

## Full Length Research Paper

# Isolation and molecular characterization of UTI associated *Serratia marcescens* TW1 from urine specimens

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Among the 100 isolates, a bacterial strain TW1 was isolated from the urine tract specimens of infected women admitted at Government hospital, Namakkal District in Tamil Nadu, India, using caprylate thalios agar medium. The organism was characterized by all biochemical tests and showed similarity with *Serratia marcescens*. The genomic level confirmation done with 16s rDNA primer by submitting the genomic sequence to Gene Bank under ACC.No-GU046545 after comparing, showed 98% sequence similarity with *S. marcescens* and thus, the strain was named *Serrtia marcescens* TW1.

**Key words:** Urinary tract specimens, *Serratia marcescens* TW1, 16s rDNA genomic analysis.

## INTRODUCTION

In hospital, patients are frequently infected with *Serratia marcescens*, a well known nosocomial species of *Serratia*. It is an opportunistic pathogen causing infection in immunodeficient patients. The ability of *Serratia* cells to attach human buccal epithelial cells (Ismail and Som, 1982) and to human urinary bladder surfaces (Yamamoto et al., 1985) poses a serious public health threat by showing resistance against beta lactam ring antibiotics (Choi et al., 2007). The incidence increases with advancing age and males are at a higher risk, in which case, 92% of the isolates were *S. marcescens* (Laupland et al., 2008). *S. marcescens* has increasingly been recognized as a cause of infection in adult pediatric and neonatal intensive care units (Teertstra et al., 2006). It is found that insulin and sedative solutions used for the patients were contaminated with *S. marcescens* which was proof as a source of outbreak in intensive care unit (Alfizah et al., 2004).

## MATERIALS AND METHODS

### Isolation of bacterium

Urine samples were collected from urinary tract infected women admitted at general Government Hospital, Namakkal District in

Tamil Nadu. The organism was isolated (Matsumoto et al., 1984) using Caprylate-thalios agar medium. The urine samples were serially diluted and spread over the medium then incubated at 37°C for 24 h.

### Identification of the bacterium

The morphology and biochemical characterization of isolated strain TW1 was performed according to method of Holt et al. (1994).

### Microscopic morphology

Microscopic morphology was observed by gram staining, flagella staining and by hanging drop method.

### Biochemical test

Biochemical tests such as indole, methyl red, voge proskeaur, citrate and triple sugar iron were carried out. The carbohydrate fermentation tests such as L-arabinose, fructose, glucose, lactose, L-rhamnose, D-sorbitol, sucrose and D-xylose were carried out to find the biochemical characters of strain TW1

### Enzyme hydrolysis test

Various enzymatic hydrolysis tests such as deoxyribonuclease test, gelatinase test, casein hydrolysis test, decarboxylase test and lipid hydrolysis test were carried out to find out organism.

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## **Molecular confirmation by 16s rDNA**

### **Extraction of bacterial DNA**

The DNA was extracted from the bacterial isolate by following the method of Sambrook et al. (2001). The single colony of bacterial strain TW1 was inoculated into the 50 ml of Luria Bertani broth and incubated at 20°C on rotatory shaker, overnight. Then, 1.5 ml of culture was transferred into eppendroff tubes and spun at 8,000 rpm for 10 min. The supernatant were discarded and drained on tissue paper. The pellet was resuspended into 400  $\mu$ l of TE buffer and 32  $\mu$ l of lysosome was added then incubated at 37°C for 30 min. After incubation at 37°C for 30 min, 100  $\mu$ l of 0.5 mM EDTA was added followed by 60  $\mu$ l of 10% SDS and 1.5  $\mu$ l of proteinase K (50  $\mu$ l/1 ml) respectively then incubated at 50°C for 60 min. After incubation at 50°C for 60 min, tubes were brought at room temperature and 250  $\mu$ l of phenol: chloroform: isoamylalcohol in the ratio of 25:24:1 were added and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to another eppendroff tubes and RNAase was added at final concentration of 50  $\mu$ l/ml and mixture was incubated at 60°C for 1 h. After incubation, DNA was precipitated with ice cold ethanol and precipitated DNA was collected by centrifugation at 5,000 rpm for 10 min. Finally, the pellet was washed with 70% ethanol and completely air dried and resuspended in 100  $\mu$ l of TE buffer at pH 8.0.

