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# Surveillance for clinical pathogens from swab samples collected from different hospitals

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**ABSTRACT**

*The study was based on surveying the occurrence of clinical pathogens in two public health care centers in Vellore. 27 Samples were collected from different areas and were inoculated into nutrient broth immediately and brought to the laboratory for incubation. After successful incubation (24 h) samples were streaked on to nutrient agar plates, which upon incubation showed growth but in order to get isolated pure colonies they were serially diluted, followed by sub culturing on nutrient agar plates which upon further incubation was sub-cultured on nutrient agar slants and on differential media. Various biochemical tests like catalase, oxidase, MRVP, etc. were also performed for the identification of the organisms isolated from public health care sectors. Our main objective was to check different types of pathogens which could be possibly present in a hospital and their resistance towards several different antimicrobials, by conducting antimicrobial susceptibility test. The work was also aimed at determining increased resistance and to identify multi-resistant forms of various pathogens by 16S rRNA sequence method, which is becoming an increased threat to mankind acquired during hospital visits.*

**Keywords**: pathogen, biochemical, antimicrobial, 16S rRNA sequence.

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## INTRODUCTION

Hospital acquired infections and diseases are becoming an increasing threat to human beings and nearly most of the dreadful diseases are spread through hospitals such as *Salmonella typhi*, *Mycobacterium tuberculosis*, *Neisseria gonorrhea*, *Pseudomonas aeruginosa*, Methicillin resistant *Staphylococcus aureus*, etc. In addition to these existing infections there are many new strains which are adapting themselves to survive in the environment of hospitals and increasing their resistance towards antimicrobials which have been used before for inhibiting or killing the growth of microorganisms. More advanced antimicrobials are being used to check for the resistance but in certain cases there are reports of multi-drug resistance microorganisms which are posing an immense threat worldwide with an increasing frequency. Our work was mainly focused on identification of pathogenic strains from different areas in two hospitals by performing various tests and trials, and then susceptibility towards various antibiotics was tested.
These microorganisms which are highly pathogenic can cause severe diseases and infections such as *Staphylococcus aureus* causing surgical site infections (SSI), including diseases caused by severe resistant forms of MRSA (Methicillin Resistant *Staphylococcus aureus).* Moreover there are increased incidences of vancomycin resistant enterococcus as nosocomial infections globally [1]. These enterococci are among the normal gut microbiota and the two most common species which are responsible for causing diseases are *E. faecalis*, and *E. faecium* and their

increased rate of survival is thought mainly due to their resistance towards several antimicrobials including glycopeptides or due to transfer of plasmids and transposons by horizontal gene transfer mechanism [1].

Antimicrobial resistance can be due to either evolution of a new drug or variation in the existing drug, and also it has been found that decrease in the population size of pathogens and an increase in the number of antibiotic resistant microorganisms has characterized the evolution of infectious disease [2]. The reason behind increased resistance could be due to improper drug use, drug overuse, population overgrowth, increasing urbanization, wars and natural disasters are other factors responsible for increased drug resistance. Incidences of multi-drug resistant forms are increasing randomly, such as multi-drug resistant strains of tuberculosis and also some extensively drug resistant forms have emerged, referred to as 'super bugs'. They are becoming difficult to treat due to interference into the immune system resulting in switching over of the genes of some non-resistant forms to resistant forms of microorganisms, or recombining the resistant forms of genes with one another generating multi-antibiotic resistant genes which have been endemic for years now. In 1944, after the discovery of Avery, MacLeod, and McCarty, it was proved that it is the capsule which is responsible for conferring virulence on a microorganism and thereby protecting it from the host's defense system [3]. Apart from genetic factors there are many other factors which can be responsible for causing virulence, and among them type III secretion system is popular, which has been identified in a variety of fully sequenced bacteria ranging from *Chlamydia trachomatis* to the genome of *M. loti*. Biofilm formation is also one of the factors behind increased resistance of microbes in which they can form aggregates under unfavorable conditions of the environment in order to survive. These biofilm help in forming channels which facilitates diffusion of nutrients into and removal of wastes away from it [4]. So our main motive was to highlight the resistant forms of pathogens from a hospital and their corresponding antibiotics to which they are resistant.

