# Isolation and Identification of a Klebsiella SPP from a Clinical Samples

**Bhanupriya Khatkar**

Research Scholar, Shri Venkateshwara University, Gajraula

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## INTRODUCTION

Nosocomial infections are an important cause of morbidity and mortality among hospitalized patients. Epidemiologists frequently rely on the laboratory identification and typing of nosocomial pathogens to provide evidence for the biologic and genetic relatedness of these organi,sms as an ai,c1 in the epidemiological investigation (Abe i a/ ,,,.)-7-,,--,-.•.41,7,-. i -r-' 1 981; Bjorvati i al. 1 985; Wachsmuth,1986). In many cases, just the species identification and antimicrobial susceptibility pattern (antibiogram) are sufficient to confirm the epidemiological relationship between different isolates; however, there is an increasing need for more detailed subspecies delineation or typing of nosocomial pathogens. The rationale for such strain delineation is that repeated isolation of an organism with identical markers from one or more patients suggests that the organism may have originated from a single source and therefore is more likely to represent infection or transmission from patient to patient from a common source or by a common mechanism.

In the Indian subcontinent the frequent occurrence of diseases such as malaria, hepatitis, typhoid, gastroenteritis and pneumonia is a major concern. The emergence of multi drug resistance in the causative microorganisms poses serious challenge and various studies have shown that resistance rates range from go-90%. A typical example is the resistance exhibited by Salmonella iyphi to chloramphenicol. Most S,ophi isolates from cases in India are found to have plasmid borne multi-drug roistanqe, to•cbloramphenicol, ampicillin, and trimethoprim/sulfamethoxazole (CiplaDoc.com., 2000). Even though several infectious diseases are prevalent, most studies in India are confined to a small number of bacterial species, such as Salmonella and which are very common pathogens. (Faruque0 ci cde00). In the present study, fifteen clinical isolates obtained from a local hospital were investigated for prevalence of antibiotic resistance.

## METERIALS AND METHODS

**Materials:**

All the chemicals and the media for the identification of bacteria were procured from 1-limedia/Bombay, INDIA. Antibiotics and tine chemicals were purchased from Sigma Chemicals Co.St.Louis2USA.A.

**Methods:**

Isolation of bacteria from hospital environment (from pus, jrinecland sputum):

Clinical samples were obtained from the laboratory of a local hospital, which were submitted for the testing. The samples were diluted in saline and serially diluted. The different dilutions of this were plated on LB agar plates and the plates were incubated overnight at 37°C. Based on their colony morphology, live colonies from each set (5 from pus samples, 5 from urine samples and 5 from sputum sample) were selected and replated on LB agar plates to get isolated pure colonies. These isolates were numbered from VM001 to VMO 15 and subjected to further analysis.

**Culturc Maintenance:**

For continuous cultures the isolated bacteria was maintained with and without antibiotics in LB agar plates while for long term storage aliquots of bacterial cultures were stored at -70°C in LB medium supplemented with 15% glycerol.

Antimicrobial susceptibility testing (Antibiogram): The cultures grown overnight(20p1) were (15 isolates numbered VM001 to VM015) inoculated in to 5m1 LB medium containing different antibiotics: ampicillin (50µg/ml), tetracycline (l 21.tg/m1), spectinomycin (0014m1), streptomycin (30µg/m1), kanamycin (301Ag/m1), gentamycin (30µg/ml) and chloramphenicol (151.tg/m1). The inoculated medium was incubated at

37°C overnight with shaking at 200rpm.

Isolation of plasmid DNA from clinical isolates: The Fifteen isolates (VM001 to VM015) were subjected to plasmid profile analysis. LB medium (5m1) was inoculated with a single colony of different isolates, incubated overnight at 37°C with shaking (250rpm). The cells grown overnight (1.5m1) was taken in an eppendorf tube and spun at 5000rpm to pellet the cells. The cells were resuspended in 1 00vtl of Glucose/ Tris/ EDTA solution and kept on ice. To this 200vil of freshly prepared 1310%--lysing solution (1% SDS and 0.2% NaOl-1) was added and mixed by inverting the tubes gently, and then kept on ice for 10min. 150111 of 5M-Potassium acetate solution was then added, mixed gently and then kept on ice for a further period of 10min. The lysed cells were centrifuged (4°6 at 0,00 The supernatant was then decanted into a clean centrifuge tube and extracted twice with phenol: chloroform Followed by chloroform. The nucleic acid was precipitated by adding two volumes of ethanol, mixing and keeping it at - 70°C for one hr. The DNA was recovered by centrifugation for 10min at 15,00*. The pellet was then washed with 200p1 of 70% ethanol, and after centrifugation, the pellet was dried under vacuum and later resuspended in 201_11TE buffer (10mM Tris-1-ICI, I mM EDTA). The aliquot of this preparation was analyzed on 0.8% agarose

Identification of Clinical isolates (Biochemical Tests):

The following biochemical tests were performed for the identification of the clinical isolates. [The Himedia Manual, 1998. HiMedia Laboratories. Bombay, India; Bergey's manual of determinative bacteriology. 7th Ed.I987; Microbiological methods.4111 Ed. Collins, C.H. Butterworth& Co (1976)].

