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Targeting of Virulence Factors and Plasmid Profiling of *Klebsiella pneumoniae* Causing Urinary Tract Infection in Sylhet City of Bangladesh

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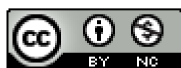
ABSTRACT

Studies were conducted to characterize Klebsiella pneumoniae isolates from urinary tract infection (UTI) patients in Sylhet city of Bangladesh. At the same time, all isolates were screened for some common virulence genes and four significant isolates were searched for plasmid number and sizes by mini alkaline-lysis method. Among five tested isolates from female UTI patients, gyrase subunit B2 (gyrb2) amplified in all isolates, lipase and nuclease detected in three isolates and serine protease amplifies in two isolates and gave the expected band of 1130 bp, 517 bp, 1055 bp and 211 bp respectively. Two of four isolates showed 9.82 kb plasmid band on agarose gel. Isolates bearing 9.82 kb plasmid were found to be resistant to multiple commercial antibiotics. At the same time all isolates were screened for in-vitro plate assay for proteolytic, lypolytic and hemolytic activity. Isolates with positive plasmid and more than one virulent gene with gyrB2 showed positive result in in-vitro culture plate with clear zone of proteolysis, hemolysis or lipolysis. This study will be helpful for further study in finding correlation or pattern of virulence properties for K. pneumoniae associated UTI in Bangladesh.

Keywords: *K. pneumoniae*, urinary tract infection, virulence factors, plasmid profiling, correlation study



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INTRODUCTION

Urinary tract infection (UTI) is the most commonly encountered hospital-acquired infection. *Klebsiella pneumoniae* is the second leading cause of urinary tract infection (UTI) after *E. coli* [1]. However, although quite low prevalence, the pathogenicity of *K. pneumoniae* associated UTI is much higher than *E. coli* [1,2]. It is estimated that 150 million people affected by UTI each year in which approximately 12% of UTI caused by *K. pneumoniae* and the number is increasing alarmingly in East Asia [2,3]. In Bangladesh, same trend for UTI infection also observed and significant numbers of isolates became antibiotic resistante [4]. Yet any study conducted on gene-virulence factors and plasmid profiling for *K. pneumoniae* in order to find out molecular mechanism in disease progression and correlation among virulence factors [5,2]. Targeting of virulence gene is the key factor in determining the pathogenicity and disease causing ability of any isolate because this gene act multi-functionally and multi-factorially. In recent time, molecular detection of virulence genes provide a promising tool for understanding pathogenicity of an isolates as well as in disease diagnosis and prophylaxis [6]. Several virulence genes i.e. nuclease, protease, lipase, hemolysin, gyrase etc. encode products that indulge/influence the organism in expressing its virulence properties in the host cell [7]. DNA *gyrase* (*gyrB*) is an ATP requiring enzyme help in replication by unwinding DNA super coil by an unusual mechanism [8,9]. DNA gyrase subunit two (*gyrB2*) is essential for cell viability and poses beta hemolytic activity: ability to lyse blood agar might contribute to the pathogenicity of any isolates [10]. Lipase (*lipA*) shows a wide variety of substrate and reaction specificity which catalyze hydrolysis of triglycerol. Increasing serum *lipase* activity results in higher chance of pancreatic inflammation and other disorders [11]. Nuclease (*nucl*) is capable of cleaving phosphodiester bonds between the nucleotide subunit of nucleic acids. Some of these are sequence specific endonuclease that recognizes and cut specific sequences within a particular point of base pairs. Nuclease showed to be very important virulence factors in many clinical isolates i.e. *Strepcoccus* sp. [12]. Serine protease (*serP*) is an enzyme that cleaves peptide bonds in protein in which serine serve as the nucleophilic amino acid at the enzyme's active site [13]. Plasmid profiling involves the study of sizes and numbers of plasmid in a particular organism. After the cell lyses, the nucleic acids are subjected to electrophoresis to visualize the size and copy number plasmid [14]. Plasmid profiling sometimes may not provide useful information because some species may contain variable number of plasmids or even unrelated bacteria may harbor similar number of plasmids. However, plasmid profile analysis is very important in analysis of gene-virulence relationship study [15,16].