## MATERIALS AND METHODS

### Sample Collection

Samples were collected from the two hospitals in Vellore using sterile swabs from different places mainly from different places such as hospital bed surfaces, doors and lift surfaces, nooks and crannies of hospital bed, nurse's desk, kitchen area of the hospital- both washing area as well as serving area, walls of common ward, main door handle, soap near wash basin of the hospital, from the bed and dustbin of operation theatre, procedure room table, chair of the nurse, etc., were inoculated into nutrient broth immediately, and then brought to the laboratory for 24 h of incubation at 37˚C.

### Isolation and culturing of the sample

The samples were cultured on nutrient agar medium by spreading on the agar medium gently and then all the plates were incubated for 24 h. The plates were named serially as $H_1$ to $H_9$ for first and $K_1$ to $K_{14}$ for the second hospital depending on the number of samples collected from different areas. After incubation growth was observed and a serial dilution of all the samples was performed, followed by sub-culturing on nutrient agar medium by spread plate method. After overnight incubation few isolated growths were observed in the Petriplates.

### Sub-culturing and serial dilution

Consecutively for 5-6 days sub-culturing on agar slants and serial dilution were done for all the samples. Differential media such as SS - agar (Salmonella- Shigella agar) for isolation of specifically *Salmonella* and *Shigella* species, and Blood agar plates for classifying and characterizing specific bacterial species, EMB agar media and blood agar media were prepared. The microorganisms were subcultured on MacConkey agar plates to distinguish between lactose fermenters and non fermenters.

### Identification by Gram Staining

Gram staining was performed for morphological identification of all the isolates as primary identification test.

### Biochemical Tests

Biochemical tests were performed for further identification of the isolates based on indole test, MRVP test, triple sugar iron test, catalase, coagulase, oxidase and mannitol motility test.

*Indole test*
Tryptone broth was prepared, inoculated with the test isolate followed by incubation at 37℃ for 24 h. After incubation period is over, 0.5 ml of Kovac's reagent was added to the tube. Pink colored ring formation is an indicative of positive result of indole production.

*MRVP test*
It includes two tests, the methyl red test and the Voges-Proskauer test and is useful in differentiating Enterobacteriaceae and some Streptococcus. Peptone broth was prepared in two tubes and inoculated with the test organism. For MR test, add 5 drops of methyl red reagent into the incubated tube and observe for the red color at the surface of broth without mixing the reagent.

For VP test, 15 drops of Voges-Proskauer A reagent was added and mixed well to aerate the sample followed by addition of 5 drops of Voges-Proskauer B reagent and solution was mixed thoroughly. Development of pink-red color after 10-15 min indicated the positive test.

*Oxidase test*
Oxidase discs (bought from HiMedia) were taken and the isolates from a fresh plate were rubbed onto it. Color change to dark purple within 5-10 sec proves the respective isolate to be oxidase positive.

*Catalase test*
A sterile glass slide was taken and a drop of $H_2O_2$ was placed on it. Further test isolate was taken from a fresh plate using a stick and mixed gently to make a solution. Formation of bubbles in the mix indicates positive result for catalase production.

*Mannitol motility test*
Motility test medium (3 g/l beef extract, 10 g/l pancreatic digest of casein, 5 g/l sodium chloride, 4 g/l agar and 5 ml 1% triphenyltetrazolium chloride solution) was prepared and dispensed in 5 ml aliquots into tubes and autoclaved. The tubes were cooled and stab inoculated the culture upto 1 cm of the bottom of the tube. A positive motility test was indicated by a red turbid area extended away from the line of inoculation.

**Antibiotic susceptibility test**
All the 9 samples were checked for antimicrobial activity against 13 different antibiotic discs; vancomycin, erythromycin, clindamycin, ciprofloxacin, ampicillin, kanamycin, gentamycin, streptomycin, tigecycline, tetracycline, oxacillin, methicillin, and chloramphenicol. The isolates were spread on Mueller- Hinton Agar (MHA) plates using sterile cotton swabs. Then the antibiotic discs were placed with the help of a sterile forceps at equidistant positions and gently pressed on to the media. The plates were left for overnight incubation and the diameter of the zone of inhibition was recorded for further inference. The organisms showing proper zone of inhibition around the discs were considered susceptible and the ones which showed absence of zone of clearance were considered to be resistant.

