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Original Research Article

Isolation Identification and Charecterization of Pencillinase Producing Bacteria from Soil

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Abstract: ***Aim*:** To identify the penicillin resistance organisms in soil sample collected from the hospital area. ***Methods***: Bacteria were grown on nutrient agar medium and sub cultured in nutrient broth with different concentrations of Penicillin. The resistance organisms were characterized by 16s rRNA gene sequencing and blast analysis. ***Results***: A total of 21 cultural were identified as penicillin resistance organisms. Out of 21 cultures we have characterized four cultures and identified as Pseudomonas putida, Pseudomonas fluorescens, Comamonas sp. KBB4, Bacterium SM2-6. ***Conclusion***: Pseudomonas putida, Pseudomonas fluorescens, Comamonas sp. KBB4, Bacterium SM2-6. Four cultures were identified as penicillin resistance organisms. Remaining 17 cultures need to characterize.

Keywords: Penicillin resistance, Soil microorganisms, Hospital environment, Bacterial identification, Nutrient agar.

1. INTRODUCTION

Bacteria are everywhere; in and on our bodies and on everything that we touch. Many species of bacteria cause a wide range of illnesses, with many different symptoms. With the discovery of antibiotics, bacterial infections are not as deadly as they used to be. Penicillin is an antibiotic that has saved many lives since it was discovered. But today it is not as useful as when it was discovered. Penicillin was once used to treat many different bacterial infections, but because of resistance, it is no longer as effective.

Alexander Flemming discovered the antibiotic penicillin in 1928. Flemming was doing research on the bacteria that causes a staph infection. He forgot to clean a petri dish with the staph bacteria on it and left it out. When he returned to his laboratory, he found the dish and noticed that a ring of mold had grown on it. Where the mold had grown, he noticed that the staph bacteria had died. He then began his work on developing the antibiotic penicillin (Huemer and Challem 1997). Initially after this discovery, not much was done with the penicillin. The first person ever to receive penicillin was a man with blood poisoning. He received doses of penicillin and his health began to improve. But at this time, it was hard to culture the penicillin and there was not enough to give the sick man. Because the doses stopped, the man's symptoms worsened and he died.

The next chance to show what penicillin could do was in 1942 when a dance club in Boston caught on fire. Many people were burned and penicillin was used to treat infections in the victims (P. Offit, B. Offit, and Bell 1999). During World War II, penicillin was mass-produced and used to treat soldiers in the war. This was the first time penicillin was used successfully and afterwards became well known and widely used ("The Story of an Antibiotic" 2001).

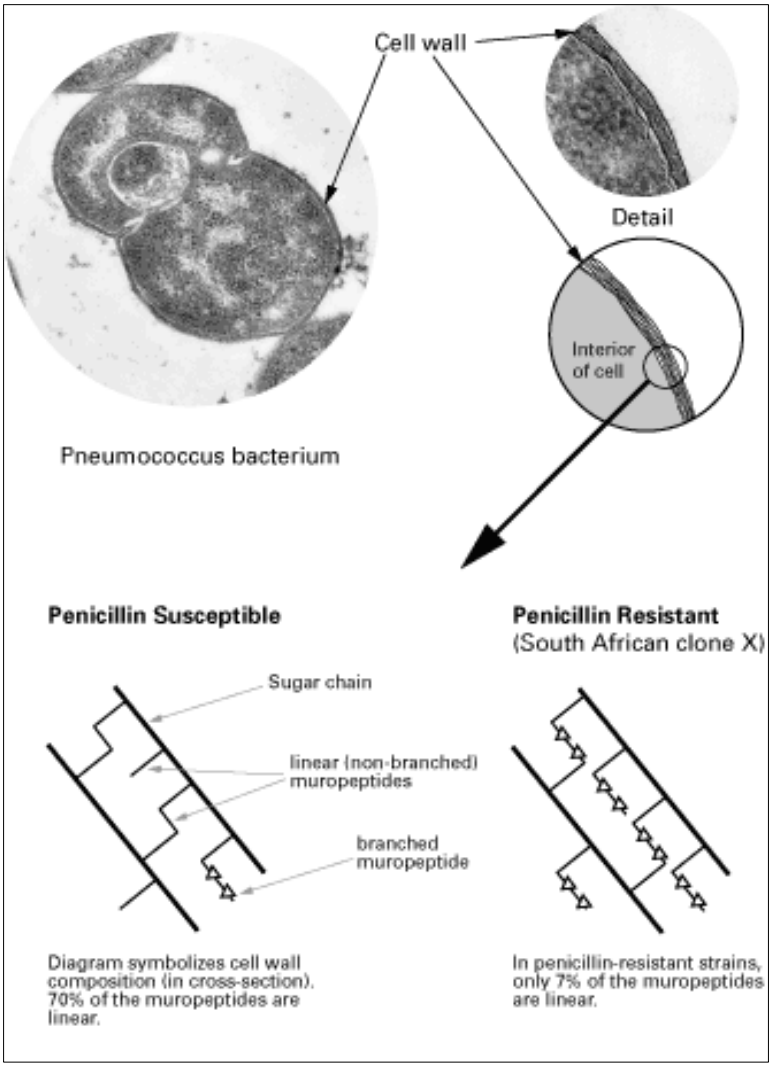
There are now 50 antibiotics that are classified as penicillin (B. Zimmerman and D. Zimmerman 1996). Penicillin antibiotics include phenoxymethylpenicillin,

ampicillin, and amoxicillin (Huemer and Challem 1997), to name a few. These different antibiotics are just modified versions of the original penicillin (P. Offit, B. Offit, and Bell 1999). Penicillin is then split into four generations. These different generations contain many variations of penicillin, but not all of these forms are used today because of the developing resistance (Lane and Read 1999).

Penicillin kills bacteria by causing their cell walls to stop growing, so the cell dies. It does this is by resembling a protein needed for production of the bacterial cell wall. Penicillin is in the shape of a ring, called a beta-lactam ring. Bacterial walls are kept strong and healthy by peptide chains. If the cell is not able to keep repairing and producing the peptide chains, it lyses, or dies. Penicillin prevents these peptide chains from forming, killing the cell (P. Offit, B. Offit, and Bell 1999;"The Story of an Antibiotic" 2001).

To better understand how penicillin works, it helps to know more about bacteria. Bacteria can reproduce in as fast as twenty minutes, which helps explain how resistance has already evolved. Bacteria have a single chromosome, a structure that contains genetic material, and plasmids, which are circular rings of genetic material. If there is an alteration in the genetic material of the plasmid that gives the bacteria an advantage over other bacteria, it will make copies of the plasmid and pass it on to others when it reproduces (Huemer and Challem 1997). Because bacteria reproduce so quickly, a genetic advantage would soon become present in many bacteria.

Penicillin resistance became evident in the 1940's (Huemer and Challem 1997). Bacteria can be resistant to penicillin in different ways. Some can break down the penicillin or they make an enzyme to disguise themselves from it (B. Zimmerman and D. Zimmerman 1996). Staph bacteria developed the ability to cut the beta-lactam ring or the shape of the penicillin. After this resistance was discovered, the beta-lactam ring was altered in 1960 so that the staph bacteria were no longer resistant. This brought about a new form of penicillin called methicillin, but resistance soon started to show and this antibiotic was altered again to create vancomycin (P. Offit, B. Offit, and Bell 1999).



Normal bacterial cells have single muropeptides in their cell walls, which help keep it strong. Because the muropeptides are single, the penicillin could easily bind to the bacteria's cell wall and kill it. But some bacteria acquired branched muropeptides, which prevent the penicillin from easily binding to the cell wall. Another way that bacterium are resistant is by rebuilding their penicillin binding proteins or PBPs. Penicillin targets the PBPs of the bacteria because they are needed to help build the cell wall of the bacteria. Without the PBPs, the bacteria will die. Some bacteria can rebuild the PBPs that are necessary for its survival ("Novel Penicillin-Resistant Gene"). This picture depicts the single and branched muropeptides ("Novel Penicillin-Resistance"). http://www.rockefeller.edu/pubinfo/tomasz042500.nr.htm

An important discovery was made by Sergio Filipe, Ph. D and Alexander Tomasz, Ph. D in 1980. They discovered two genes that code for branched muropeptides; murM and murN. They found that bacteria that are resistant have these genes activated. If the genes are inactivated, the bacteria are no longer resistant. They also found that in order for the penicillin to be resistant it is necessary to have the activated murM and murN genes as well as altered PBPs. This was a very important discovery because it opened up the option of creating a new antibiotic. Since it is now known what genes affect resistance, an antibiotic could be given along with penicillin that would inactivate or shut off the murM and murN genes. This prevents the branched muropeptides from forming and allow the penicillin to bind to the cell wall of the bacteria ("Novel Penicillin-Resistance")

There are many bacteria that are now resistant to penicillin. "From 1989 to 1991 the proportion of resistant isolates rose in the U.S. dramatically from 0.02% to 1.3% ("Penicillin Resistance"). Some of them are *Staphylococcus aureus*, *Streptococcus pneumonia*, and the bacterium that cause syphilis, gonorrhea, and gangrene. So far, no resistance has been found in the strep bacteria. "Ninety percent of *S. aureus* strains are resistant to penicillin-family antibiotics . . ." (Huemer and Challem 1997). *S. pneumoniae* was found to be resistant in 1960 and staph began to show resistance in 1945 (P. Offit, B. Offit, and Bell 1999).

