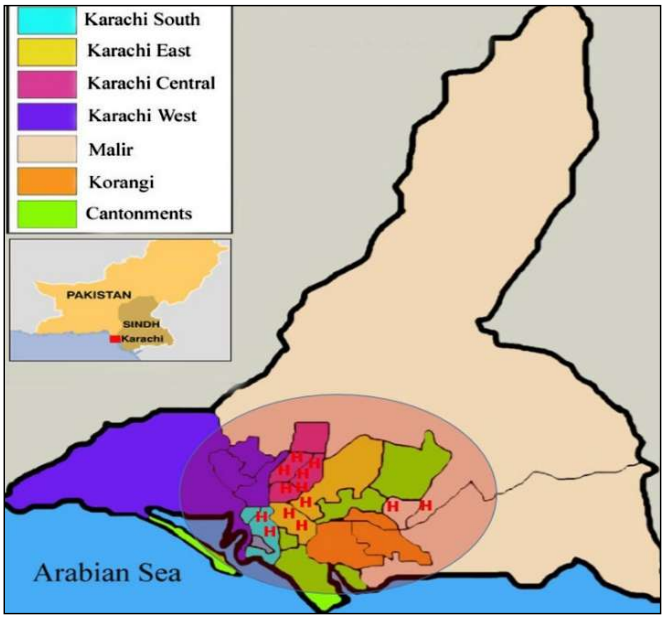

Fig1. Karachi hospitals and catchment area from which blood culture isolates of BCC were randomly selected. Each hospital has a catchment area of ~ 15 km radius. Pink circle demarcates the catchment area. Inset (above) shows Karachi districts and inset (below) shows the location of Karachi within Pakistan. Hospitals are shown as 'H'.

recA PCR-based identification and differentiation into genomovars:

All 138 isolates were identified as BCC on the basis of recA conserved PCR for BCR1. Of the 138 clinical isolates, 93.4% were identified as BCC genomovar I (*B.cepacia*) making this the most prevalent genomovar causing neonatal bacteremia. On sequential testing of the remaining 9 isolates, 4 were identified as B.cenocepacia (genomovar III) with 3 testing positive for recA cluster III- A, and 1 for cluster III- B, and 2 tested positive for *B.stabilis* (genomovar IV). Genomovars for 3 isolates remained unresolved on recA PCR testing. Figure 2 shows distribution of genomic species and respective genomovars among the 138 isolates tested.

Biochemical reactions of BCC on API 20 NE and susceptibility: All isolates were identified as *Burkholderia cepacia* by API NE. Susceptibilities against tested antibiotics were: ceftazidime 92.8%, (n=128), co-trimoxazole 85.5% (n=118), meropenem 52.2% (n=72), and levofloxacin 60.3% (n=73 of 121 isolates tested). All isolates non-susceptible to co-trimoxazole were

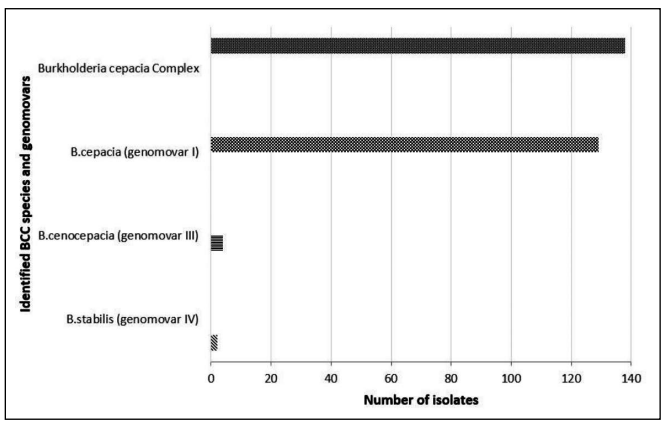

Fig 2. Distribution of BCC genomovars among neonatal bacteremia isolates from Karachi, Pakistan. There is predominance of genomovar I among prevalent strains.

also non-susceptible to levofloxacin and meropenem, but remained susceptible to ceftazidime.

Biochemical reactions on API NE did not discriminate among genomovars. However, as very few isolates belonged to genomovars other than 1, biochemical profiles and phenotypic susceptibility patterns were not correlated for discriminatory power.

Discussion

Our analysis of BCC isolates recovered from neonates provides strong valuation of the relative frequencies with which respective genomovars cause neonatal bacteremia and sepsis. We report the dominance of BCC genomovar I among neonatal bacteremia episodes in Karachi, Pakistan. Despite their genetic similarity, there is great variation in genomovar-specific disease epidemiology in different populations, and in different parts of the world, for instance, between CF and non-CF patients.[8] The reason for this could be better adaptation of some species to infection of the CF lung and vice versa.[9]

B.cenocepacia(genomovar III) was considered the most common species in CF patients but with the changing epidemiology and improved infection control measures the rates have declined, making environmentally acquired *B. multivorans* most commonly isolated BCC species from CF patients.[8,9] Since *B.cepacia*genomovar I is an environmental species, it is likely that this organism is transmitted to neonates from the healthcare environment. In contrast, B.cenocepacia (genomovar III) prevalence among our neonatal bacteremia cases was low, suggesting either low prevalence in the nosocomial environment in our setting, or low colonization potential of this genomovar among neonates. A detailed study of hospital microbiome with special reference to BCC can resolve these hypotheses.

BCC is an emerging pathogen and source and transmissibility of strains involved remain questionable. Molecular characterization of bacterial isolates is vital for epidemiological breakdown of bacterial pathogens and subspecies. It has been hypothesized that the transmissibility varies for different genomovars of BCC. While transmission characteristics of *B.cenocepacia* and *B. multivorans*[15] have been studied, little is known about the person-to-person transmission potential of *B.cepacia*genomovar I. Our next steps comprise molecular fingerprinting of *B.cepacia* genomovar I isolates and sequencing in an effort to demonstrate genetic similarity and determine putative transmission chains among neonates.

BCC infections, especially bacteremia and sepsis also have significant impact on clinical outcome of patients. Case fatality rates of 17-28% among infants have been reported from populations where CF is a rare condition.[4,16] High mortality rates can be attributed to limited therapeutic options and emerging antimicrobial resistance (AMR) as well as virulence factors. BCC virulence factors include flagellar motility, superoxide dismutase, lipopolysaccharide, and siderophore production factors. Genomovar I strains also produce melanin and exopolysaccharide which may contribute to pathogenicity and virulence. BCC phenotypic AMR profiles in our study show high resistance rates against meropenem which is commonly used in neonatal sepsis. Although resistance rates against ceftazidime and cotrimoxazole remain low, continuous monitoring of AMR rates in BCC remains essential to capture emerging resistance.

Our study has limitations. BCC strains studies are predominantly from one city and cannot be taken to represent genomovar distribution across other areas of Pakistan or low resource settings. We were also unable to correlate bacteremia with treatment details clinical outcomes as medical records were not available from all hospitals.

We provide preliminary evidence of epidemic spread of BCC genomovar I among hospitalized neonates in Karachi, Pakistan. Our study is the first to systematically evaluate BCC genomovar distribution in a neonatal population. Larger prospective studies are required to investigate and understand transmissibility and pathogenic potential of BCC genomovar with special reference to the neonatal population.

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