16S rRNa gene sequence
Further for complete identification of the two isolated showing highest susceptibility and resistance pattern 16S rRNA gene sequencing was done. The obtained gene sequence was searched in BLAST and submitted to NCBI to match with the already present database. The phylogenetic tree was constructed using Mega 5 software and reported in the paper.

## RESULTS AND DISCUSSION

**Sample collection and Isolation**
In total 27 samples were collected including both the health care sectors and processed immediately for the isolation and identification of the pathogens grown on the media surface. Further, slants were prepared for each isolate and stored at 4℃.

**Gram staining**

The results of Gram staining confirmed the shape of the isolates as well as their charge being positive or negative (Table 1). Based on Gram staining and morphological characters 9 different isolates were identified and further characterization was performed accordingly.

**Table 1. Gram staining of the clinical isolates**

| S. No. | Sample Code | Gram Staining | Shape |
|---|---|---|---|
| 1 | H1 (a) | + | Cocci in cluster |
| 2 | H1 (b) | + | Cocci in cluster |
| 3 | H3 (a) | + | Rods in chain |
| 4 | H3 (b) | + | Rods in chain |
| 5 | H4 (a) | - | Minute rods, Scattered |
| 6 | H4 (b) | - | Small rods |
| 7 | H5 (a) | + | Long rods in chain |
| 8 | H5 (b) | - | Small rods, scattered |
| 9 | H9 | + | Minute cocco-bacilli |
| 10 | K1 (a) | + | Rods in chain |
| 11 | K1 (b) | - | Rods in chain |
| 12 | K2 | + | Cocci in clusters |
| 13 | K3 | + | Cocci in clusters |
| 14 | K4 | + | Cocci in clusters |
| 15 | K5 (a) | + | Cocci in clusters |
| 16 | K5 (b) | + | Rods in chain |
| 17 | K6 | + | Scattered rods |
| 18 | K7 (a) | + | Cocci in chain |
| 19 | K7 (b) | + | Rods in clusters |
| 20 | K8 | - | Minute rods |
| 21 | K9 | + | Rods in chain |
| 22 | K10 | - | Rods in chain |
| 23 | K11 | + | Cocci in cluster |
| 24 | K12 | - | Rods in chain |
| 25 | K13 | + | Rods in chain |
| 26 | K14 (a) | + | Minute rods in groups |
| 27 | K14 (b) | - | Minute rods in groups |

**Biochemical characterization**

All the biochemical tests were performed for the 9 clinical isolates identified to be different based on Gram staining. All the biochemicals performed are listed below in table 2.

**Table 2. Biochemical tests of the clinical isolated**

| S. No. | Isolate | Indole test | Methyl red test | Voges-Proskauer test | Catalase test | Coagulase test | Oxidase test | Mannitol motility test |
|---|---|---|---|---|---|---|---|---|
| 1 | H1(a) | - | - | - | + | - | + | Motile |
| 2 | H3 (b) | - | - | - | - | - | + | Motile |
| 3 | H4 (b) | - | - | - | + | - | + | Non motile |
| 4 | H5(b) | + | - | - | + | - | + | Motile |
| 5 | K1 (b) | - | - | - | + | - | - | Motile |
| 6 | K7 (b) | - | - | - | + | - | + | Non motile |
| 7 | K9 | - | - | - | + | - | - | Motile |
| 8 | K13 | - | - | - | + | - | - | Motile |
| 9 | K14 b) | - | - | - | + | - | + | Motile |

From different biochemical tests which were done we can interpret the various groups of microorganisms present in the sample like Gram positive *Staphylococcus* sp., gram negative *Pseudomonas* sp., Gram negative *Serratia* sp., etc. But regarding identification of specific microorganisms more work has to be done.