Many different factors brought about penicillin resistance. After penicillin started being mass-produced in the 1950's, it could be purchased without a prescription. This contributed to resistance because any one could go and buy penicillin, regardless if they needed it or not. Someone could take penicillin for an illness it cannot cure. Many people confuse viral infections with bacterial infections because their symptoms can be very similar. Antibiotics are not effective towards viral infections, so if people take penicillin when they have a viral infection, they are helping the bacteria evolve resistance. Once resistance started to show, penicillin was available only with a doctor's prescription (P. Offit, B. Offit, and Bell 39 1999). After penicillin was available only with a prescription, doctors still over-prescribed for it, giving out penicillin more often than needed. Since so much penicillin was used, the few bacteria that developed a resistance were favored and selected for (B. Zimmerman and D. Zimmerman 1996). As quoted in The Guide to Beating Supergerms from *Natural Health* magazine, "By taking antibiotics, you are constantly taking out the weaker bacteria and selecting for the stronger ones." By 1980, no one was researching for new antibiotics, which meant that bacteria just became more and more resistant to penicillin (Huemer and Challem 1997).

Contributing to the overuse of penicillin, in the 1940's penicillin was used to treat gonorrhea. Prostitutes in other countries would receive monthly injections of penicillin to prevent the spread of gonorrhea. Most of the time, they were probably receiving the penicillin when they were not sick and did not need it, so resistance began to develop in gonorrhea bacteria (P. Offit, B. Offit, and Bell 1999).

Meat eaten from livestock that received penicillin also contributes to resistance in humans. Livestock are regularly injected with penicillin to prevent disease and enhance growth. If the cattle have *E.coli*, for example, and then someone eats the infected meat, it may be hard to treat if the person who got sick from it. The *E.coli* from the cattle already may have developed some resistance from the penicillin, so when the human is treated with penicillin after they eat the meat, it will be hard to cure because the bacteria are already resistant (Huemer and Challam 1997).

Many studies have been done in the United States and other countries around the world to see just how resistant some bacteria are. There is a scale that is used to measure resistance: Minimal Inhibitory Concentration, or MIC. "Resistant strains are categorized into intermediate (MIC 0.1-2.0 ug/ml) and highly resistant (MIC greater then 2.0 ug/ml)" ("Penicillin Resistance"). "The annual prevalence of penicillin resistance . . . among *S. pneumoniae* isolates in New York City increased from 7.2% in 1993 to 15.1% in 1995; the percentage with high-level resistance . . . increased from 1.5% to 6.3% in 1995" ("Penicillin-Resistant *Streptococcus pneumoniae*").

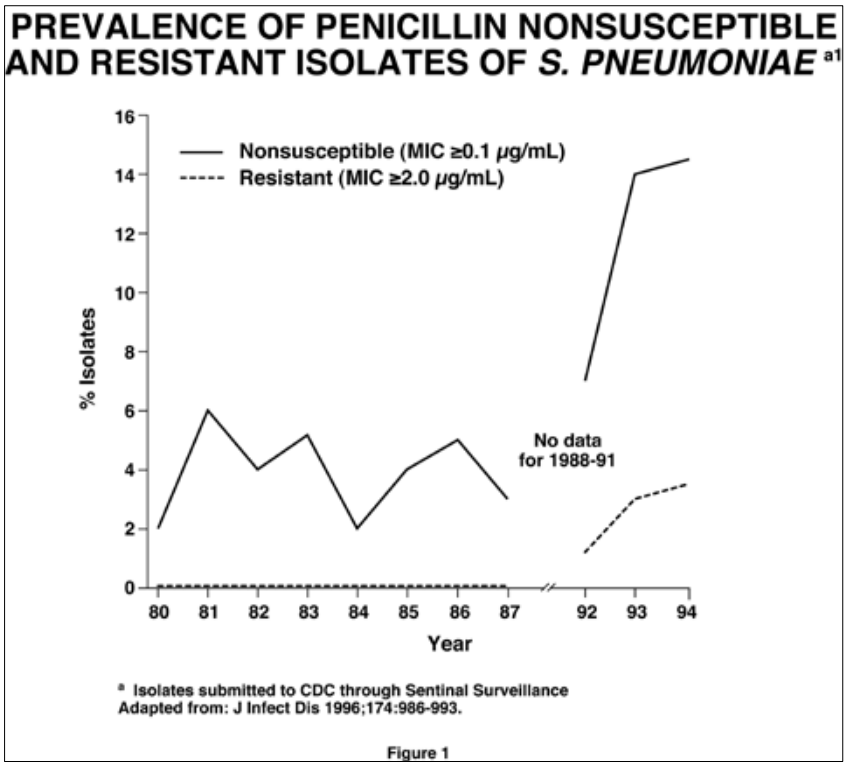


Table 1
Results of tests for penicillin resistance in pneumococci at hospitals participating in IMPACT

Year	Total Isolates	Oxacillin Screening Test		Penicillin MIC µg/mL			
		No. Resistant/No. Tested	%	No. Tested	< 0.10	0.10 - 1.0	≥ 2.0
1991	233	4/219	1.8	78	75	3	0
1992	263	9/252	3.6	93	86	6	1
1993	225	3/218	1.4	72	71	1	0
1994	234	10/233	4.3	101	93	5	3
TOTAL	**955**	**26/922**	**2.8**	**344**	**325**	**15**	**4**

http://www.nfid.org/publications/clinicalupdates/id/pneumococcal.html
http://www.hc-sc.gc.ca/hpb/lcdc/publicat/ccdr/96vol22/dr2219ea.html

The chart above summarizes the data from a study done in Canada at different children's hospitals to test Pneumococcal bacteria for resistance. It showed that 2.8% of 344 people tested had resistance to penicillin ("Invasive Pneumococcal Isolates").

Different groups of people are at higher risk of getting sick from resistant bacteria. Out of all places that show the highest resistance, hospitals are at the top. There are many people that are sick in the hospitals, many different bacteria are being spread, and large amounts of antibiotics being given to patients. The bacteria are spread very easily from patient to patient, and since there are different antibiotics being used, the bacteria develop multi-drug resistance very quickly (Huemer and Challem 1997).

Another group that is at high risk are white upper- and middle-class families, especially children. Upper- and middle-class families are more likely to take their children to the doctor when they are sick. If the children are going to the doctor more often, they are more likely to be prescribed penicillin or another antibiotic when they do not need it, like when they have a viral infection. Also, wealthier children are more likely to be put in daycare. If a sick child is in daycare, they are going to spread the bacteria easily to other children there. As mentioned before, they are also more likely to go to the doctor for medication. So bacteria that are more likely to be resistant because of antibiotic use will be spread between the children in day care (P. Offit, B. Offit, and Bell 1999).

There are different measures that should be taken to help prevent any further resistance from developing or to stop resistance all together. First, doctors should only prescribe medications when they are needed, like for bacterial infections, not viral infections. Second, the use of penicillin should decrease or stop altogether. Gradually bacteria that are not resistant will

be more common then bacteria that are resistant. This would happen because many bacteria have to use more of their energy to make a protein that helps them be resistant. When antibiotics are stopped, the number of people with resistant bacteria in their bodies drops. If there is no penicillin present to kill of the non-resistant bacteria, they will be selected over the resistant bacteria because they are not wasting energy making a protein they do not need. Also, the amount of penicillin given to livestock needs to be decreased (P. Offit, B. Offit, and Bell 1999). There are also some alternatives to penicillin, even though penicillin is still the best treatment for some bacterial infections. Sandalwood oil can be used to treat laryngitis and soar throats and garlic could be used to treat wound and throat infections (B. Zimmerman and D. Zimmerman 53; Heumer and Challem 1996). Those at high risk of aquiring a resistant strain should be vaccinated to prevent the infection ("Pneomococcal Resistance").

Penicillin resistance is not just a problem in one area, it is a problem all over the world. Resistance resulted from unnecessary use and overuse of penicillin. Now, there are many different bacteria that are resistant to penicillin, which makes it an even bigger problem. Everyone is at risk of getting sick from a resistant strain, but especially those in hospitals, white upper and middle class families, and children in daycare. Those at high risk should be vaccinated. Because resistance began to show rather quickly and it's presence is increasing, measures need to be taken now to prevent it from going any further.

2. REVIEW OF LITERATURE

2.1 Antibiotic Resistance in Hospitals

It is puzzling that sewage and water experts who promote the use of reclaimed water and sewage sludge for irrigation and fertilizer pretend ignorance about pathogenic antibiotic resistant E. coli and other members of the Enterobacteriacea family (coliforms and fecal colifroms) that cause over 40% of hospital acquired infections. They claim E. coli (the primary coliform and fecal coliform) does not generally cause disease even though it was one of the first documented killers identified in hospitals.