### **Polymerase chain reaction**

The polymerase chain reaction was carried out by following a method of Sambrook and Russel (2007). The 16s rDNA forward primer 5' TAG GGA AGA TAA TGA CGG 3' Reverse primer 5' CCT CTA TCC TCT TTC CAA CC3' was used to obtain amplification. The 50  $\mu$ l reaction mixture was transferred into 0.5 ml microfuge amplification tube containing mixture in the following order: 10x amplification buffer (5  $\mu$ l), 20 mM solution of four dNTPs, pH 8.0 (1  $\mu$ l), 20  $\mu$ M forward primer (2  $\mu$ l), 20  $\mu$ M reverse primer (2  $\mu$ l), Taq DNA polymerase (2  $\mu$ l), nuclease free water (33  $\mu$ l), DNA sample (5  $\mu$ l), and total volume (50  $\mu$ l). The reaction mixture was centrifuged at 4,000 rpm for 5 min then placed in the thermal cycler fitted with heated lid. The nucleic acids amplified by setting denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 1 min in the thermal cycler and number of denaturation cycles repeated to 30 cycles. After polymerase chain reaction, 15  $\mu$ l of amplified DNA product was run in an agarose gel electrophoreses by preparing 1.5% agarose gel and DNA bands were viewed under the UV transilluminator.

### **Automated sequencing**

The sequencing of the genomic DNA amplicon coding for strain TS1 was carried out at scientific synergy company, Canal Bank Road, Chennai, India using an instrument ABI3130, Amersham Biosciences United kingdom by following method of Sambrook et al. (2001).

### **Purification**

1  $\mu$ l of PCR reaction mixture was aliquated into a 0.6 ml microfuge tube and diluted to a total volume of 20  $\mu$ l with distilled water. Then, 20  $\mu$ l of 4M-ammonium acetate was added and mixed well. To this, 40  $\mu$ l of isopropanol was added and mixed well. It was left at room temperature for 10 min and after that, the tubes were centrifuged at 12,000 g for 10 min to obtain the DNA precipitate. The supernatant was removed, discarded, and the pellets washed with 70% ethanol

to obtain pure DNA. Then, pellet was dried under vacuum and resuspended in 20  $\mu$ l TE buffer.

### **Materials for cycle sequencing**

The materials which were used for the DNA sequencing are: Prism Tm ready reaction dideoxy terminator premix, I: ABI mix 1.5  $\mu$ M dd ATP viz 94.7  $\mu$ M dd TTP, 0.42  $\mu$ M dd GTP, 43.3  $\mu$ M dd CTP, 168.43 mM Tris p<sup>H</sup> 9.0, II: 4.2 mM (NH<sub>4</sub>)SO<sub>4</sub>, III: 0.42U/ $\mu$ l Ampli Taq DNA polymerase.

### **Cycle sequencing of PCR products**

The following reagents were mixed in 0.6 ml microfuge tube: 5  $\mu$ l of DNA template, 1  $\mu$ l of primer used for PCR, and 4.4  $\mu$ l sterile water. To this, 9.5  $\mu$ l of ABI prism ready mixture ddNTP primer was added. The reaction mixture was placed in a thermocycler and the following conditions were employed: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min, total reaction cycle – 30, and samples were kept at 4°C.

### **Materials for cycle sequencing products**

Phenol: water: chloroform (17:18:15), 2 M sodium acetate (p<sup>H</sup> 4.5), 100 and 70% ethanol, sequencing gel (6%), 10x TBE buffer contains; 890 mM Tris borate, 890 mM boric acid, 20 mM EDTA (p<sup>H</sup> 8.3), 12 ml of 40% urea/acrylamide (W/V), (19:1) acryl amide/bisacrylamide, 20 ml distilled water, 1 g mixed bed ion exchange resin, TEMED, 10% W/V ammonium per sulphate.

