NUSANTARA BIOSCIENCE
Vol. 6, No. 2, pp. 111-117
November 2014

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n060202

Genetic regulation of allolysis in response to sub-lethal antibiotic stress in *Streptococcus pneumoniae*

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Manuscript received: 2 June 2014. Revision accepted: 17 July 2014.

Abstract. *Dash M, Dash HR, Das S. 2014. Genetic regulation of allolysis in response to sub-lethal antibiotic stress in Streptococcus* pneumoniae*. Nusantara Bioscience 6: 111-117.* Allolysis is the phenomenon of cell lysis induced by other cells of the same species. Gram-positive bacterium *Streptococcus pneumoniae*, a major human pathogen exhibits competence induced allolysis that increases the genetic recombination and enhances the virulence. During allolysis, a group of non-competent bacterial cells are lysed by another group of competent cells in the same culture. This process is regulated by *com* operon as well as bacteriocin. In this study, allolysis was induced in *Streptococcus pneumoniae* MTCC655 by sub-lethal dose of antibiotic (chloramphenicol) and the mechanism of allolysis has been deduced by amplification of *lyt*A, *lyt*C and *cbp*D genes in the bacterium. The strain was found to be resistant to a number of antibiotics including amoxicillin, cefpodoxime, erythromycin and vancomycin. The early onset of allolysis induction from 7-9 h under normal conditions to 2-3 h by sub-lethal dose of chloramphenicol was observed.

Key words: Antibiotic, *cbpD* gene, DNA transformation competence, gene expression, *Streptococcus pneumoniae.*

INTRODUCTION

Streptococcus pneumoniae, a gram-positive, alpha-hemolytic, aerotolerant bacterium, is the major cause of huge death rate during late 19thcentury (Ryan and Ray 2004). The disease caused by this pathogenic organism is pneumonia that infects the upper respiratory tract and can spread to blood, lungs, middle ear and nervous system. In addition to that, this organism is responsible for many other diseases including sinusitis, otitis media and bacterial meningitis (Brandenburg et al. 2000). The major symptoms associated with *S. pneumoniae* infection include persistent cough, excess production of sputum, shortness of breath and fever and most of the patients show evidence of a pulmonary infiltrate (Kadioglu and Andrew 2004; Calbo and Garau 2010). In spite of the action of antibiotics and effective immunizations, pneumococcal infection is responsible for many respiratory failure, hospitalization and mortality in large number of people at the age group of young children, the elderly as well as the individuals with underlying medical conditions. As per one of the reports, more than one million childhood deaths are associated with pneumococcal pneumonia each year (Ortqvist et al. 2005).

The major treatment of *S. pneumoniae* is the use of antibiotic penicillin; however, the worldwide emergence of antibiotic resistance among *S. pneumoniae* has rapidly changed this approach of treatment. The rapid development and spread of penicillin resistance and multiple antibiotic resistances is due to many factors and the majority of the risk factors involve young age and day care attendance post antibiotic use (Kaplan et al. 1998). Increase in treatment facility as well as infrastructure could not delineate the streptococcal infection and *S. pneumoniae* is reported to be the primary cause for community acquired lower respiratory infections (LRI) among children of developed countries accounting for 12-45% of the cases (Michelow et al. 2002).

In this regard, competence shown by many species of *Streptococcus* can be explored for the in vivo treatment of disease onset. In *S. pneumoniae*, competence is the population density dependent mechanism induced by competence stimulating pheromone peptide (CSP). 1-10 ng/mL of CSP is able to induce competence in liquid culture that works via quorum sensing mechanisms which is a population stress response mechanism in the organism (Steinmoen et al. 2003). During the process of competence, *S. pneumoniae* encodes for seven important proteins required for the start of the gene cassette i.e. *com*AB: secretion apparatus of CSP, *com*C: the precursor of CSP, *com*DE: the two-component regulatory system where *com*D: a histidine kinase and *com*E: cognate response regulator of *com*D, *com*X (encoded by two identical genes, *com*X1 and *com*X2): the alternative sigma factor and *com*W: the recently discovered positive regulator of competence. The *com*X regulon encodes for late genes that secrete proteins involved in DNA uptake, binding and recombination process (Steinmoen et al. 2002; Peterson et al. 2004; Kausmally et al. 2005; Prozorov and Danilenko 2011).