In 1945, G. Johnson, *et al*., reported on the "ISOLATION OF TRICHOMONAS VAGINALIS WITH PENICILLIN." They said, "Exposure of Trichomonas vaginalis in vaginal discharge for 60 hours to 5,000-10,000 units of penicillin in 10.0 ml of a medium containing cysteine (0.15 per cent.), peptone, liver infusion, maltose and human serum was adequate to destroy the associated bacteria. Seven strains were isolated from seven women without a failure. This technic widens the field of investigation by offering a means of obtaining additional strains of bacteria-free Trichomonas vaginalis for comparative study. Efforts to isolate Trichomonas vaginalis by such laborious methods as washing, micropipetting and migration have all failed to yield bacteria-free cultures at this and other laboratories. The adherence of bacteria to the trichomonads, which appear to have sticky surfaces, and the relatively slow speed with which these protozoa swim are probably in large measure responsible for failure. Further trials by these methods now seem unnecessary. In the cases reported above the success of the penicillin method may be attributed to several factors. Resistance of Trichomonas vaginalis to penicillin in the concentrations reported above, the absence from the specimens of vaginal discharge of bacteria resistant to penicillin in these same concentrations, and the destruction of bacteria adherent to the sticky surfaces of the trichomonads perhaps played a large part in assuring successful isolation of the protozoa." In 1949, C. Phillip Miller and Marjorie Bohnhoff, reported on the "Effect of streptomycin therapy on the bacterial flora of the throat." They said, "Specimens from the throats of patients receiving streptomycin were cultured onto streptomycin media in order to detect the presence of streptomycin-resistant and streptomycin-dependent bacteria. Streptomycin-resistant bacteria in large numbers were cultured from the throats of 98.4 per cent of sixty-one patients who were receiving 1 to 4 Gm. of streptomycin per day. They began to appear during the first thirteen days of treatment in the twenty-four patients who were followed from the beginning of streptomycin therapy. Results of a single survey of another series of patients receiving small doses of streptomycin (0.5 to 0.75 Gm. per day) suggested that resistant flora appeared more slowly. These streptomycin-resistant bacteria all belonged to species normally inhabiting the human throat. Yeast-like forms (Monilia) were found in unusually high incidence. Streptomycin-dependent bacteria were found in two-fifths of the patients receiving large doses of streptomycin, i.e., 1 Gm. or more per day. Streptomycin-resistant bacteria in small numbers were recovered from only 4 per cent of 157 members of the hospital staff, student body and clerical personnel and from 10 per cent of untreated patients. The highest incidence of positive cultures in the control series, 21 per cent, occurred in the nursing and ward personnel. Strongly positive cultures were found in four nurses who were caring for patients receiving streptomycin. Streptomycin-dependent micro-organisms were recovered from the pharynx and large bowel of mice and rabbits after one week of treatment with large doses of streptomycin."

In 1949, P. N. Coleman and S. Taylor, Townleys Hospital at Bolton, Lancashire, reported on "COLIFORM INFECTION OF THE URINARY TRACT." They said, "This paper concerns the investigation of the types of coliform organisms encountered in the urines of one hundred consecutive cases of pyuria admitted to Townleys Hospital, Bolton, during 1948. Recently Warner (1948), discussing urinary infection in paraplegic patients, has drawn attention to the frequency with which Bact. Aerogenes was found. This organism was insensitive both to sulphanilamide and to penicillin, and Warner considered that these drugs

may be of only limited value in urinary infection. It was thought that it would be interesting to discover if a similar high incidence of Bact. aerogenes would be found in other types of urinary infection and to consider all the types of coliform organisms found from the point of view of chemotherapy. -- Of the eighteen strains of Bact. aerogenes tested, six produced gas at 44°C. After 48 hours' incubation though not after 24 hours'. – It was found that in group 1 Bact. coli was the predominant organism (49 strains compared with 12 of other organisms) whereas in group 2 Bact. coli was relatively uncommon (7 strains compared with 56 of other organisms,). Its place was taken in roughly equal proportions by P. vulgaris, P. morgani, and Bact. Aerogenes. – On the other hand, Warner found that the 12 Bact. coli strains tested as well as the Bact. Aerogenes strains were insensitive to 50 mg./100 ml. of sulphathiazole. – In cases of primary urinary infection Bact. coli was the predominant organism; in cases secondary to urinary obstruction Bact. aerogenes, P. vulgaris, and P. morgani predominated. – Because of the high incidence of non-sensitive strains found in cases of urinary infection secondary to obstruction, no benefit from treatment with sulphonamnides or penicillin is likely."

In 1954, Dr. R. Meyer, Jerusalem, reported on a "BONE ABSCESS CAUSED BY BACTERIUM COLI." Meyer found an abscess (pus pocket) in the knee of an 18 year old girl. There was no history of the girl being infected by E. coli. Meyer found a pus sac in the knee joint. Pure E. coli cultures were grown from the pus. The E. coli was resistant to streptomycin and pencillin. However, it was sensitive to cholomycetin. He noted the literature suggested that while abscesses, conjunctivitis and invasion of body tissues had been observed, they were unusual.

In 1967, Susumu Mitsuhashi, *et al.,* reported on "Drug Resistance of Enteric Bacteria IX. Distribution of R Factors in Gram-negative Bacteria from Clinical Sources." They said, "Many isolates belonging to the Enterobacteriaceae were collected in 1965 from the inpatients at geographically scattered hospitals in Japan. Among 2,650 Shigella strains examined, 58.4% were found to be drug-resistant; 95.0% of these resistant strains were multiply resistant. Among 434 resistant strains examined, 81% carried R factors that were transferable by cell-to-cell contact. Of 160 isolates of other enteric bacteria, drug-resistant strains included 84.2% of the Escherichia coli, 93.0% of the Klebsiella, and 90.0% of the Proteus cultures. Among these resistant strains, 70.3% of the E. coli, 66.7% of the Klebsiella, and 52.0% of the Proteus were multiply resistant. Of these resistant strains, 84.0% of the E. coli, 88.0% of the Klebsiella, and 50.0% of the Proteus strains carried R factors. These results indicate that R ors are widespread among gram-negative bacteria of clinical significance."

By 1969, Naomi Datta, Hammersmith Hospital in England, reported on "Drug Resistance and R Factors in the Bowel Bacteria of London Patients before and after Admission to Hospital." Datta said, "The content of drug-resistant coliform bacteria in faecal specimens collected before admission from patients awaiting non-urgent surgery were compared with specimens collected in hospital. Resistant strains of Escherichia coli were isolated from 52% of preadmission specimens and were present in large numbers in 28%. Tetracycline, sulphonamide, and streptomycin resistance were commonest: 60% of resistant strains carried transmissible R factors and multiple resistance was commoner than single. No characteristically resistant intestinal bacteria of any genera were found in hospital specimens as compared with those from outside." She said, "resistant bacteria which are harmless in the bowel may infect the urinary tract or cause other parenteral infections." Eighty-one different resistant E. coli strains as well as 30 strains of resistant Klebsiella species, 11 Proteus species, 4 Enterobacter, 7 Citrobacter, and 5 Pseudomonas aeruginosa.were found in the study. Moreover, she said, "In hospital medicine there is no doubt of the importance of coliform infections of the urinary tract, of surgical wounds, and of the blood stream and outside hospital E. coli in the urinary tract is one of the commonest causes of infective illness."

In 1976, T.D. Fontaine, 3rd, and A. W. Hoadley, reported in the study, "Transferable drug resistance associated with coliforms isolated from hospital and domestic sewage" that "The incidence of antibiotic-resistant fecal coliforms in raw and treated hospital and municipal wastes was investigated to determine whether such wastes may serve as reservoirs for the spread of resistant bacteria and resistance transfer factors. Multiple resistance occurred in 87.8% of isolates from hospital and 42.6% of isolates from municipal wastes. Antibiotic resistance was transferable to Escherichia coli and Salmonella cholerae-suis recipient strains from 62.3% of resistant isolates from hospital and 90.9% of resistant isolates from municipal wastes, and from 56.2% of all isolates from hospital and 45.9% of all isolates from municipal wastes. Numbers of multiply-resistant fecal coliforms decreased during passage through a sewage treatment plant, but their proportion did not change appreciably, although proportions exhibiting resistance to 3, 4, 5, 6, and 7 drugs decreased. A study of transfer in sewage indicated that transfer of resistance from donors present in sewage to pathogenic Salmonella strains can occur under appropriate conditions. The data suggest that both raw and treated wastes, and especially those from hospitals, may serve as reservoirs for the spread of antibiotic-resistant bacteria and transferable resistance in the environment.

In the 1981 study "An R plasmid of broad host-range, coding for resistance to nine antimicrobial agents endemic in Gram-negative nosocomial isolates" at the Hines VA hospital, S. Tantulavanich, *et al*., said, "These

results show that a particular R plasmid has established itself among the Enterobacteriaceae at Hines VA Hospital. This R plasmid appears to be the predominant genetic element responsible for linked resistance to carbenicillin, gentamicin and to bramycin among these hospital-associated bacteria." Furthermore, "Of 3952 clinical isolates of Enterobacteriaceae, 246 exhibited resistance to at least carbenicillin, gentamicin and tobramycin. All these isolates, representing eight genera, were resistant to at least nine antimicrobial agents in common, including the three key antibiotics and streptomycin, kanamycin, sisomycin, ampicillin, cephalothin and sulphonamide. The strains could be subdivided into seven groups depending upon additional resistance traits and some were resistant to as many as 15 antibiotics. When mated with a standard strain of Escherichia coli, 85% of 123 randomly selected donors transferred resistance to at least the nine core antibiotics. Some donors occasionally transferred resistance to two additional antibiotics, neomycin and tetracycline, while one Citrobacter freundi donor always transferred linked resistance to all 11 drugs.

In 1982, Marylse Devaud, *et al*., University of Zurich, reported on "Transposon-Mediated Multiple Antibiotic Resistance in Acinetobacter Strains," an unusually resistant strain of Acinetobacter calcoaceticus subsp. Anitratus, that caused an epidemic of respiratory tract infections in an intensive care unit. They said, "Only recently has this organism been found to be a possible cause of nosocomial infections in compromised hosts. – We suggest that a plasmid resistant to multiple antibiotics was transferred from the hospital flora into Acinetobacter sp. but could not be maintained stably in this host. Instead, a multiply resistant DNA sequence was transposed and stably integrated into the Acinetobacter chromosome. The occurrence of such multiply resistant transposons on conjugative plasmids contributes greatly to the genetic variability of bacteria and may sometimes have serious epidemiological and therapeutic consequences.