### **Extraction of cycle sequencing products**

80  $\mu$ l of sterile water was added to PCR DNA sample, to this 100  $\mu$ l of phenol: water: chloroform (68:18:14) mixture was added and vortexed well, then sample was centrifuged at 12,000 g for 1 min. The supernatant was transferred to a clean eppendroff tube and 15  $\mu$ l of 2 M sodium acetate, 300  $\mu$ l of 100% ethanol were added and mixed well. The mixture was centrifuged at 12,000 g for 15 min at room temperature. The supernatant was removed and pellet was washed with 70% ethanol then dried under vacuum and resuspended in 20  $\mu$ l of TE buffer.

### **Sequencing sample preparation**

4  $\mu$ l of deionizer foramide: 50 mM EDTA having pH 8.0 was added to sequencing product tubes and centrifuged briefly. Before loading, the sample was heated at 90°C for 3 min and transferred immediately to ice. Then, sample was loaded on to automated DNA sequence (ABI3130) fitted with 6% polyacrylamide gel and run, then results were analyzed for determining the phylogenetic relationship by submitting to gene bank and accession number were obtained.

### **Phylogenetic tree analysis**

Nucleotide sequence was compared to those in the Gene Bank database with Basic Local Alignment Search Tool (BLAST) algorithms to identify known closely related sequences. CHROMO SOFTWARE analyzed sequences. The tree was generated by the neighbouring algorithm (Saitou and Nei 1987) joined to it by implementation with phydit. The assemblage of 16s rDNA gene sequences in each library was analyzed by rarefaction analysis

using Ecosim (Gotelli and Entsminger, 2006) to assess the extent to which the diversity of microbial communities was represented by the library at the class and species level. The number of species in each clone library was determined (Tatusova and Madden, 1999) by comparing closely related sequence using b12 seq (<http://www.ncbi.nlm.nih.gov/blast/b12seq/wblast2.cgi>). The 16s rDNA sequences exhibiting a percentage of similarity of 97% or lower (Devereux et al., 1990) were considered for species authentication.

## RESULTS

### Colony morphology of bacterial isolate

After 48 h of incubation in caprylate thallicous agar medium,  $1.82 \times 10^7$  CFU/ml were found by ten fold dilution method from the urine specimens collected from the infected women by ten fold dilution method. All isolates showed visible growth at 37°C. The purified colony morphology appears as non-pigmented convex and relatively opaque centre effuse, colorless, almost transparent periphery, and irregular crenate edge surrounded by a clear zone around the colonies were observed.

### Microscopic and biochemical tests

The microscopic examination showed gram-negative rods in gram staining, peritrichous flagella, and were active motile. The various biochemical tests were performed and it was found that isolated strain TW1 belonged to genus *S. marcescens* by showing indole negative, methyl red negative, voges proskauer positive, citrate positive, triple sugar iron reaction showed yellow colour butt (acid) and red colour slant (alkaline) and there was no black colour precipitate in the medium, so did not produce hydrogen sulphide. The strain TS1 has fermented the glucose, sucrose, sorbitol, fructose, and did not fermented the xylose, rhamnose, lactose, and arabinose (Table 1).

### Enzyme tests

The organism *S. marcescens* led the liquification in the gelatin agar medium thus showed an ability to produce gelatinase. When deoxyribonuclease agar medium was observed, there was big zone around the colonies, which means *S. marcescens* had an ability to produce an extracellular enzyme. In the mollers decarboxylase lysine and ornithine broth medium, a colour change took place from yellow to blue, so, showed decarboxylation reactions. While in ornithine broth medium, no colour change took place so showed no decarboxylation reaction. The strain produced an extracellular enzyme lipase in the lipid in the medium and showed a clear zone around the colonies.