When competence is induced in a population of *S. pneumoniae*, a sub-fraction of the cell population are killed. The death of the cells is caused by some differentiated cells of the same population (Steinmoen et al. 2002). Since cells of the same strain are involved in killing, the process is termed as allolysis or fratricide i.e. killing of siblings (Prozorov and Danilenko 2011). This phenomenon can be explored with the early onset of competence for the treatment of the disease associated with this infection.

Induction of competence will lower the population number of the *S. pneumoniae* which can be taken care of by the host immune system. Thus, the current work aims to establish the relationship between the sub-lethal dose of antibiotic stress and onset of competence in *S. pneumoniae* for the alternative treatment of Streptococcal infection.

MATERIALS AND METHODS

Strain selection and culture medium used

The type strain of *S. pneumoniae* MTCC655 was obtained from Microbial Type culture collection (MTCC), India. The strain was pathogenic in nature and was retrieved from the lyophilized culture by re-suspending in 5 mL of Casein Soyabean Digest broth (pancreatic digest of casein 17 g/L, papaic digest of soyabean meal 3 g/L, sodium chloride 5 g/L, dipotassium hydrogen phosphate 2.5 g/L, dextrose/glucose 2.5 g/L) and incubated at 37°C for 24 h with shaking at 180 rpm. For further experiments, Luria Bertani agar (tryptone 10 g/L, yeast extract 5 g, NaCl 10 g/L, agar- 15 g/L) medium was used.

Characterization of the strain

Gram's staining

A thin smear of overnight grown culture was prepared on a clean and dry slide, air dried and heat fixed. Crystal violet was flooded to the smear for 1 min followed by gentle washing and addition of Gram's iodine for 1 min. The slide was gently washed and Gram's de-colorizer was added until the disappearance of violet color from the slide. Again the slide was rinsed with tap water and counter stained with saffranin for 1 min. Slide was washed with tap water and air dried. Then it was observed under oil immersion microscope.

Blood agar haemolysis

The isolated colony of *S. pneumoniae* MTCC655 was taken with the help of a sterile inoculum loop grown on Tryptic Soy Agar plate and was streaked on a Blood Agar Plate (Hi-Media, India). The plate was incubated at 37°C for 12 h and observed for the pattern of haemolysis.

Bile solubility test

One (1.0) mL of saline (0.8% NaCl dissolved in sterile mili-Q water) solution was prepared and suspension was divided into 2 tubes with approximately 0.5 mL in each tube. In one tube, a suspension of *Streptococcus pneumoniae* MTCC655cells was prepared from overnight grown culture on an agar plate. As a negative control to this experiment, in another tube a suspension of *Pseudomonas* sp. was prepared. Turbidity equal to that of 1.0 to 2.0 McFarland density standards was used. 0.5 mL of 2% sodium deoxycholate (bile salts) was added to each tube and was mixed by vigorous shaking. The tubes were incubated at 37°C for up to 2 h and analyzed.

Biochemical characterization of the strain

Biochemical characterization was done by using Hi-Media KB009 kit containing 12 carbohydrates in the order inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α methyl D glucoside and ribose. 50 µL of overnight grown culture was added to each well and incubated at 37°C for 12 h and examined by observing the change in colour and matching with the chart provided by the manufacturer.