In 1983, Gordon L. Archer and C. Glen Mayhall, Medical College of Virginia, Virginia Commonwealth University, reported on the "Comparison of Epidemiological Markers Used in the Investigation of an Outbreak of Methicillin-Resistant Staphylococcus aureus Infections." They said, "The incidence of nosocomial [hospital] infections caused by methicillin-resistant (MR) Staphylococcus aureus strains is increasing dramatically in hospitals in the United States. MR S. aureus isolates have been shown to be fully virulent, causing staphylococcal endocarditis [inflammation heart lining and heart valves] and septicemia [bacteria in the blood] at a frequency similar to that of methicillin-sensitive S. aureus isolates. They said, "An outbreak of nosocomial infections was caused by a single strain of methicillin-resistant (MR) Staphylococcus aureus. This strain was followed as it was transmitted from the index caseto17 patients, 3 hospital personnel, and 12 items in the hospital environment – The epidemic strain was resistant to beta-lactam antibiotics, gentamicin, erythromycin, clindamycin, and rifampin. Resistance to rifampin was the only unique marker in the antibiogram which distinguished the epidemic strain from the indigenous strains – we found that plasmid pattern analysis was a useful epidemiological tool for fingerprinting MR S. aureus strains. It has the following advantages. First, the plasmid pattern is stable. We found that all 32 of the epidemic (rifampin-resistant) MR S.aureus isolates obtained over 7 months had an identical pattern. Furthermore, the pattern remained stable for up to 1 year of storage at -70°C."In 1983, Shunro Kohbata, *et al*., Gifu University School of Medicine at Tsukasa-Machi, reported on "Lactose-Fermenting, Multiple Drug-Resistant Salmonella typhi Strains Isolated from a Patient with Postoperative Typhoid Fever. They said, "Since 1959, frequent incidences of salmonellosis due to lactose-fermenting strains [coliforms] have been reported from the United States, Brazil, Canada, and Japan (1, 9, 14, 24). In these cases, including two outbreaks in Canada and Brazil, the isolates belonged to serogroups B, C1, C2, D, E1, or E4. – Among the lactose-fermenting isolates, strain ST-2 was the only strain of naturally occurring Salmonella typhi hitherto reported in the literature. Strain ST-2 was first reported by Baron *et al.,* (4) in 1959 as a high frequency recombinant strain compatible with many strains of Salmonella, Shigella, and Escherichia species. – Two lactose-fermenting Salmonella typhi strains were isolated from bile and blood specimens of a typhoid fever patient who underwent a cholecystectomy due to cholelithiasis. One lactose-fermenting S. typhi strain was also isolated from a pus specimen which was obtained at the tip of the T-shaped tube withdrawn from the operative wound of the common bile duct of the patient. These three lactose fermenting isolates: GIFU 11924 from bile, GIFU 11926 from pus, and GIFU 11927 from blood, were phenotypically identical to the type strain (GIFU 11801 = ATCC 19430 = NCTC 8385) of S. typhi, except that the three strains fermented lactose and failed to blacken the butt of Kligler iron agar or triple sugar iron agar medium. All three lactose-fermenting strains were resistant to chloramphenicol, ampicillin, sulfomethoxazole, trimethoprim, gentamicin, cephaloridine, and four other antimicrobial agents. The type strain was uniformly susceptible to these 10 drugs. The strain GIFU 11925, a lactose-negative dissociant from strain GIFU 11926, was also susceptible to these drugs, with the sole exception of chloramphenicol (minimal inhibitory concentration, 100,ug/ml).

In 1995, R.R. Reinert, *et al*., Technical University of Aachen, reported on "Recurrent bacteremia due to Brevibacterium casei in an immunocompromised patient." They said, "A case of an immunocompromised patient who experienced two episodes of septicemia caused by a coryneform bacterium is reported. Biochemical characteristics and

analysis of cellular fatty acids and of cell wall components showed two identical strains of Brevibacterium casei to be responsible for these infections. The lack of easy-to-perform methods for identification may have led, in the past, to an underestimation of the role of this bacterium, especially in immunocompromised patients.

In 1998, Piero Galieni and Catia Bigazzi, Università di Siena, reported on the "Recurrent Septicemia in an Immunocompromised Patient Due to Probiotic Strains of Bacillus subtilis." They said, " Bacillus subtilis is a gram-positive, aerobic, spore-forming soil bacterium ubiquitous in the environment. The beneficial effects of B. subtilis spores on the balance of the intestinal microflora is the rationale for its general use as a probiotic preparation in the treatment or prevention of intestinal disorders. B. subtilis spores are available in Italy as a pharmaceutical preparation for oral use. Each dose contains a mixture of 109 spores of four distinct antibiotic-resistant derivatives of ATCC 9799 (Enterogermina; distributed by Sanofi Winthrop, Milan, Italy) (1, 4) per vial. The pathogenic potential of B. subtilis is generally described as low or absent (2). Data on the general importance of infections due to B. subtilis are incomplete, since it is a general practice of most microbiological laboratories to discard these strains or to report them as contaminants. Also, in the cause-of-death statistics of the World Health Organization no data on B. subtilis infections are present since, even if reported, they would be "invisible" at the international comparative level due to the coding used for classification of death causes (2a). In the literature, only a few cases of infections due to B. subtilis are reported (3, 6–8, 10) and only one retrospective study describes the isolation of antibiotic-resistant strains of B. subtilis (6).

In 1998, M.K. Glynn, *et al*., Centers for Disease Control and Prevention, reported on the "Emergence of multidrug-resistant Salmonella enterica serotype typhimurium DT104 infections in the United States." They said, "Strains of salmonella that are resistant to antimicrobial agents have become a worldwide health problem. A distinct strain of Salmonella enterica serotype typhimurium, known as definitive type 104 (DT104), is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline and has become a major cause of illness in humans and animals in Europe, especially the United Kingdom. – we analyzed data collected by local and state health departments and public health laboratories between 1979 and 1996 in national surveys of the antimicrobial-drug resistance of salmonella. Selected typhimurium isolates with the five-drug pattern of resistance were phage typed. – The prevalence of typhimurium isolates with the five-drug pattern of resistance increased from 0.6 percent in 1979-1980 to 34 percent in 1996. In 1994-1995, such isolates were identified in samples from 36 of the 46 surveillance sites (78 percent). Thirty-nine of 43 typhimurium isolates with the five-drug pattern of resistance identified in 1994-1995 and 1996 were phage [virus] type DT104 or a closely related phage type. – Multidrug-resistant typhimurium DT104 has become a widespread pathogen in the United States.

In 1998, Tessy A. Joseph, *et al*., Cook County Children's Hospital at Chicago, reported on the "Neonatal Early-Onset Escherichia coli Disease – The Effect of Intrapartum Ampicillin." They said, "Early-onset E coli infection was diagnosed in 30 of 61498 live births. The overall infection rate (0.49 per 1000 live births) did not change significantly during the 2 time periods (0.37 per 1000 live births during period 1 vs 0.62 per 1000 live births during period 2, P=.21; 2 test); however, there was an increase in the infection rate in neonates weighing between 1501 and 2500 g. Infected neonates had a clinical syndrome that was indistinguishable from early-onset group B streptococcal infection; respiratory distress was the single most frequent finding in 73% (22/30) infected neonates. An increase in the proportion of infections caused by ampicillin-resistant E coli was observed during period 2 (12/18) compared with period 1 (3/12, P=.03; Fisher exact test). During period 2, 61% (11/18) of mothers of infected neonates received intrapartum ampicillin compared with 17% (2/12; P=.02) during period 1. Overall, a higher proportion of neonates born to ampicillin-treated women had ampicillin-resistant infection (12/13 vs 3/17; P<.001). Mothers of 10 of 15 neonates with ampicillin-resistant infection had received more than 2 doses of intrapartum ampicillin. The difference between the prevalence of intrapartum fever in mothers with sensitive organisms (40%, or 6/15) and resistant organisms (93%, or 14/15) was also significant (P=.003). All 6 early-onset E coli– related deaths were due to ampicillin-resistant organisms; 4 of the 6 mothers received intrapartum ampicillin. – We have shown a shift of early-onset E coli infection from a less fulminant disease caused by ampicillin-sensitive organisms to a more fulminant disease caused by ampicillin-resistant organisms. Increased use of maternal intrapartum ampicillin therapy may account for these changes. In the absence of evidence for group B streptococcal disease, clinicians should consider the possibility of ampicillin-resistant E coli infection in critically ill neonates born to women with a history of intrapartum fever and treatment with intrapartum ampicillin.

In 2000, G. Prats, *et al*., Hospital de la Santa Creu i Sant Pau, Universitat Autònoma at Barcelona, reported on "Antibiotic resistance trends in enteropathogenic bacteria isolated in 1985-1987 and 1995-1998 in Barcelona." They said, "Trends in resistance to antimicrobial agents used for therapy have been evaluated with 3,797 enteropathogenic bacteria, Campylobacter, Salmonella, Shigella, and Yersinia, between 1985-1987 and 1995-1998. The greater increase in the rate of resistance was observed in Campylobacter jejuni for quinolones (from 1 to 82%) and tetracycline (from 23 to 72%) and in gastroenteric salmonellae for

ampicillin (from 8 to 44%), chloramphenicol (from 1.7 to 26%), and trimethoprim-sulfamethoxazole and nalidixic acid (from less than 0.5 to 11%). Multidrug resistance was detected in several Salmonella serotypes. In the 1995-1998 period, 76% of Shigella strains were resistant to trimethoprim-sulfamethoxazole, 43% were resistant to ampicillin, and 39% were resistant to chloramphenicol. Seventy-two percent of Yersinia enterocolitica O3 strains were resistant to streptomycin, 45% were resistant to sulfonamides, 28% were resistant to trimethoprim-sulfamethoxazole, and 20% were resistant to chloramphenicol.