Antibiotic sensitivity assay is generally done concurrently with plasmid profiling in order to correlate resistance pattern with their amplified plasmid [17]. Large plasmid confer broad antibiotic resistance pattern including extended spectrum beta-lactamase (ESBL) which significantly associated with virulence properties including a number of virulence functions in a human infection [17,18]. *In-vitro* plate assay to analyze proteolytic, hemolytic and lypolytic activity have been shown to be a promising approach to indentify co-relation between amplified gene and corresponding virulence factors [19,20]. Thus the present study was conducted in order to i) detect the major putative virulent genes, size and copy number of plasmid in *K. pneumoniae* causing UTI in Eastern part of Bangladesh; ii) to find out correlations among virulence factors and plasmid of UTI causing *K. pneumoniae.*

MATERIALS AND METHODS

Collection of bacterial isolates

Six bacterial isolates were collected from two different hospitals and diagnostic centre of Sylhet city, Bangladesh, Popular Hospital and Diagnostic Centre of Bangladesh (isolates K1, K5 and K8) and M. A. G. Osmani Medical College and Hospital (isolates K10, K11 and K12). Isolates were cultured in ESBL medium overnight and transported immediately after culture to USDA-project laboratory of Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh by maintaining cool chain. All isolates were collected from UTI patients of different aged persons, older than 40 years except K10, which collected from 12 years old girl.

Bacterial culture condition

In laboratory, individual bacterial isolates were streaked in nutrient agar plate for pure culture and incubated at 37 ºC overnight. After incubation, single colony was picked for different biochemical tests for *K. pneumoniae*. For the isolation of DNA, bacterial isolates were cultured in nutrient broth (NB) at 37ºC in a shaker incubator at 150 rpm speed. For profiling of plasmid DNA, five isolates were cultured in Luria broth (LB) medium and incubated overnight at 37ºC in a shaker incubator with same speed used for DNA extraction.

Biochemical characterization

Freshly cultured bacterial isolates were tested for further confirmations belong to *K. pneumoniae* species. A series of biochemical test were done according to Burgey's manual of determinative bacteriology [21]. Major tests were; gram's test, gram staining, catalase, oxidase, oxidative-fermentative, indole, hydrogen sulfide, methyl red, voges-proskauer and, motility and so forth. All results were matched with Burgey's manual and only sole characteristics belong to *K. pneumoniae* species were taken for further study.

Bacterial DNA extraction

A total of five bacterial isolates were cultured in nutrient broth overnight at 37 ºC in a shaker incubator. Bacterial genomic DNA was extracted by following instructions of commercial genomic DNA extraction kit (Bio Basic Inc., 160 Torbay Road, Markham Ontario, Canada) and extracted DNA stored initially at - 20 ºC for further use. DNA of five isolates was quantified by gel electrophoresis with lambda DNA as well as in spectrophotometer as a ratio of DNA-protein absorbance.

PCR reaction mixture set-up

The PCR was performed in 25 µl master mixtures containing DNA template (150 µg of genomic DNA of *K. pneumoniae*) of 1.2 µl, 1 µl of 25 mM $MgCl_2$, 5 µl of 10x colorless reaction buffer, 0.5 µl concentration of deoxynucleotide triphosphate (dNTP), 1.2 µl of each forward and reverse primer with the concentration of 0.5 µM (Table 2) and 0.15 µl Taq DNA polymerase. The amplifications were carried out in a MultiGene gradient thermal cycler (Labnet International Inc. USA).

PCR amplification condition

PCR amplification condition was optimized after several laboratory trials with the following parameters: an initial denaturation run of 94°C for 4 min; denaturation step of 94°C for 1 min, annealing 1 min at 64 °C for *nucl*, *lipA* and *serP*, 62°C for *gyr* B2, and an extension at 72°C for 90 s; and a final extension step of 72°C for 10 min. For all genes, 35 serial cycles of amplification reaction was performed.

Profiling of bacterial plasmid DNA

Five bacterial isolates were inoculated in freshly prepared LB medium and incubated at 37ºC in a shaker incubator at 150 rpm for overnight. Plasmid DNA was isolated and purified by alkaline lyses method by using following reagents: solution 1 (50 mM glucose, 25 mM Tris HCL, and 10m MEDTA), solution 2 (0.2% NaOH, 1% SDS) and phenol-chloroform [22].