## Molecular characterization of the strain

The genomic DNA was amplified with 16s rDNA primer, Forward primer: 5' TAG GGA AGA TAA TGA CCG3' Reverse primer: 5' CCT CTA TCC TCT TTC CAA CC 3'. The amplified PCR product when run in agarose gel electrophoresis, strain TS1 was found having a molecular weight approximately of 870 bp when compared with the DNA marker (Figure 1). The strain TW1 was identified and named as *Serratia marcescens* TW1 under Gene Bank ACC.No-GU046545. The genomic sequence obtained

are are: AA G T C GA GC

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GGTAGCACAGGGAGCTTGCTCCCTGGGTGACGACC
GGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG
ATGGAGGGGGATAACTACTGGAACGGTAGCTAATA
CCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTC
GGCCTCTTGCCATCAGATGTGCCAGATGGGATTA
GCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGA
TCCCTAGCTGGTCTGAGAGGATGACCAGCCACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGG
GTTGTAAAGCACTTTTCAGCGAGGAGGAAGGTGGTGA
ACTTAATACGTTTCATCAATTGACGTTACTCGCAGAAG
AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGG
CGTAAAGCGCACGCAGGCGGTTTGTAAAGTCAGATG
TGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGA
AACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
GGAATACCGGTGGCGAAAGCGGCCCCCTGGACGAA
GACTGACGCTCACGTGCGAAAGCGTGGGGAGCAA
CAGGATTAGATACCCTGGTAGTCCACGCTGTAACCG
ATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTCGCC
TTCCGGAGCTAACGCGTTAATCGACCGCCTGGGGGA
GTACGGCCGCAAGGTAACCTCAAATGAATTGACTG
GG
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### Phylogenetic analysis

The 16s rDNA sequences of the amplified products revealed that the strains TW1 had unique sequences which matched with *S. marcescens* present by phylogenetic tree (Figure 2).

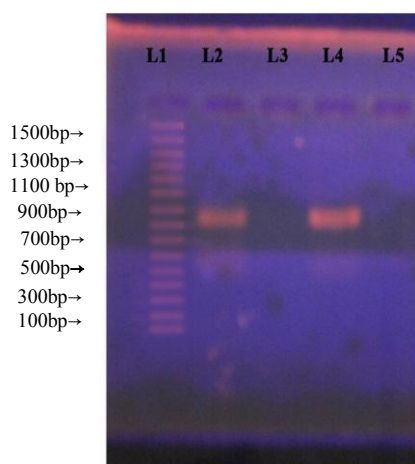
## DISCUSSION

The healthy human beings do not often become infected by *Serratia*, whereas, the hospitalized patients were frequently colonized or infected. *S. marcescens* were generally an opportunistic pathogen causing infection in immunodeficient patients. The possible pathogenic factors found in *Serratia* strains are the formation of fimbriae (Old et al., 1983), potent siderophores, presence of cell wall antigens, ability to resist the bactericidal action of serum, production of proteases, the ability of *Serratia*

**Table 1.** Biochemical identification of clinical isolates *Serratia marcescens* TW1.

Serial number	Biochemical test	Result
1	Indole	N
2	Methyl Red	N
3	Voges Proskauer	P
4	Citrate	P
5	Triple sugar iron	A/K G <sup>-ive</sup>
6	Hydrogen sulphide production	
7	Glucose	F
8	Sucrose	F
9	D- Sorbitol	F
10	Fructose	F
11	D-Xylose	NF
12	L-Rhaminose	NF
13	Lactose	N
14	L-Arabinose	NF

N-negative, P-positive, A/K – Acid/Alkaline, G<sup>-ve</sup> –gas negative, F- fermentation, NF- no fermentation.