In 2002, David M. Livermore, Antibiotic Resistance Monitoring and Reference Laboratory, Central Public Health Laboratory, Colindale, London, reported on the "Multiple Mechanisms of Antimicrobial Resistance in Pseudomonas aeruginosa: Our Worst Nightmare?" He said, Pseudomonas aeruginosa carries multiresistance plasmids less often than does Klebsiella pneumoniae, develops mutational resistance to cephalosporins less readily than Enterobacter species, and has less inherent resistance than Stenotrophomonas maltophilia. – A few isolates of P. aeruginosa are resistant to all reliable antibiotics, and this problem seems likely to grow with the emergence of integrins that carry gene cassettes encoding both carbapenemases and amikacin acetyltransferases. – The original emergence of multidrug resistance in association with plasmids and integrins is less predictable than mutational resistance because it depends on the random escape of genes to mobile DNA. However, once such resistance has emerged, either the host strain can spread among patients or the resistance can disseminate among strains. – Many acquired β-lactamases and aminoglycoside-modifying enzymes have been noted in P. aeruginosa. Some of these are widely prevalent among isolates from southern Europe, Turkey, and Southeast Asia, although they are not widely prevalent in the United Kingdom. – Permeability mutations are widely blamed for increased resistance to β-lactams and luoroquinolones, but, again, much of what was once attributed to impermeability is now understood to reflect up-regulated efflux. – In a hospital in Thessaloniki, Greece, a serotype O:12 strain with a VIM β-lactamase and cross-resistance to aztreonam, aminoglycosides, and ciprofloxacin persisted for 3 years, with µ211 isolates of this strain recovered. In South Korea, VIM-2 producers are widespread in P. aeurginosa, with the enzyme being found in organisms at 9 of 29 hospitals surveyed.

In 2002, Philip S. Stewart, Montana State University at Bozeman, reported on the "Mechanisms of antibiotic resistance in bacterial biofilms." He said, "Bacteria that attach to a surface and grow as a biofilm are protected from killing by antibiotics. Reduced antibiotic susceptibility contributes to the persistence of biofilm infections such as those associated with implanted devices. The protective mechanisms at work in biofilms appear to be distinct from those that are responsible for conventional antibiotic resistance. In biofilms, poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multi-layered defense. The genetic and biochemical details of these biofilm defenses are only now beginning to emerge. Each gene and gene product contributing to this resistance may be a target for the development of new chemotherapeutic agents. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments.

In 2003, Thien-Fah Mah, *et al*., Dartmouth Medical School, Hanover, reported in "A genetic basis for Pseudomonas aeruginosa biofilm antibiotic resistance" that, "Biofilms are surface-attached microbial communities with characteristic architecture and phenotypic and biochemical properties distinct from their free-swimming, planktonic counterparts1. One of the best-known of these biofilm-specific properties is the development of antibiotic resistance that can be up to 1,000-fold greater than planktonic cells2. We report a genetic determinant of this high-level resistance in the Gram-negative opportunistic pathogen, Pseudomonas aeruginosa. We have identified a mutant of P. aeruginosa that, while still capable of forming biofilms with the characteristic P. aeruginosa architecture, does not develop high-level biofilm-specific resistance to three different classes of antibiotics. The locus identified in our screen, ndvB, is required for the synthesis of periplasmic glucans. Our discovery that these periplasmic glucans interact physically with tobramycin suggests that these glucose polymers may prevent antibiotics from reaching their sites of action by sequestering these antimicrobial agents in the periplasm. Our results indicate that biofilms themselves are not simply a diffusion barrier to these antibiotics, but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents.

In 2003, James A. Karlowsky, *et al*., Focus Technologies and Clyde Thornsberry, Merck Research Laboratories, reported on "Trends in Antimicrobial Susceptibilities among Enterobacteriaceae Isolated from Hospitalized Patients in the United States from 1998 to 2001." They said, "Longitudinal surveillance of Enterobacteriaceae [coliform & thermotolerant fecal coliform] for antimicrobial susceptibility is important because species of this family are among the most significant and prevalent human pathogens. – Members of the family Enterobacteriaceae are among the most important bacterial human pathogens. They comprise approximately 80% of gram-negative bacteria and 50% of all isolates identified in hospital laboratories in the United States. Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter spp., and Serratia marcescens account for the majority of Enterobacteriaceae isolated from clinical specimens. –

Antimicrobial resistance is increasing in many species of Enterobacteriaceae as well as in other gram-negative, gram-positive, and anaerobic bacteria. Current antimicrobial resistance issues for Enterobacteriaceae include the emergence and proliferation of extended-spectrum ß-lactamases, ß-lactamase-inhibitor-resistant TEM enzymes, stably derepressed and plasmid-encoded AmpC cephalosporinases, fluoroquinolone resistance, and the dissemination of multidrug-resistant (MDR) strains.

Also, in 2003, Maria Sjölund, *et al*., University Hospital Uppsala, Swedish Institute for Infectious Disease Control and New York University School of Medicine, reported on the "Long-Term Persistence of Resistant Enterococcus Species after Antibiotics To Eradicate Helicobacter pylori." They said, "Antibiotic treatment selects for resistance not o nly in the pathogen to which it is directed but also in the indigenous microflora. – In treated patients, all enterococci isolated immediately after treatment showed high-level clarithromycin resistance due to erm(B). In 3 patients, resistant enterococci persisted for 1 to 3 years after treatment. No resistance developed among controls. – Conclusion: A common H. pylori treatment selects for highly resistant enterococci that can persist for at least 3 years without further selection.

In 2004, Abigail A. Salyers, *et al*., University of Illinois at Urbana, reported on "Human intestinal bacteria as reservoirs for antibiotic resistance genes." They said, Human intestinal bacteria have many roles in human health, most of which are beneficial or neutral for the host. In this review, we explore a more sinister side of intestinal bacteria; their role as traffickers in antibiotic resistance genes. Evidence is accumulating to support the hypothesis that intestinal bacteria not only exchange resistance genes among themselves but might also interact with bacteria that are passing through the colon, causing these bacteria to acquire and transmit antibiotic resistance genes. http://thewatchers. us/Antibioticresistants/NormalFlora-Resistant-transfer.pdf

In 2004, Edwin D. Charlebois, *et al*., Division of Infectious Diseases, San Francisco, reported on the "Origins of Community Strains of Methicillin-Resistant Staphylococcus aureus." They said, "To characterize methicillin-resistant Staphylococcus aureus (MRSA) strains circulating in the community, we identified predictors of isolating community MRSA and genotyped a sample of MRSA collected from a community-based, high-risk population. Computerized databases of the Community Health Network of San Francisco and the Clinical Microbiology Laboratory were searched electronically for the years 1992–1999 to identify community-onset infections caused by MRSA. Sequential analyses were performed to identify predictors of MRSA strains. The majority (58%) of infections were caused by strains traceable to the hospital or to long-term care facilities. Injection drug use was associated with infections that were not associated with health care settings. Genotypes for 20 of 35 MRSA isolates recovered from injection drug users did not match any of >600 genotypes of clinical isolates. In a nonoutbreak setting, the hospital was the main source of community MRSA; however, the presence of genetically distinct and diverse MRSA strains indicates MRSA strains now also originate from the community."

In 2004, C. A. Fux, *et al*., Montana State University at Bozeman, reported on the "Detachment Characteristics and Oxacillin Resistance of Staphyloccocus aureus Biofilm Emboli in an In Vitro Catheter Infection Model." They said, "Catheter-related bloodstream infections due to Staphylococcus aureus are of increasing clinical importance. The pathophysiological steps leading to colonization and infection, however, are still incompletely defined. We observed growth and detachment of S. aureus biofilms in an in vitro catheter-infection model by using time-lapse microscopy. Biofilm emboli were characterized by their size and their susceptibility for oxacillin. Biofilm dispersal was found to be a dynamic process in which clumps of a wide range of diameters detach. Large detached clumps were highly tolerant to oxacillin compared with exponential-phase planktonic cultures. Interestingly, the degree of antibiotic tolerance in stationary-phase planktonic cultures was equal to that in the large clumps. The mechanical disruption of large clumps reduced the minimal bactericidal concentration (MBC) by more than 1,000 times. The MBC for whole biofilm effluent, consisting of particles with an average number of 20 bacteria was 3.5 times higher than the MBC for planktonic cultures. We conclude that the antibiotic resistance of detached biofilm particles depends on the size and could be attributed to nutrient-limited stationary-phase physiology of cells within the clumps. We hypothesize that the detachment of multicellular clumps may explain the high rate of symptomatic metastatic infections seen with S. aureus.

In 2005, Maria Sjölund, *et al*., University Hospital at Uppsala, reported on the "Persistence of Resistant Staphylococcus epidermidis after Single Course of Clarithromycin." They said, "We examined how a common therapy that includes clarithromycin affects normally colonizing Staphylococcus epidermidis. Samples from the nostrils of 5 patients receiving therapy were collected before, immediately after, 1 year after, and 4 years after treatment. From each patient and sample, S. epidermidis strains were isolated and analyzed for clarithromycin susceptibility and presence of the erm(C) gene. We show that macrolide-resistant strains of S. epidermidis were selected during therapy and that the same resistant strain may persist for 4 years, in the absence of further antimicrobial treatment.