Antibiotic sensitivity of the strain

Antibiotic sensitivity pattern of the strain was determined by disc diffusion method (Bauer et al. 1966). Briefly, the lawn culture of the bacterium was prepared with cotton swab. The inoculum was dried for some time and antibiotic discs (amoxycilin, cefpodoxime, cephotaxime, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, tetracycline, vancomycin) (HiMedia, India) were placed on the plates followed by incubation at 37°C for 12 h and the examined for the development of zones of inhibition.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by micro-broth dilution method in a 96 well micro-titer plate following clinical and laboratory standard institute (CLSI 2006) guidelines. Antibiotic stock solution of Chloramphenicol (10 mg/mL) was prepared in Dimethylsulphoxide (DMSO). 150 µL of Muller Hinton Broth medium (Beef infusion 300g/L, Casein acid hydrosylate 17,5g/L, starch 1,5g/L) was added in all wells (except 1^{st} and 12^{th} column), followed by addition of 300 µL and 150 µL of antibiotic solution in 1^{st} and 12^{th} column respectively. The antibiotic solution was two-fold serially diluted from 1^{st} to 10^{th} column (i.e. A1 to A10) and 150 µL was discarded from the 10^{th} column. 10 µL of culture was added to each well except 12^{th} column. Thus, 10^{th} column in the plate containing MHB and culture acts as a positive control whereas, 12^{th} column containing MHB and antibiotic solution without bacterial culture acts as the negative control. The plate was incubated in a moist chamber at 37°C for 24 h. MIC was determined by analyzing the growth in each well by measuring the OD_{595} in micro-titer plate reader.

In-vitro allolysis testing

By viable cell count

A novel approach was carried out to determine the effect of allolysis on viable cell concentration under normal growth condition and sub-lethal antibiotic stress in *S. pneumoniae* MTCC655. Briefly, culture of *S. pneumoniae* MTCC655 was grown in Brain Heart Infusion (BHI) medium (M210, Hi-Media, India) at 37°C with shaking at 160 rpm for 24 h. 1 mL of the grown culture was inoculated to the flasks containing LB broth with antibiotic and without antibiotic. Immediately after the transfer, 1 mL of solutions was taken from each flask to 1.5 mL micro-centrifuge tubes. Then cultures were serially diluted and spread on Nutrient agar (Peptic digest- 5,0 g/L, NaCl- 5,0 g/L, Beef extract- 1,5 g/L, Yeast extract- 1.5 g/L, agar- 15,0 g/L, pH- 7.4±0.2) plates. The plates were incubated at 37°C for 12 h and colonies on each plate were counted. This process was continued up to 9 h of incubation at an

interval of 2 h. Simultaneously, the growth was measured by determining OD_{595} in micro-titer plate reader.

By quantization of extracellular DNA and haemolysis pattern

Two BHI tubes, one with sub-lethal dose of chloramphenicol stress and another without any antibiotic stress were inoculated with overnight grown culture of *S. pneumoniae* MTCC655 and incubated at 37°C with shaking of 180 rpm. At an interval of 1 h, 1 mL of the culture was aliquoted to 1.5 mL micro-centrifuge tube and centrifuged at 6.000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh vial and the concentration of extracellular DNA was measured using a Nanodrop. In addition, to observe the haemolysis pattern, 1 µL of culture was spot inoculated on blood agar plates and incubated at 37°C for 24 h and observed.

Amplification of *cbp*D gene

In order to deduce the mechanism of competence in the test organism, *cbp*D gene was amplified using polymerase chain reaction. The primers for this gene was designed in NETPRIMER Software and the obtained primer sequences were *cbp*DF (5'GCTCGTCGGGAAGGTTATCG3') and *chp*DR (5'GGATGGTGGCAGTGTTGGGA3'). Template DNA was prepared by boiling lysis of the bacterial culture and PCR was carried out using 1X PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 pM of both forward and reverse primers, 1 U of Taq polymerase and 5 µL of template DNA. The PCR programmed was carried out with an initial denaturation of 95ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 1 min and extension at 72ºC for 1 min, final extension for 10 min at 72°C and final hold at 4°C. The banding pattern was observed in 1% agarose gel under UV light in a gel documentation system.

RESULTS AND DISCUSSION

Bacterial culture and strain selection

The collected strain of *S. pneumoniae* MTCC655 is pathogenic with the characteristic properties of a type strain (Figure 1.A). The infections associated with this organism can be divided classically into two forms one invasive and another non-invasive form (CDC 2010). The non-invasive forms include sinusitis, acute otitis media and pneumonia whereas; the invasive forms are associated with the isolation of organism from a normally sterile site mostly causing bacteremic pneumonia and meningitis. In this regard, the mortality rates and the incidence of *S. pneumoniae* infections are related to bacterial virulence factors. Though, the routine immunization and introduction of the heptavalent pneumococcal vaccine has decreased the onset of these invasive diseases in younger children, however, the incidence rate in older patients has increased dramatically (Martinot et al. 2014). Antibiotic resistance in *S. pneumoniae* is increasing throughout the world creating the problem in the treatment of the infection (Van Bambeke et al. 2007). Thus, allolysis has been explored as a new therapy to treat these antibiotic resistant *S. pneumoniae* infections.