**Antibiotic Suceptibility Test**

The isolated samples were checked for the antibiotic susceptibility pattern. The antibiotic disc's vancomycin, clindamycin, erythromycin,and chloramphenicol were found to be resistant in few cases, whereas oxacillin and methicllin were found to be resistant in almost all the cases , indicating high degree of resistance towards these antimicrobials. The results indicate that there is increased resistance pattern among micro-organisms to various

antibiotics. Therefore resistance is a matter of serious concern which can jeopardize lives of several individuals. The susceptibility pattern is given in table 3.

**Table 3. Antibiotic sensitivity test**

| S. No | Antibiotic | Isolate (Zone of Inhibition in mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | H1 (a) | H3 (b) | H4 (b) | H5 (b) | K1 (b) | K7 (b) | K9 | K13 | K14 (b) |
| 1 | Ampicillin | - | 10.5 | - | - | 10.25 | - | 9.25 | 10.0 | - |
| 2 | Erythromycin | 10 | 20.25 | - | - | 11.25 | - | 29 | 26.0 | - |
| 3 | Tetracycline | 9.5 | 21.5 | 10.25 | 17.0 | 20.0 | - | 26.5 | 24.0 | - |
| 4 | Streptomycin | 13.0 | 19.25 | 17.0 | - | 18.75 | 16.75 | 21.75 | 18.0 | - |
| 5 | Vancomycin | 12.50 | - | - | 20.25 | 18.0 | - | 14.75 | 14.25 | - |
| 6 | Methicillin | - | - | - | - | - | - | - | - | - |
| 7 | Cloramphenicol | 11.5 | - | - | - | 23.75 | - | 19.5 | 19.5 | - |
| 8 | Ciprofloxacin | 21.5 | 27.25 | 24.75 | - | 24.25 | - | 24.5 | 24.5 | 28.0 |
| 9 | Clindamycin | - | 20.5 | - | - | - | - | 23.25 | 21.5 | - |
| 10 | Oxacillin | - | - | - | - | - | - | - | - | - |
| 11 | Kanamycin | 9.5 | 23.25 | 9.25 | - | 19.25 | 9.0 | 19.5 | 20.0 | - |
| 12 | Tigecycline | 6.75 | 16.75 | - | - | 13.5 | - | 20.25 | 20.0 | - |
| 13 | Gentamycin | 13.75 | 16.25 | 13.75 | 13.5 | 13.5 | 14.5 | 17.25 | 16.5 | 16.75 |

So far we have mainly focused on the antibiotic susceptibility test was conducted in order to understand better which antibiotics are becoming more tolerable microorganisms present in different hospitals. This could be a positive impact on the health of patients by helping them to avoid such drugs and new effective methods could be established for administering drugs into their bodies. More research has to be done in making of drugs and also awareness is required in hospitals and health centers regarding proper administration of specific drugs. The increased resistance towards drugs like vancomycin and methicillin should be an important matter of discussion. Especially vancomycin which has been found to obey "concentration dependent" kinetics is required in large doses to treat infections and this widespread use of vancomycin has lead to an increased VRE prevalence worldwide[1].

**16S rRNA gene sequence**

Two isolates were found to be resistant to multiple antibiotics, hence molecular identification by 16S rRNA gene sequence was done for H5 (b) and K14 (b). As H5 (b) showed highest susceptibility against vancomycin and was found to be resistant to most of the antibiotics except gentamycin, tetracycline and vancomycin, it can be further studied at the gene level to explore the behavior and epidemiology of the organism. Thus the phylogenetic tree of the organism is given in figure 1 which was identified as *Escherichia coli* strain JASR1 with accession number JN966995.