Antibiogram profile of the isolates

Resistance or sensitivity of the *K. pneumoniae* isolates to 12 commercial antibiotic discs was determined by disk diffusion assay [23]. The antibiotic disks used in this study were ampicilin (10 µg/disk), kanamycin (30 µg/disk), erythromycin (15 µg/disk), amoxycillin (30 µg/disk), chloramphenicol (30 µg/disk), levofloxacin (5 µg/disk), ciprofloxacin (30 µg/disk), azithromycin (30 µg/disk), cefradine (25 µg/disk), gentamicin (10 µg/disk), streptomycin (10 µg/disk) and sulphamethoxazole (25 µg/disk). Overnight bacterial culture (30 µl) was spread inoculated on Isosensei Test Agar plates (Micromaster, India) and disks were aseptically placed on the culture. After 24h of incubation at 37ºC, the zone of inhibitions were measured and resistance or sensitivity of the strains were estimated according to the disk manufacturers instruction.

***In -vitro* plate assay for study of virulence factors**

Bacterial isolates were cultured *in-vitro* in culture plate with casein, tween20 and sheep blood agar supplementation for analyzing proteolysis, lipolysis and hemolysis. Proteolytic activity was determined by using casein as a protein source and positive isolates can hydrolyze casein and produce zone of hemolysis [24]. Casein agar media was prepared as follows: 0.5% of casein, 0.5% of glucose, and 2% of agar (w/v), pH 7.0. Bacterial isolates were streaked into tween20 agar containing 10 (g/L) peptone; 5 (g/L) NaCl, 0.1 (g/L) $CaCl_2$, 2% agar and 1 (ml/L) tween20 for measuring the halos of precipitation for determine lipolytic activity [25]. Hemolytic activity was calculated by culturing isolates on TSA supplemented with 5% (v/v) sheep's blood [25] and the diameter of the halos of hemolysis was measured [19].

RESULTS

Identification of *K. pneumoniae* isolates

Although all bacterial isolates were initially supplied as *K. pneumoniae* culture on selective agar media supplemented with ornithine, raffinose and koser citrate [26]; however, all bacterial isolates were assayed for their morphological, physiological and biochemical properties for further confirmation. Among six isolates, five isolates were confirmed belong to *K. pneumoniae* species according to Burgey's manual [21] for identification of bacteria (Table 1). Isolates were numbered as K1, K5, K8, K10 and K11. Isolate K12 showed confusing result in oxidative-fermentative test and this was excluded from this study.

Table 1. Biochemical characteristics of presumptive isolates

Biochemical tests	Results
Gram's test	-
Gram's staining	-
Catalase	+
Oxidase	-
Oxidative-Fermentative	F
Lactose fermentation	+
Glucose fermentation	+
Sucrose fermentation	+
Indole test	-
H_2S production	-
Methyl red	-
Voges-proskauer	+
Gelatin hydrolysis	-
Urease	+
Motility	-
Aerobic growth	+
Anaerobic growth	+

Note: - =Negative, + =Positive, F =Fermentative

PCR amplification of virulence genes

After PCR run, products were electrophoresed in 1.5% agarose gel in TBE buffer for visualization of amplified gene(s) in tested isolates. Gel stained with ethidium bromide solution (10 mg/mL) for 40 min and visualized under gel documentation system. Among five tested isolates, *nulc* and *lipA* amplified in K1, K5 and K8 whereas *serP* found in K1 and K10 and gave expected product length of 517 bp, 1055 bp and 211 bp respectively. Gyrase subunit B2 (*gyrB*2) amplified in all five isolates with PCR product size of 1130 bp in agarose gel (Figures 1-4).