Characterization of the strain

Gram staining result confirmed the gram-positive coccus form of the strain. The strain was confirmed to be positive for alpha haemolysis on blood agar plates i.e. incomplete haemolysis after 12 h of incubation and development of green colored zone (Figure 1.B). In a similar fashion, after 12 h of incubation, a clear solution in the tube containing *S. pneumoniae* MTCC655 was observed indicating the lysis of the cells whereas, the tube containing negative control as *Pseudomonas* sp. showed turbid solution (Figure 1.C) confirming the bile sensitivity of *S. pneumoniae* MTCC655. Streptococci has been reported to occur intracellularly or extracellularly as gram-positive lanceolate diplococcic and in certain instances it also occurs as single cocci or in short chains of cocci showing alpha haemolysis (Tamura and Nittayajarn 2000; Ruoff 2002) which has been well established in the present study. It has been clinically important to distinguish between catalase negative, alpha haemolytic and non-haemolytic gram-positive cocci in pairs and chains as enterococci i.e. group D streptocci and non-group D viridans group streptococci (Patterson et al. 1995). In this regard, bile esculin test is widely recommended to distinguish between enterococci and group-D streptocci (Chuard and Reller 1998). Group D streptocci are bile tolerant and thus the pathogenic nature of the *S. pneumoniae* MTCC655 has been established due to its bile salt sensitivity nature.

Biochemical characteristics of the strain

Biochemical characterization of the strain reveals the preliminary identification and nature of pathogenicity of the strain. During this study, *S. pneumoniae* MTCC655 was found to be positive for sugar fermentation such as arabinose, inulin, lactose, raffinose, ribose, trehalose, starch, glycogen, positive hydrolysis for arginine, esculin and production of alkaline phosphatase, alpha galactosidase, beta glucuronidase, beta galactosidase, leucine arylamidase and alpha haemolysis (Table 1). Similar result was obtained for pathogenic strains of *S. pneumoniae* in many other studies (Pagliero et al. 2004; Ruiz-Maso et al. 2006; Calix et al. 2012; Nagata et al. 2012).

Table 1. Biochemical characteristics of *S. pneumoniae* MTCC655

Characteristics	Result	Characteristics	Result
Arabinose	+	Esculin	+
Inulin	+	Hippurate	-
Lactose	+	Voges-Proskauer	-
Mannitol	-	Alkaline phosphatase	+
Raffinose	+	Alpha Galactosidase	+
Ribose	+	Beta-Glucuronidase	+
Sorbitol	-	Beta-Galactosidase	+
Trehalose	+	Leucine arylamidase	+
Starch	+	Pyrrolidone arylamidase	-
Glycogen	+	Alpha haemolysis	+
Arginine	+	Beta-haemolysis	-