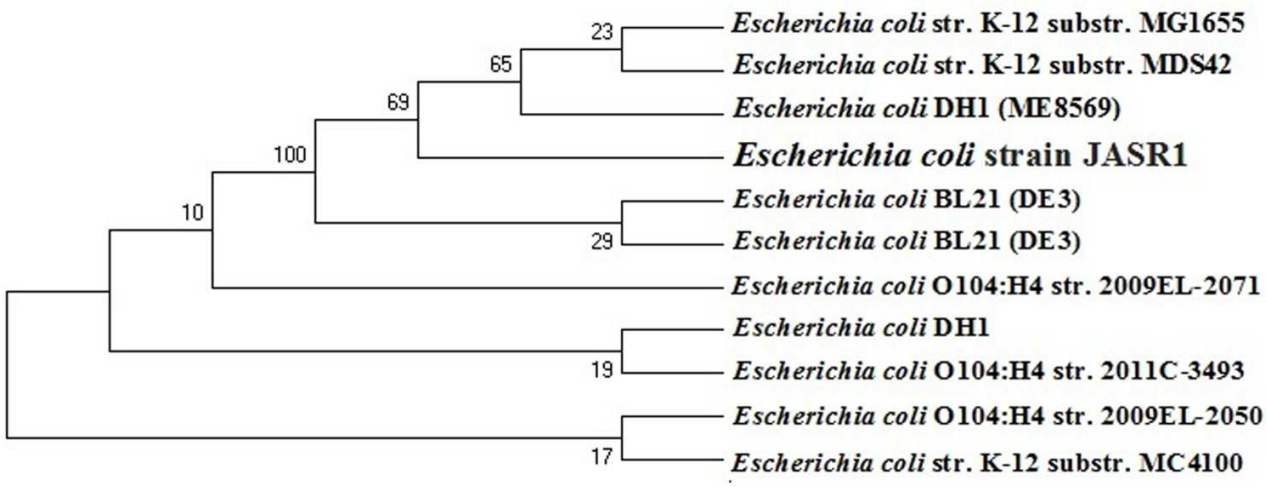


**Figure 1. Phylogenetic tree of *Escherichia coli* strain JASR1**

Similarly, K14 (b) was found to be susceptible to gentamycin and ciprofloxacin only, the gene sequencing was done to identify the organism and the result depicted it to be *Serratia marcescens* JASM1 with accession number being KF528829. The phylogenetic tree was drawn using Mega 5, is given in figure 2.

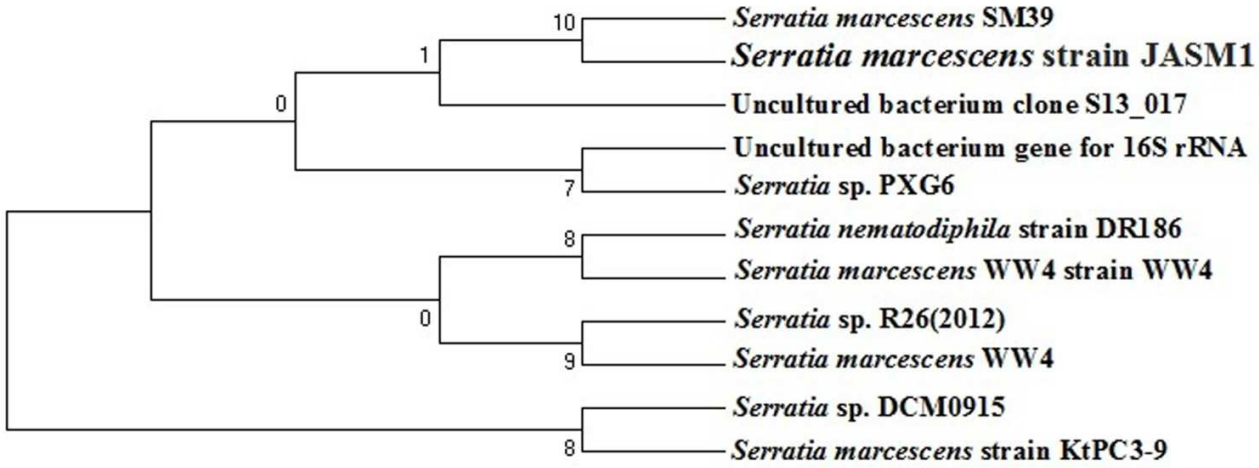


**Figure 2. Phylogenetic tree of *Serratia marcescens* JASM1**

## CONCLUSION

The present work represents a set of data concluding the availability of numerous pathogenic bacteria surrounding us in a public health care sector, leading to a number of hospital acquired diseases. So there is a need of better hospital practices and cleanliness. Moreover, awareness among patients is crucial for successful treatment. These pathogens are the major cause of most of the unnecessary infections in hospitals and they possess great deal of unrealized resistance against many antibiotics used nowadays.

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