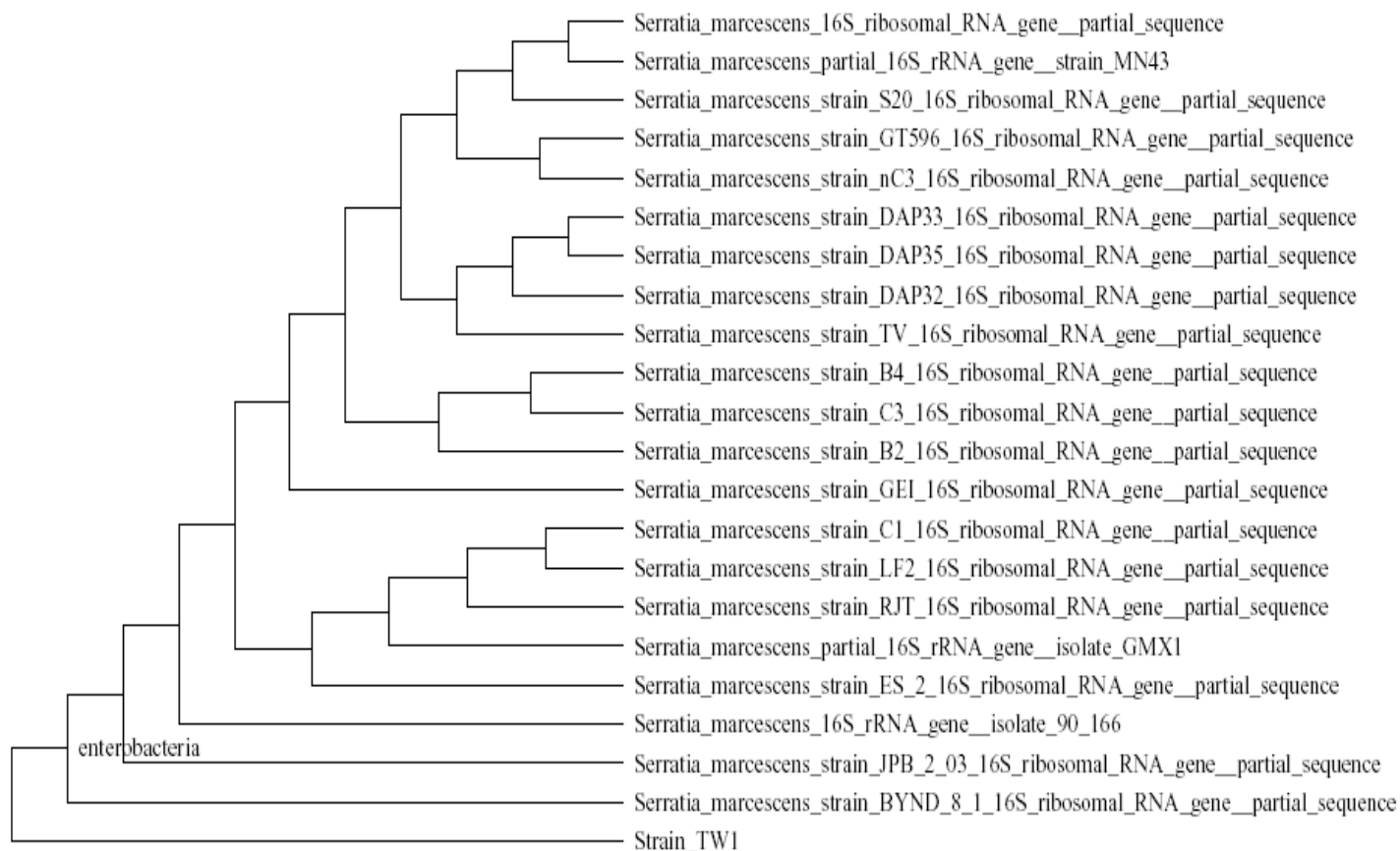


**Figure 1.** The PCR product of bacterial genomic DNA of *Serratia marcescens* TW1 where L1-Marker (1500bp), L2, L4, DNA Samples (870 bp).