Note: + = positive, - = negative

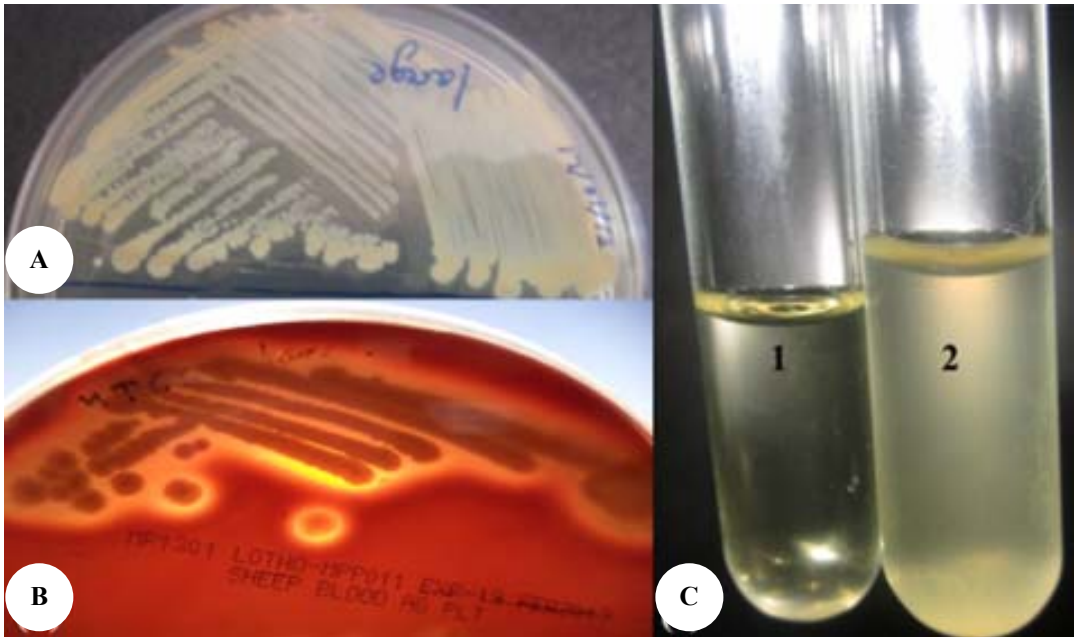

Figure 1. Phenotypic characteristics of *S. pneumoniae* MTCC655 used during the study. A. Pure culture of the strain on Luria bertani agar plate, B. Haemolysis pattern exhibited by the strain on blood agar plates, C. Bile susceptibility testing of the strain 1: *S. pneumoniae* MTCC655, 2: *Pseudomonas* sp.

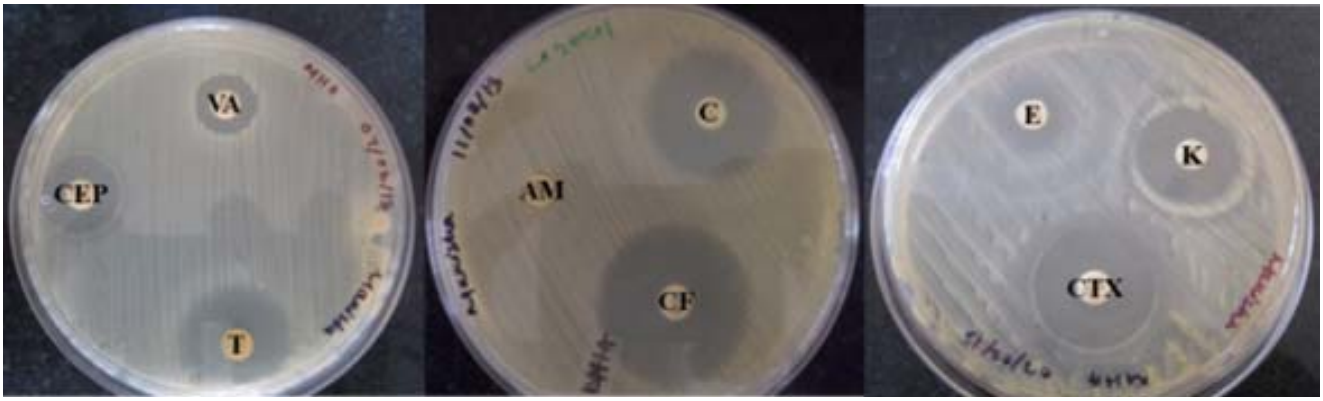

Figure 2. Antibiotic susceptibility test of *S. pneumoniae* MTCC655 by disc diffusion test. The antibiotic discs used are cefpodoxime (CEP), vancomycin (VA), tetracyclin (T), amoxicillin (AM), chloramphenicol (C), ciprofloxacin (CF), erythromycin (E), kanamycin (K), cephotaxime (CTX)

The biochemical results of the *S. pneumoniae* MTCC655 suggest the emergence of this strain by non-clonal expansion but may be due to the spontaneous mutation which is not caused due to the exposure to antibiotics because of their certain biochemical alterations.