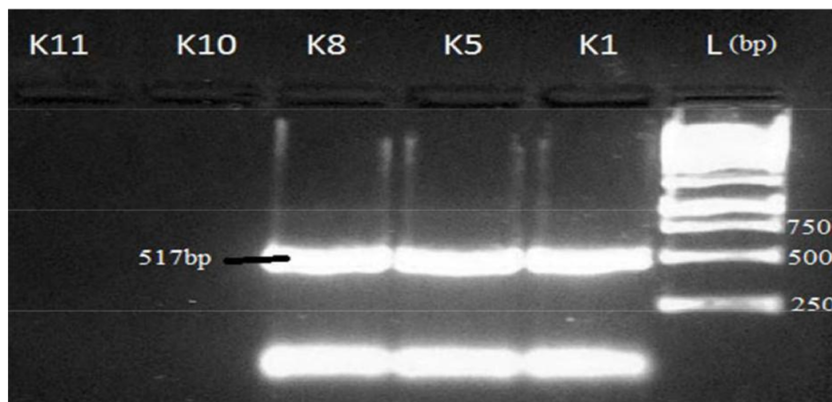

Figure 1. PCR amplification of nuclease gene in *K. pneumoniae* isolates.

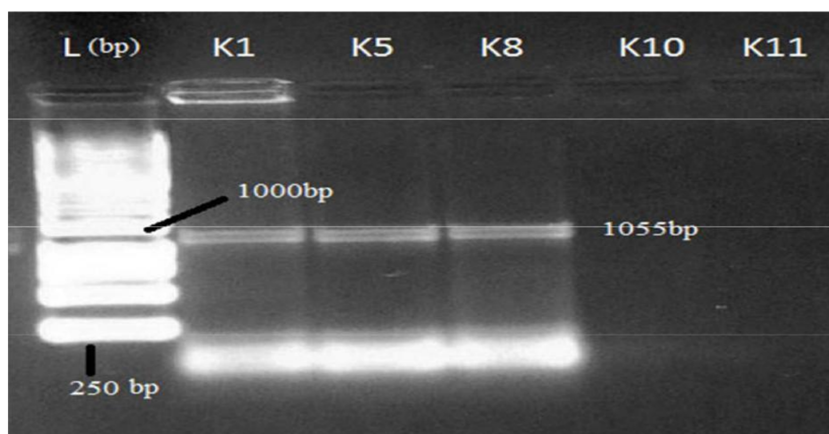

Figure 2. PCR amplification of lipase gene in *K. pneumoniae* isolates.

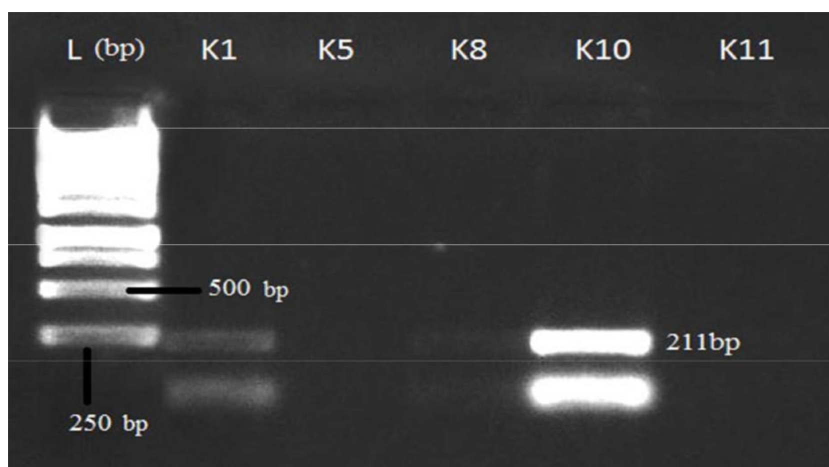

Figure 3. PCR amplification of serine protease gene in *K. pneumoniae* isolates.

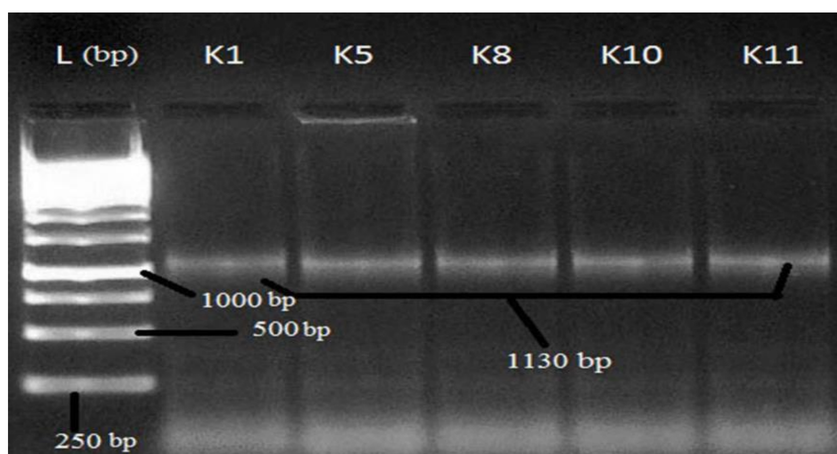

Figure 4. PCR amplification of gyrase subunit B2 gene in *K. pneumoniae* isolates.