In 2005, Carla Novais, *et al*., Universidade do Porto,Portugal, reported on the "Environmental

Contamination with Vancomycin-Resistant Enterococci from Hospital Sewage in Portugal." They said, "Vancomycin-resistant enterococci (VRE) were detected in samples of sewage obtained downstream of hospitals of the Porto area in Portugal, and in samples from the Douro Estuary. Clonal analysis, Tn1546 typing, and presence of putative virulence traits indicate the clinical origin of these isolates. This observation highlights the importance of hospital sewage in the VRE contamination of the environment. – Enterococci have been traditionally considered as indicators of fecal contamination of drinking and recreational waters, although they are usually recovered at high concentrations from natural environments lacking exposure to heavy fecal contamination, such as rivers, seawater, and nonagricultural soils. Release of antibiotic-resistant bacteria to the community is therefore of particular concern since they might proliferate in soil and surface waters, persist and spread in different environments, and transfer antibiotic resistance genes among different species. – Vancomycin-resistant enterococci (VRE) are one of the most worrisome pathogens in hospitals in the United States, and they are starting to increase in European health institutions, Portugal being the area with the currently highest VRE prevalence. – Our data suggest that both particular clones and mobile elements carrying antibiotic resistance or virulence associated to the clinical setting might be continuously contaminating the community environment through wastewater. Reducing the release of bacteria or genetic elements from the clinical setting to the community is becoming a critical issue to avoid the buildup of environmental reservoirs of antibiotic resistance.

In 2007, Z.A. Memish, *et al*., King Fahad National Guard Hospital at Riyadh, reported on the "Emergence and trends of penicillin non-susceptible Streptococcus pneumoniae in Saudi Arabia and Kuwait - perspective and outstanding issues."

They said, "For many years in the past Streptococcus pneumoniae was uniformly susceptible to penicillin until the sudden and unexpected emergence of clinical infections caused by penicillin-resistant S. pneumoniae (PRSP) in 1967. Within the following decade, reports of nosocomial and community outbreaks of infections due to PRSP became widespread all over the world. Recent reports suggest that the incidence of resistance rates is rising in many countries although there are geographical variations in the prevalence and patterns of resistance between countries. The problem of antibiotic resistance is further compounded by the emergence of resistance to many beta-lactam antibiotics. The first report of PRSP in Saudi Arabia was in 1991. Barely a year after, PRSP infection was reported in Kuwait in 1992. Since then, studies from various parts of these countries have recorded prevalence rates ranging from 6.2% in Riyadh to 34% in Jeddah and 20% to 56% in neighboring Kuwait. These suggest considerable variation in the prevalence of PRSP in different cities in the Saudi Kingdom and Kuwait. The mechanism of resistanceis due to chromosomally mediated alteration of penicillin-binding proteins (PBPs), which are target sites for beta-lactam antibiotics. It would appear that the spread of PRSP strains in Saudi Arabia is driven by the selective pressure created by excessive use and misuse of antimicrobial agents made possible by the easy availability of these agents, often frequently obtainable over the counter. In Kuwait, irrational and misguided use of antibiotics may be the major driving force favoring the spread of PRSP. The serotypes of strains encountered in Saudi Arabia and Kuwait are almost identical, with serotypes 19, 6, 15, 14 and 23 being the most common; together they constitute about 70% of the isolates circulating in these countries. In general, almost 90% of the serotypes included in the 23-polyvalent vaccine are present in the general population. However, a much lower percentage of these serotypes is found in the conjugated vaccines, which are more relevant to our communities. This paper reviews the emergence and the steady increase in the prevalence of penicillin-resistant pneumococcal strains in Saudi Arabia and Kuwait during the last 10 years. It discusses the trends, mechanisms of resistance and factors associated with the emergence, dissemination, and colonization of resistant organisms and suggests options available to clinicians for management of infections due to PRSP.

In 2008, Michaela Haas, *et al*., University of Ulm, reported on the "Detection of Resistance to Macrolides in Thermotolerant Campylobacter Species by Fluorescence In Situ Hybridization," They said, "Thermotolerant Campylobacter spp. (C. jejuni, C. coli, C. lari, and C. upsaliensis) [while heat resistant, it is not a fecal coliform] are leading causes of bacterial diarrhea. Even though most infections are self-limiting, antimicrobial therapy decreases the duration and severity of symptoms and is mandatory in the case of severe illness. Because resistance to quinolones is increasing, macrolides are currently the recommend first-line treatment. However, resistance to macrolides is an emerging problem. In human isolates, the rate of resistance is about 5%, but rates vary considerably, reaching up to 80% in animal isolates of C. coli. In 2008, S.G. Jenkins, *et al*., Mount Sinai School of Medicine at New York, reported on the "Trends in antibacterial resistance among Streptococcus pneumoniae isolated in the USA: update from PROTEKT US Years 1-4." They said, the increasing prevalence of resistance to established antibiotics among key bacterial respiratory tract pathogens, such as Streptococcus pneumoniae, is a major healthcare problem in the USA. The PROTEKT US study is a longitudinal surveillance study designed to monitor the susceptibility of key respiratory tract pathogens in the USA to a range of commonly used antimicrobials. Here, we assess the geographic and temporal trends in antibacterial resistance of S. pneumoniae isolates from patients with community-acquired respiratory tract infections collected between

Year 1 (2000-2001) and Year 4 (2003-2004) of PROTEKT US. – Over the first 4 years of PROTEKT US, penicillin and erythromycin resistance among pneumococcal isolates has remained high. Although macrolide resistance rates have stabilized, the prevalence of clonal isolates, with a combined erm(B) and mef(A) genotype together with high-level macrolide and multidrug resistance, is increasing, and their spread may have serious health implications. Telithromycin and levofloxacin both showed potent in vitro activity against S. pneumoniae isolates irrespective of macrolide resistance genotype.

In 2008, Xuan Qin, *et al*., Children's Hospital and Regional Medical Center at Seattle, reported on the "Prevalence and Mechanisms of Broad-Spectrum β-Lactam Resistance in Enterobacteriaceae: a Children's Hospital Experience." They said, "Resistant isolates of the Enterobacteriaceae have recently emerged as a problem in adults, both in hospital settings and in community settings. The Enterobacteriaceae are also major pathogens in neonates, infants, and children, although little is known about the broad-spectrum β-lactamase-producing strains in this specific age group. The spread of β-lactam-resistant Enterobacteriaceae in children is of particular importance, since fluoroquinolones are not approved for use in this age group and are not considered first-line agents for use in this age group. – The objective of this study was to investigate the trends and patterns of resistance in β-lactamase-producing embers of the family Enterobacteriaceae in a children's hospital over a 9-year period (1999 to 2007). Clinically significant isolates of the Enterobacteriaceae were screened for patterns of broad-spectrum resistance to β-lactams. The strains likely to be resistant were subsequently confirmed by an inhibitor-based disc test. The plasmid-mediated resistance determinants in these isolates were identified by PCR and by in vitro transformation, which successfully reproduced the AmpC phenotype unrestricted by the species of the host organisms. Among 8,048 Enterobacteriaceae isolates belonging to the four chromosomal ampC-negative or -nonfunctional genera, 86 (1.07%) isolates (56 Escherichia coli isolates, 22 Klebsiella species isolates, 1 Proteus mirabilis isolate, and 7 Salmonella species isolates) exhibited broad-spectrum β-lactam resistance patterns. These organisms collectively produced three classes of β-lactamases, including class A extended-spectrum β-lactamases (n = 47), class C or AmpC β-lactamases (n = 36, including 4 isolates that produced both class A and class C enzymes), and class A or B carbapenem-hydrolyzing β-lactamases (n = 3). The proportion increased from 0.46% during the first 3 years to 1.84% during the last 3 years (relative risk [RR], 4.04; 95% confidence interval [CI], 2.28 to 7.42; P < 0.001). The increase was mainly due to the emergence of a plasmid-mediated blaCMY-2 β-lactamase, the incidence of which increased from 0.11% during the first 3 years to 0.96% during the last 3 years (RR, 9.11; 95% CI, 2.76 to 30.1; P = 0.001). Class A-type resistance increased slightly during the study period, from 0.35% during the first 3 years to 0.85% during the last 3 years (RR, 2.42; 95% CI, 1.15 to 5.07; P = 0.02). A Proteus mirabilis strain was documented to possess a novel blaDHA determinant. Of special concern, three carbapenemase-producing isolates were identified between 2003 and 2006. The infections caused by resistant isolates of the Enterobacteriaceae mainly affected hospitalized patients with underlying conditions; however, 19 (22%) episodes were of community onset in otherwise well children. The rate of resistance to broad-spectrum β-lactams among isolates of the Enterobacteriaceae is increasing in children in both hospital- and community-acquired settings, and the resistance is driven largely by plasmid-mediated AmpC β-lactamases.

In 2009, Burhan Arikan and Ashabil Ayan, Cukurova University & Kahramanmaras Sutcu Imam University, reported on the "Resistance Variations of Third Generation of Cephalosporins in Some of the Enterobacteriaceae Members in Hospital Sewage." They said, "To estimate the resistance variation to 3rd generation Cephalosporin antibiotics, total 1457 strains of the Enterobacteriaceae family (Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia) were isolated from hospital sewage water. – K. pneumonia showed the highest resistance to all three antibiotics compared to the E. aerogenes and E. coli. – Total 655 bacterial samples were isolated on October, 2006-February, 2007. E. coli was not the predominant strain (38.04%), compared to the E. aerogenes (33.69%) and K. pneumonia (27.02%) in the first week of the October 2006-Fabruary 2007 period. Similar results were obtained in the second and the fifth weeks of study. But in the third week, E. coli was the more predominant strain (69.14%) than E. aerogenes (15.95%) and K. pneumonia (14.89%), while the lowest percentage of E. coli (9.77%) was obtained in the fourth week. Overall percentage of the strains isolated in first period was 35.87% (E. coli), 37.09% (E. aerogenes) and 27.02% (K. pneumonia). The water temperature was recorded as 12°C in first period and 32°C in the second period of the study. – It was found that the rates of the antibiotic resistance in the all strains vary in periods. In the first period, lmost half of the E. coli strains had resistance to Ceftriaxone (CRO), Ceftizoxime (ZOX) and Cefotaxime (CTX), but the resistance were rare in the fifth week (≤ 3%). – The existence of these resistance bacteria in sewage water, no mater where they come from, is a real risk for acquiring such bacteria in the environment. – In conclusion, the results of this study underline the importance of the water temperature for dissemination of the resistance factors between the microorganism and such a screening of antibiotic resistance may be reflecting the consequence of the drug using habits and would help to address the contribution spread of resistant bacteria to the environment.