**1. Oxidase Test (Cytoehrome Oxidase):**

Cultures were incubated on nutrient agar slopes for 24hrs at 37°C. A few tops of freshly prepared I-% aqueous p-aminodimethylanilene oxalate and I% alpha-napthol in ethanol were then added to the slant. The formation of a deep blue color indicated the presence of the enzyme cytochrome oxidase.

**2. Urease:**

The surface of a urea agar slope was inoculated with a pure culture, and incubated at 37°C for 6hrs. Urease produced by the organism hydrolyses the urea to form ammonia, resulting in the changes in the pH of the medium, indicated by the change of color of the medium From yellow to pink red.

**3. Lysine Iron Agar test:**

The lysine iron agar medium alter sterilization slanted in such a way that a short slant and a deep butt were formed. It was then inoculated with a straight needle by stabbing at the base of the butt and streaking on the slant. The slant was incubated at 37°C overnight.

The production of lysine decarboxylase from the bacteria resulted in a purple color Formation.

**4. Triple Sugar Iron Agar test:**

The triple sugar iron agar medium was allowed to set in a sloped form with a butt about one inch long and was inoculated and incubated at 37°C for 24hrs. The utilization of carbohydrates present in the medium produced large amounts of acid, keeping the medium in the acidic pH as indicated by a yellow color in the region of both slant and the butt.

**5. Gelatin Liquification:**

Nutrient broth was inoculated and denatured gelatin charcoal disc was placed in the medium and incubated at 37°C overnight. Gelatin liquilication indicated by the release of charcoal granules that fall to the bottom of the tube.

**6. Arginine decarboxylase:**

The cultures were incubated in arginine broth for 24hrs and a few drops of Nessler's -reagent were added. A brown color indicated the decarboxylating activity towards the amino acids.

**7. Nitrate reduction:**

Nitrate broth was inoculated and incubated overnight at 37°C. A dense suspension of the organism in 0.01M Sodium nitrate in phosphate buffer (pH 7.0) was made and incubated at 37° C for 24hrs. This suspension was acidified with a few drops of I, N hydrochloric acid and 0.5ml each or a 0.2% solution of sulphanilamide and 0.1% N-nal;Ihyl ethylene diamine hydrochloride owas added. The formation of a pink color denoted nitrate reductase activity.

**8. Citrate Utilization:**

Solid Simmon's citrate medium was inoculated with a straight wire and incubated at 35°C overnight. Growth produced an alkaline reaction and changed the color of the medium from green to bright blue.

**9. Oxidative/Fermentative test:**

Two tubes of dextrose medium were heated in boiling water for 1 0min to drive off the oxygen and

after cooling, the medium was inoculated with the bacterium. One tube was incubated aerobically and the other one was incubated anaerobically. The production of acid in the aerobic tube alone is indicative of oxidative growth, while acid formation in both tubes indicated fermentative growth.

**10. Malonate test:**

Malonate broth was inoculated and incubated overnight at 30°C. Growth and a deep blue color indicated malonate utilization. Organisms that simultaneously utilize sodium malonate as a carbon source and ammonium sulfate as a nitrogen source produce an alkalinity due to the formation of sodium hydroxide.

**11. MRVP test:**

MR test: MRVP medium was inoculated and incubated for five days at 30°C, and a few drops of methyl red solution (prepared by dissolving 0.1 gm of the dye in 300 ml. of ethanol and making up to 500m1 with water) were added. A red color indicates that the nil has hen mthimi in 4. Pr ig§4. has been reduced to 4.5 or less.

VP test: This is a test for the formation of acetylmethylcarbinol (acetoin) from dextrose. This acetoin is oxidized by the reagent to diacetyl, which produces a red colour with guanidine residues in the medium. MRVP medium was inoculated and incubated for live days at 30°C. A trace amount of creatine and 5 ml of 40% potassium hydroxide solution were added. A bright pink color appearing in 5min is a positive reaction.

12. Indole test: Strips of filter paper were impregnated with a solution of 5gm of p-dimethylamino benzaldehyde in 50m1 of methanol and 10m1 of orthophosphoric acid and dried. The strip was placed in the neck of the culture tube during incubation. A pink colour indicated indoie formation.

Testing for heavy metal tolerance of the isolated bacteria: Cells were grown in M9minimal medium with glucose as carbon source for a period of 2hrs. This culture was used for inoculating 1 0m1 of fresh M9 medium (0.05A(,00 /m1 initial cell density) containing different concentrations (0. lto 1 0µg/ml) of the heavy metal ions such as, lead, arsenate, bismuth, zinc, copper. After inoculation, growth was observed up to 52hrs.

Studies on herbicide degradation by isolated bacteria: In order to check if any unusual property was exhibited by these isolates, they were tested for their ability to degrade some herbicides. Five different herbicides were selected for the degradation study such as Diuron, l3utachlor, Atrazine, 2-4-D and Chlorimuron ethyl.

These herbicides were first dissolved (10% wfv) in methanol. Required concentrations of the herbicides were then added to the medium from the 1 0% stock preparation. The cells were inoculated in M9 minimal medium devoid of carbon source that containing 0.01 to 0.1% of herbicides and incubated at 37°C up to 52hrs. For control, media with similar concentrations of the herbicides were also incubated without the inoculum. At the end of the growth period the culture medium was extracted with .equal volumes of dichloromethane -and subjected to thin layer chromatography on silica gel using chloroform : acetone ( 9 : 1 ) as the solvent.