Antibiotic sensitivity pattern

The strain used during this study was found to be resistant to amoxicyllin, cefpodoxime, erythromycin and vancomycin (Figure 2), while it is susceptible to cephotaxime, chloramphenicol, ciprofloxacin, kanamycin and tetracycline. In a similar fashion, Zhao et al. (2003) reported 85% of *S. pneumoniae* to be resistant to ampicillin, 6,6% to cefazolin, 25% to cefaclor, 6,6% to ceftriaxone, 85,7% to erythromycin, 66,7% to clindamycin and 28,2% to chloramphenicol in clinical isolates from Shanghai, China. Prevalence of antibiotic resistance in *S. pneumoniae* from Saudi Arabia was also found to be in the same range for erythromycin (25%), tetracycline (29%) and trimethoprim-sulfamethoxazole (42%) (Al-Tawfiq 2004). The rapid spread of resistant clones of *S. pneumoniae* as well as the emergence of new variants of resistant mechanisms should be monitored and powerful molecular epidemiological methods can be employed to identify the antibiotic resistance reservoirs and their mode of transmission in the resistant bacteria (Tomasz 1997).

Minimum Inhibitory Concentration (MIC) for Chloramphenicol

Streptococcus pneumoniae, the major pathogen in pediatric infections, showed increased resistance towards a number of antibiotics including penicillin and other β-lactam antibiotics thus increasing the difficulty in treatment those infections (Hernández et al. 2003). Thus, rifampicin and chloramphenicol have been recommended as alternative therapies due to their less cost and high accessibility to the communities. However, many chloramphenicol resistant *Streptococcus* strains have been also reported (Widdowson et al. 2000). The resistance to chloramphenicol is mainly due to the acetylation of the antibiotic by the production of chloramphenicol acetyltransferase (CAT) (Ayoubi et al. 1991). The chloramphenicol resistant genotype harboring transposon Tn5252 is capable of conjugational transfer between pneumococci and *Streptococcus agalactiae*, *Streptococcus gordonii* and *Enterococcus faecalis* (Vijayakumar and Ayalew 1993). Thus, the result obtained for the minimum inhibitory concentration of *S. pneumoniae* MTCC655 for chloramphenicol (64 µg/mL) showed moderate resistance to the antibiotic and is well above the MIC level reported by Widdowson et al. (2000) and Hernández et al. (2003).

In-vitro allolysis test

The number of viable cells present in the culture medium after incubation with sub-lethal dose of chloramphenicol stress was found to be in decreasing order after 6 h of incubation in comparison to the higher number of cells without antibiotic stress (Table 2). In a similar fashion, the concentration of extracellular DNA increased to a large fold in comparison to without antibiotic stress incubation suggesting the early onset of allolysis in *S. pneumoniae* with sub-lethal antibiotic stress (Table 3). Though many reports are there for allolysis across the gram-positive and gram-negative isolates, the phenomenon was discovered initially in *S. pneumoniae* (Claverys et al. 2006). As allolysis phenomenon leads to the lysis of the cells thus death of the bacteria, viable cell count proves to be suitable screening method for study of allolysis phenomenon. Viable cell count of the type strain with antibiotic stress was found to be $9{,}1 \times 10^7$ CFU/mL after 2 h whereas a much higher count was observed ($4{,}54 \times 10^8$ CFU/mL) when the strain was grown without the antibiotic stress after same duration of incubation. This establishes the preliminary idea of the early onset of competence in presence of sub-lethal concentrations of chloramphenicol antibiotic. However, another approach of studying allolysis by quantifying extracellular DNA proved to be quiet useful for this study. When cell lysis occurs, the cellular DNA comes out of the cell to the growth medium, which was collected and quantified by UV absorption at 260 nm. The study reveals the early onset of allolysis after 3 h incubation as the antibiotic stress results in higher amount of extracellular DNA (255,1 ng/µL) in comparison to without antibiotic stress (227,3 ng/µL). The same phenomenon has also been observed in *Bacillus subtilis* suggesting the induction of competence is due to general response to stress in gram-positive bacteria (Claverys et al. 2006).