In 2010, K. Chawla, *et al*., Kasturba Medical College at Manipal, “Reporting Emerging Resistance of Streptococcus pneumoniae from India.” They said, “There are reports of emergence of resistant strains of S. pneumoniae showing resistance to penicillin from all over the world, and now, resistance to multiple drugs (multidrug-resistant strains) has been added to it. However, scanty reports are available so far from India, depicting such resistance. – A cross-sectional study was conducted from June 2008 to December 2008, in our tertiary care center. Fifty pathogenic clinical isolates were collected from patients suffering from lower respiratory tract infections. – Out of 50 isolates, 4% (95% Confidence Interval - 1.4, 9.4) showed total resistance to penicillin, whereas, 10% (95% CI; 1.6, 18.3) showed intermediate resistance. These penicillin-resistant pneumococci (4%) were also found to be multidrug-resistant (MDR) strains. Maximum resistance was observed for cotrimoxazole and tetracycline (24% each with 95% CI; 12.2, 35.8) followed by erythromycin and ciprofloxacin (14% each with 95%CI; 4.4, 23.6). – Increasing emergence of the resistant strains of S. pneumoniae in the community set up requires continuous monitoring and a restricted use of antibiotics to keep a check on its resistance pattern, for an effective treatment plan.In 2010, J.S. JAGAI, *et al*., reported on the EPA Research Project “Trends and Seasonality in Antibiotic Resistance among Elderly Patients with Clostridium Difficile-Associated Disease.” They said, “In the US,over 300,000 cases of Clostridium dijficile-associated disease (CDAD) occur annually in hospitals or long-term care facilities and incidence has risen over the past two decades potentially due to increased antibiotic use. A primary risk factor for CDAD is previous antibiotic exposure therefore, temporal patterns in CDAD hospitalizations may be driven by pathogens for which antibiotics are prescribed. We evaluate differences in patterns between hospitalization rates of C. dijficile and percentage of C. dijficile with antibiotic resistance (AR) by gender, geographic distribution, annual trends, and seasonality. All 1,054,125 hospitalization records for the US elderly for C. difficile were abstracted from the Centers for Medicare and Medicaid Services MedPAR database for a l-l-year period (1991-2004). In the population over 65, hospitalization rates of CDAD increased from 13.71 per 10,000 in 1993 to 45.18 per 10,000 in 2004. Of all cases 19,654 (1.86%) exhibited co-morbid AR. The overall number of AR cases increased and the percentage of cases demonstrated an increasing trend from 0.06% to 2.35% over the study period. As expected, the highest rate of hospitalizations was observed in the 85+ years old individuals (48.2 per 10,000 population vs. 11.9 in 65-74 y.o., and 26.0 in 75-84 y.o.). Counts were organized into weekly time series and the highest rates of CDAD were observed in mid-March (week 10) for all age groups. Cases of CDAD with AR did not show a clear seasonal pattern. These results suggest that C. dijficile and antibiotic resistance is a significant and growing concern in the elderly population and is associated with environmental characteristics that require further research.

In the 2011 CDC Research Article, "Global Distribution and Epidemiologic Associations of Escherichia coli Clonal Group A, 1998–2007", James R. Johnson, *et al*., stated, "Escherichia coli clonal group A (CGA) was first reported in 2001 as an emerging multidrug-resistant xtraintestinalpathogen. Because CGA has considerable implications for public health, we examined the trends of its global distribution, clinical associations, and temporal prevalence for the years 1998–2007. We characterized 2,210 E. coli extraintestinal clinical isolates from 32 centers on 6 continents by CGA status for comparison with trimethoprim/sulfamethoxazole (TMP/SMZ) phenotype, specimen type, inpatient/outpatient source, and adult/child host; we adjusted for clustering by center. CGA prevalence varied greatly by center and continent, was strongly associated with TMP/SMZ resistance but not with other epidemiologic variables, and exhibited no temporal prevalence trend. Our fi ndings indicate that CGA is a prominent, primarily TMP/ SMZ-resistant extraintestinal pathogen concentrated within the Western world, with considerable pathogenic versatility. The stable prevalence of CGA over time suggests full emergence by the late 1990s, followed by variable endemicity worldwide as an antimicrobial drug–resistant public health threat. CGA has been recognized primarily as a cause of community-acquired cystitis and pyelonephritis in adult women mainly in the United States.

The recommended disinfectants, depending on application, are chlorine, chloramines, ozone, chlorine dioxide, metals and Ultraviolet light/radiation. While the vegetable type Enterobacteriacea coliforms are relatively easy to inactivate (not destroy), other organisms are much more hardy. All may develop antibiotic resistance to some degree. Any treatment system injures some bacteria leaving them viable but non culturable as well as antibiotic resistant. It would seem the experts forget bacteria have an autonomous repair system. They do know it takes longer than the 24 hours duration of the tests to repair the damage.

AIM: To identify the penicillin resistance bacteria from soil surrounding to Tertiary care multi-specialty hospital.

Objectives:

1. Culturing of microorganisms from soil sample
2. Identification of Penicillin resistance strains
3. Species level characterization of bacteria to identify the new strains which are penicillin resistance.

3. MATERIALS AND METHODS

3.1 Isolation of Pencillinase Producing Bacteria from Soil Sample

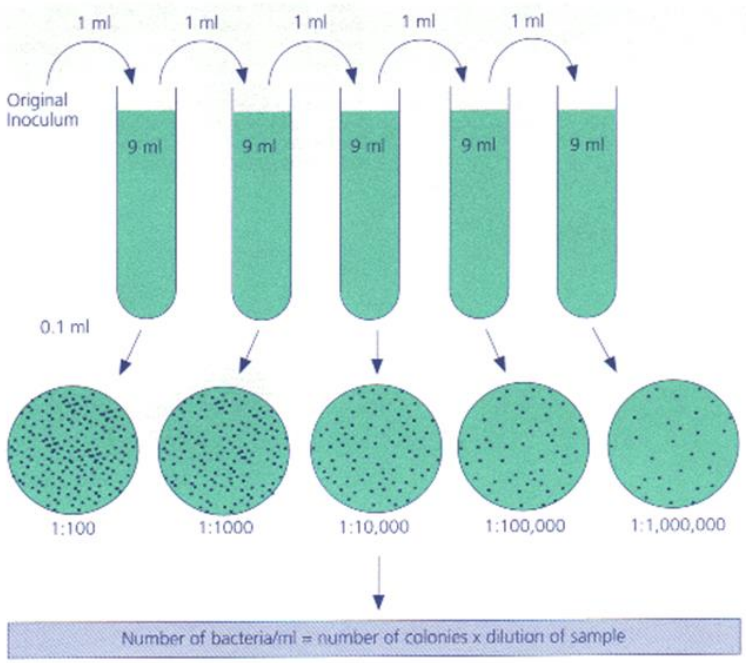
Location of Study: The soil sample is collected from the surroundings of Multi-speciality Hospitals, Hyderabad.

3.2 Sterilization of Materials:

All the glass wares used were washed, dried and sterilized in hot air oven at a temperature of 160°C for 1 h according to the method described by Adibe and Eze (2004). Culture media used were sterilized in an autoclave at a temperature of 121°C for 15 min. The wire loop was also sterilized using spirit lamp.

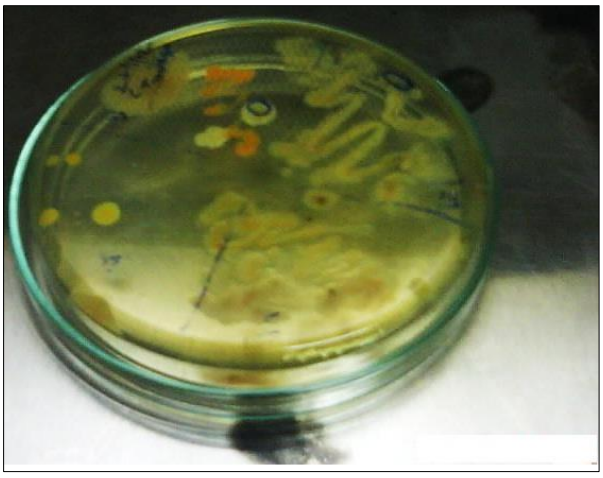
3.3 Preparation of Serial Dilution

- Serial dilution was performed by taking Nine milliliters of sterile water aseptically into five tubes each.
- 1 ml of the original sample was added to the first test tube and mixed thoroughly.
- Another 1 ml was taken from the first tube and added to the second test tube and mixed very well.
- From the second test tube, another 1 ml was taken and introduced into the third test tube and mixed very well. This procedure continued until the fifth test tube.