Antibiotic sensitivity of *K. pneumoniae* isolates

To know the antibiogram profile of the isolates, we screened 12 antibiotics representing different antibiotic groups. All of the isolates exhibited 80-100% resistant to ampicillin, erythromycin, and chloramphenicol and also for cephradine, kanamycin and sulphamethaxazole. Sensitivity was observed for only four antibiotics *viz.,* streptomycin, gentamycin, ciprofloxacin and levofloxacin except for isolates K1 and K5 which showed sensitivity to only one and resistant to three other antibiotics. Sensitivity results for other antibiotics varied but neither one showed 100% sensitivity alone to all tested

isolates. All of the isolates exhibits resistance to multiple antibiotics tested (Table 2).

Table 2. Antibiotics susceptibility pattern of *K. PNEUMONIAE* isolates from urine samples of UTI patients

Isolates	Amp	Kan	Ery	Amx	Chl	Lev	Cip	Azt	Cef	Gen	Stp	SXT
K1	R	R	R	R	R	S	R	R	R	R	R	R
K2	R	S	R	R	S	S	R	R	R	S	R	R
K3	R	R	R	R	S	S	S	R	R	S	S	S
K4	R	R	S	S	S	S	S	S	R	S	S	R
K5	R	R	R	R	R	R	R	R	R	S	R	R
K6	S	R	R	R	S	S	S	R	S	S	R	R
K7	R	R	R	R	S	S	S	R	S	R	R	R
K8	R	R	R	R	R	S	R	R	R	S	R	R
K9	R	R	S	S	S	S	S	R	R	S	S	R
K10	R	R	R	R	S	S	R	R	R	R	R	R

R=Resistant; S=Sensitive

Profiling of plasmid DNA

Plasmid isolation and analysis was done for isolates K1, K5, K10 and K11, those showed positive result in virulent gene amplification for *nulc, lipA* and *serP*. Plasmid DNA was amplified in two isolates (K1 and K5) and showed 9.82 kb size on 1.2% agarose gel under gel documentation system while others showed negative result for plasmid DNA (Figure 5).

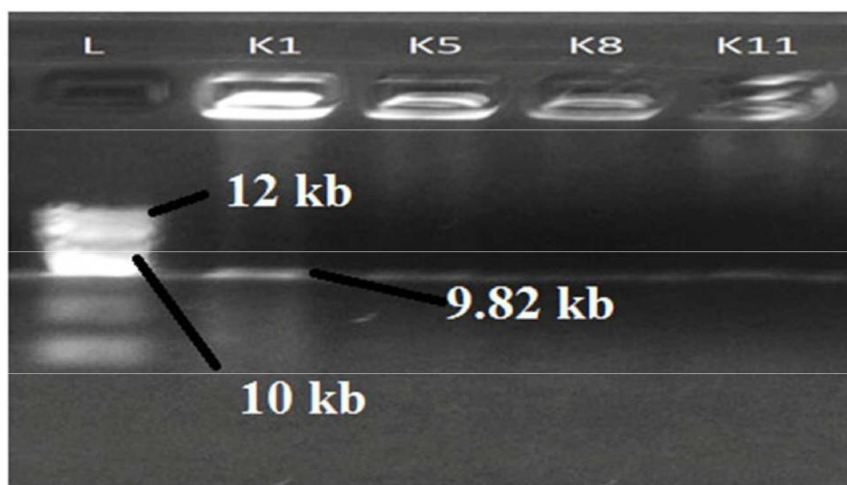

Figure 5. Plasmid profiling of *K. pneumoniae* isolates causing UTI.