Table 2. Viable cell count of *S. pneumoniae* MTCC655 with and without antibiotic stress of chloramphenicol

Time interval (in h)	Viable cell count (CFU/mL)	
	With chloramphenicol	Without antibiotic stress
0	2.3×10^7	2.1×10^7
2	9.1×10^7	4.54×10^8
4	1.22×10^8	3.0×10^9
6	3.2×10^7	5.22×10^9
7	2.7×10^7	3.0×10^{10}
8	2×10^7	1.6×10^{10}
9	2×10^7	7×10^{11}
10	3×10^7	3×10^{13}

Table 3. Dynamics of extracellular release of DNA at various time intervals with and without antibiotic stress in *S. pneumoniae* MTCC655

Time interval (in h)	Concentration of DNA (ng/mL)	
	With chloramphenicol	Without antibiotic stress
0	236.1	330.1
2	194.0	229.6
3	255.1	227.3
4	194.2	185.2
5	140.9	128.9
6	155.3	121.4
7	116.7	90.3
8	167.9	213.3
9	262.1	200.2

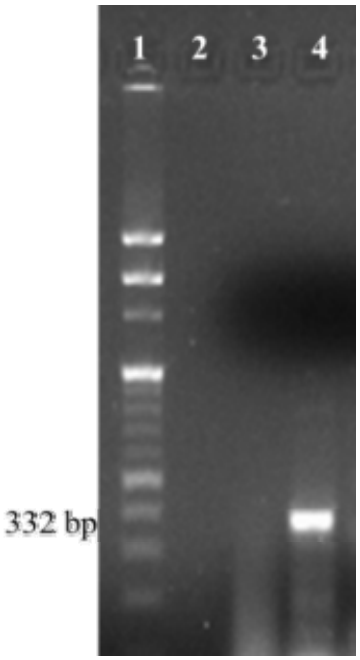

Figure 3. Amplification of *cbp*D gene in *S. pneumoniae* MTCC655, 1: 100 bp ladder, 2: negative control, 3: Non-pathogenic strain of *Bacillus cereus*, 4: *S. pneumoniae* MTCC655.

Amplification of *cbp*D genes

The genetic analysis of the isolate showed the presence of *cib*A, *cib*B, *cbp*D and *ply* genes in its genomes by giving a clear distinct banding pattern when observed after PCR amplification and agarose gel electrophoresis (data shown for only *cbp*D gene here) (Figure 3). As all these genes contribute positively for the onset of allolysis in bacteria, *S. pneumoniae* MTCC655 isolate is proved to be a suitable model for studying allolysis. As autolysins like *lyt*A, *lyt*C and a murine hydrolase choline binding protein D (*cbp*D) play key roles in hydrolyzing the non-competent cells (Prozorov and Danilenko 2011), analysis of *cbpD* gene expression pattern provided to be useful for a complete understanding of the genetic mechanism and early onset of allolysis under antibiotic stress (Gonzalez-Pastor 2010).

CONCLUSION

In the 21st century the greatest threat to human beings is the prevalence of multi-drug resistant bacteria by nosocomial infections. Mere discovery of new antibiotics may not solve this global problem. Hence, suitable alternative medications may be put forward to deal with this health associated problem. In this regard, allolysis phenomenon in many clinical isolates may be given attention. As this study clearly demonstrates the early onset of allolysis by *S. pneumoniae* in presence of sub-lethal dose of antibiotic, it may be used for therapeutic purpose. The early onset of allolysis may decrease the number of populations of the antigen, which may be taken care of by the host's immune system. The decrease in time period for induction of allolysis from 7-9 h to 2-3 h, as confirmed by viable cell count and extracellular DNA estimation is highly significant. Though this study established the phenomenon in case of *S. pneumoniae*, it may be tested against other bacterial pathogens experimentally, suitable clinical trial prior to therapeutic application in large scale. Though this pathogen was resistant to a number of antibiotics, it showed allolysis and decrease in cell population by the sub-lethal dose of an antibiotic confirmed the potential of this phenomena in therapeutic application.

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