cells to attach human buccal epithelial cells (Ismail and Som, 1982) and human urinary bladder surfaces (Yamamoto et al., 1985). These are factors which causes the infection in the human beings. The relationship between *Serratia* strains and patient may be in the form of an ephemeral association (in gut or throat or on hand or skin), long term colonization (in gut or urinary tract or on the skin) or a localized or a generalized infection. The form of relationship may depend on the species or the strain of *Serratia*, entry route or ecological advantage or the patient's physiological status. One cell of *Serratia* is able to cause complete lysis in ten human erythrocytes within two hours in a liquid assay or haemolysis assay by

suspending erythrocytes in citrate buffer (Marty et al., 2002). The community acquired pneumonia caused by gram negative bacilli was more frequently associated within septic shock, malignancy, cardiovascular disease, smoking, hyponatremia and dyspnea (Kang et al., 2008). The antimicrobial susceptibility profile of *S. marcescens* showed reduced susceptibility to ciprofloxacin, gentamicin, and piperacillin-tazobactam led an outbreak of multiple resistant *S. marcescens* (Knowles et al., 2000). The clinical isolate *S. marcescens* TW1 were non-pigmented when incubated at 37°C for 24 h, but normally, when grown at 28°C for 24 h, it produces the pigment (William and Qadri, 1980). The microscopic observation of the strain TW1 appeared as gram negative rod shaped bacteria (Grimont and Grimont, 1981) and in flagella staining, it appeared dark black with peritrichous tiny weavy threads of greater length than cell. The flagella help in motility. Swarming motility is a type of population migration behavior characteristic of some bacterial species on solid medium (Eberl et al., 1999). Extracellular nuclease produced by bacteria, are usually reported as deoxyribonuclease and ribonuclease.

In present study, *S. marcescens* strain TW1 produces an enzyme deoxyribonuclease. The genomic DNA from the strain TW1 (Sambrook et al., 2001) showed orange colour sharp bands in agarose gel were due to the intercalating of ethidium bromide. The PCR technology has the advantage of not only higher sensitivity and specificity but also a reduction in time and work load. The amplified product of genomic DNA was obtained with 16s rDNA by forward primer 5' TAG GGA AGA TAA TGA CGG 3' and reverse primer 5' CCT CTA TCC TCT TTC CAA CC 3'. The molecular weight was about 870 bp in the strain TSI (Zhu et al., 2007, 2008). Whole denaturation cycle was repeated for 30 cycles and



**Figure 2.** The gene sequence of Strain TW1 was deposited in the National Centre for Biotechnology Information, Bethesda, United States and the number was obtained under the gene bank account number GU046545. The phylogenetic tree was drawn by multiple sequence alignment with neighbour joining method. The sequences were submitted to Treetop, online tree construction tool, A.N.B Institute, Russia and strain TW1 showed 97% similarities with that of *Serratia marcescens*.

amplified product was obtained (Sambrook and Russel, 2007). Nucleic acid based diagnostic systems including polymerase chain reaction methods as well as the application of DNA and RNA probes are well known sensitive techniques for more rapid detection and specific identification of an organism, therefore, hold promise for sensitive and specific detection within much shorter time (Lahmann et al., 2008). The genotyping is the easiest technique as reproducible as ribotyping and with almost the same ability to discriminate different strains (Parvz et al., 2002). The DNA was used as template to amplify polymerase chain reaction by designed two primers; that is, forward and reverse primer, with reaction mixtures at appropriate conditions (Ruiz-sanchez et al., 2005). The PCR product that was run in agarose gel electrophoresis showed 870 bp for strain TW1. The DNA sequence of the strain TSI having Gene Bank ACC.NO.GU046545 when compared to those in Gene bank data bases with BLAST were found to have 98% similarities with *S. marcescens*. This strain is named as *S. marcescens* TS1 (Gene bank ACC. No. GU046543). The phylogenetic tree showed resemblance with *S. marcescens*.

## Conclusion

This research was carried out in order to understand the clinical importance of the gram negative bacteria, especially those responsible for infections in urinary tract system of women. This research work provides a means of accounting the infective role of *S.marcescens* TW1 with that of other gram negative bacteria which are also responsible for urinary tract infection in women. The identification showed that newly isolated strain of *S. marcescens* TW1 was non-pigmented, having a molecular weight of DNA 870 pb. This strain was responsible for causing urinary tract infection and the findings of this study will help to cure the urinary tract infection in advance by prescribing the combined dosage of antibiotic.

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