***In -vitro* assay for virulence factors**

In-vitro plate assay showed divergence pattern of activity on culture plate. Better *In -vitro* proteolysis and lipolysis activity was observed for isolates K1, K5 and K10 isolates while other isolates hardly hydrolyze casein and tween20 on culture plate. No significant the halo of hemolysis was noticed for four isolates of *K. pneumoniae* except for K1, which, slightly hydrolyze blood agar only around the center (Table 3).

Table 3. In-vitro plate assay of *K. pneumoniae* isolates

Isolates	Proteolysis	Lipolysis	Hemolysis
K1	+++	+++	++
K2	+	+	+
K3	-	+	-
K4	-	+	-
K5	++	++	+
K6	+	+	-
K7	-	+	-
K8	+	++	+
K9	-	+	-
K10	+++	++	+

DISCUSSION

K. pneumoniae reported to cause wide range of infections in human and other organisms and the pathogenicity depends on a variable number of factors directed by an array of genes and virulence factors [27,28,29]. Among those, urinary tract infection (UTI) is one of the most common diseases that cause potential threat to human health; especially for pregnant women and aged persons [12,30]. Present study also reveals that, women's during pregnancy and people after age of 50 generally more vulnerable to UTI causing *K. pneumoniae.* Six out of ten isolates were collected from adult patients of age range between 35 and 65, in which three were pregnant women. Nuclease (*nulc*) and lipase (*lipA*) gene amplified in three isolates both of which encode plasmid of same molecular weight. In a most recent study, nuclease like protein, colibacin toxin, a putative cytotoxin in the outer membrane identified as one of the virulence factors of *K. pneumonia* [31]. In addition, a protein from phospholipase D family (PLD1) was also reported as a virulent gene in *in-vivo K. pneumoniae* mouse model after expression of its mRNA [31]. Increasing serum lipase activity also responsible for inflammation and other disorders by altering the structure of cytoplasmic membrane of host and thus exacerbates its pathogenicity [11,32]. Serine protease, a protein that triggers proteolysis by binding and inhibiting the enzymatic activity of elastase and cathepsin G in *in-vitro,* has been reported as a virulence factors for *K. pneumoniae* in recent times [33]. DNA gyrase subunit B on the other hand, encode sequences essential for DNA replication, exhibits beta-hemolytic activity in mouse model and contribute to the progression of enterobacteriaceae associated disease in human [10,34]. In our present study, two isolates (K1 and K2) of *K.pneumoniae* had plasmid molecular weight of 982 kb. This plasmid likely to bear high antibiotic resistance encoding beta-lactamse enzyme confer resistance to ampicilin, kanamycin, chloramphenicol [35,36]. Our present study found majority of isolates (80%) resistant to antibiotic ampicilin, 70% resistant to chloramphenicol and kanamycin. Data results expose a correlation in between 9.8 kb plasmid and antibiotic resistant pattern which suggest involvement of this plasmid in pathogenicity of *K. pneumoniae.* We conducted *in-vitro* plate assay of respective gene viz. nuclease, lipase, protease and gyrase to analyze their activity (proteolysis, lipolysis and hemolysis) extracellularly. This assay proved to be very useful in determining virulence associated factors, analyzing virulence level and predicting the correlation of these two with the amount of enzymes and toxins produced by the pathogens. Our present study showed a unique correlations: isolate K1 bearing *nucl, lipA* and *serP*, exhibited strong activity to hydrolyze casein, tween20 and moderate activity to blood agar whereas,

K5 which had *nucl, lipA,* hydrolyze tween20 effectively but not casein and sheep blood. Finally, isolate K10 amplified with *serP* gene was powerful hydrolyser of casein in extracellular environment but barely utilize tween20 and blood agar. Therefore, a strong correlation was revealed among putative virulence factors and bacterial plasmid in our study that contribute to the pathogenicity of *K. pneumoniae* associated urinary tract infection (UTI) in Bangladesh.

CONCLUSION

K. pneumoniae pathogenicity depends on three factors: number and type of virulent genes, size of the plasmid and result of *in -vitro* hydrolysis assay for virulence factors. In this study, we found a strong correlation among these factors in the pathogenicity of *K. pneumoniae* associated urinary tract infection (UTI) in Bangladesh. Further studies i.e. sequencing of genes, analysis of the sequence for any mutation are currently going on in our research laboratory. Investigation need to develop animal model for further analysis of *in-vivo* relationship of these three factors.

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