3.4 Inoculation of Plates

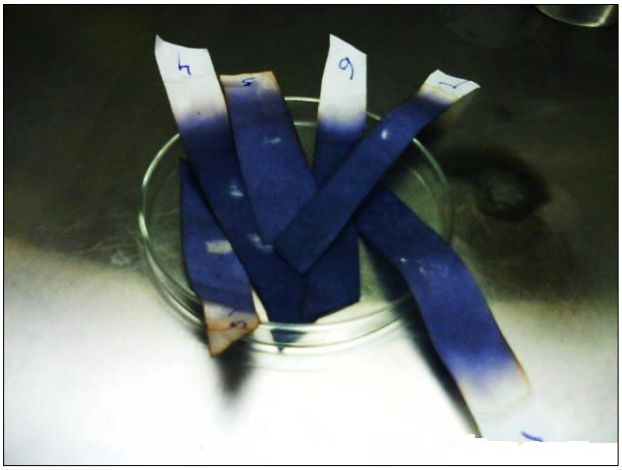
Duplicate plates of nutrient agar were inoculated with 0.1 ml of the diluted solution (10^{-2} to 10^{-5}) using glass spreader technique. All plates were incubated at a temperature of 37°C for 24 hour before colony enumeration and isolation. The temperature was chosen to differentiate the mesophiles which constitute most medically important pathogenic bacteria (Baker and Silverton, 1985). All isolates were sub-cultured by streaking method to get pure cultures.



Well separated deferential colonies were identified and subcultured again on LB agar to obtain pure cultures. These subcultures were again grown on LB broth to perform a Pencillinase assay.

Requirements

- Soluble starch 1% (1gm starch in 100ml distilled water) dissolved in boiling water
- Whatman no1 paper strips
- Grams iodine
- Pseudomonas fluorescens

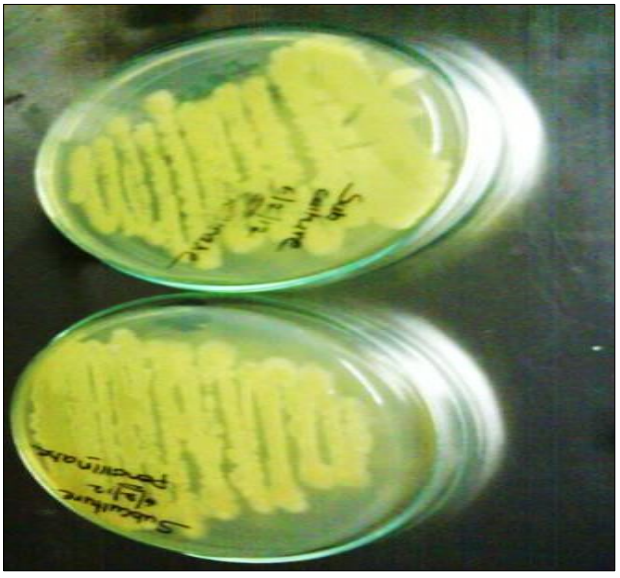


Procedure

- The what man no1 paper strips are dipped in Pseudomonas culture
- After air drying, the strips were dipped in grams iodine followed by starch and allowed to air dry
- A loopful of culture is rubbed to the what Mann no1 filter paper
- If the area becomes colorless then it is conformed as positive result

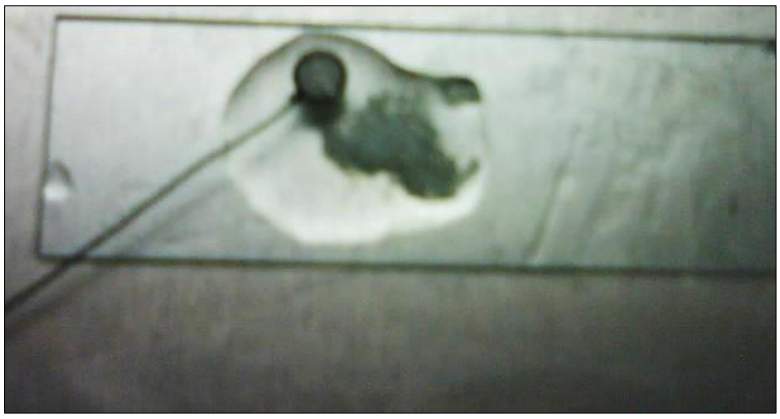
Isolation of High Penicillinase Producing Bacteria

All cultures which have shown positive result were grown on penicillin containing media to isolate high Pseudomonas produing organisms. 10 mg/ml of penicillin G was incorporated into LB medium and all the Pseudomonas positive cultures were inoculated. Of the 5 isolates, 3cultures were positive for Pseudomonas and again out of which only two could grow on the penicillin containing media. Pseudomonas test was re-performed on these isolates.



Biochemical Tests

1. The Catalase Test: Take the clean slide & take loop full of culture in to it & add two or three drops of hydrogen peroxide if it gives bubbles then it is taken as positive.



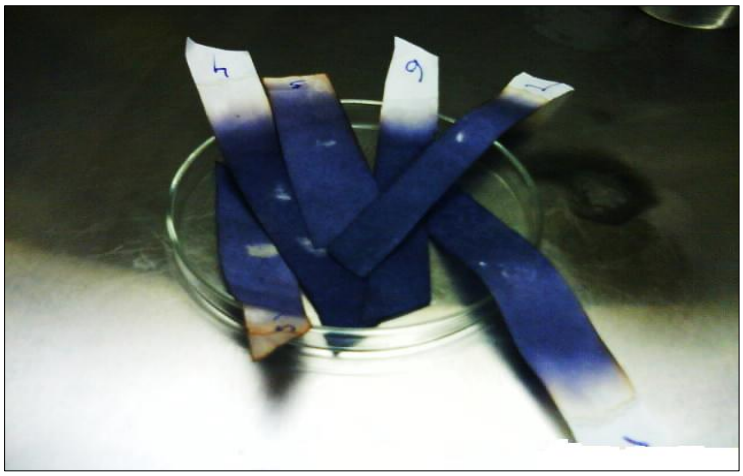
2. Triple Sugar Iron Agar (TSI AGAR)

For the identification of Enterobacteriaceae according to Sulkin and Willett (1940), modified acc. to Hajna (1945).

Use the medium in slanted tubes with good depth and short slant. Inoculate by streaking on surface and stabbing deeply. It is advisable to use tubes with cotton plugs, in order to allow a redoxidation of the indicator. If screw caps are used, they must be loose.

3. Oxidase Production Test

For this test a piece of filter paper was placed in a Petri dish and moistened with freshly prepared cold 1%w\v of tetramethyl-p-phenylenedamine dehydrochloride solution. Subsequently a bacterial colony was smeared over the moistened paper by means of platinum loop. Appearance of dark purple color with in 30 sec indicated positive reaction.



4. Indol Production Test

For this test 1% (w\v) peptone water was inoculated with the bacteria and incubated for 7 days. Then a few drops of kovac's reagent were added to the peptone water. The appearance of pink or red color indicated the positive reaction.

5. Methyl Red (MR) Vages Proskaver (V-P) Test

For MR test the bacteria were inoculated on MR-VP medium and incubated at 37c for 2-5 days. Then 2 drops of methyl red solution was added. Appearance of red color indicated the positive reaction which was confirmed later on by V-P test. After completion of methyl test 0.6 ml of napthol solution and 0.2 ml of 40% KOH aqueous solution was added. Appearance of strong red color after 1 hour indicated the positive reaction.

6. Urease Production Test

For this test Urease medium was inoculated with the bacteria and incubated for about 24 hours, appearance of reddish color indicated the positive reaction.

7. Gelatin Liquifaction Test:

For this test gelatin agar was inoculated with the bacteria and incubated at 30 c for the 3 days. Then the medium was flooded with 5-10 ml acid mercuric solution. Appearance of clear zone indicated the area of gelatin hydrolysis.

8. Simmon's Citrate Agar Test:

Simmons Citrate agar tests show the ability of organisms to utilize citrate as a carbon source.

Simmons Citrate agar contains sodium citrate as the 'C' source and ammonium di hydrogen phosphate as the nitrogen source. A Simmons Citrate agar slants is streaked with the organism and incubated at 37 c for 48 hours.

9. Glucose Fermentation Test:

This test is released with the measurement of acid production from glucose metabolism under aerobic conditions in the basal medium of Hugh and Leifson 1953. The appearance of yellow color after 1, 2 and 7 days incubation was taken as an indication of oxidation reaction. In case the yellow color didn't appear, after 1, 2 and 7 days incubation it as taken as an indication of fermentation reaction.

Preparation of Genomic DNA From Bacteria:

Bacteria from a saturated liquid culture were lysed and the proteins were removed by digestion with the proteinaseK. Cellwall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

MATERIAL

1. TE BUFFER: 1ml of 1M tris (pH 8) + 200micro/lit 0.5 M EDTA in 100ML of deionized water. Incubator or water bath at 50c, with shaker.
2. 10% sodium dodecyl sulfate (SDS) (10g of SDS to make 100ml of solution).
3. 20mg/ml proteinaseK (5micro/lit of 20mg/ml proteinase K per 1ml.
4. 5M Nacl (29.25g to make 100ml of solution).
5. CTAB/Nacl Solution (10% CTAB in 0.7M Nacl): Dissolve 4.1 Nacl in 80ml water and slowly add 10g CTAB while heating and stirring. If necessary, heat to 65c to dissolve. Adjust final volume to 100ml withequal volume of .24:1 ratio of chloroform/isoamyl alcohol.

Isopropanol, 70% ethanol (70ml Ethanol + 30ml deionized water).

Protocol:

Spin 1.5ml of the culture in a microcentrifuge for 2min, or until a compact pellet forms. Discard the supernatant.

Resuspend the pellet in 567micro/lit TE buffer by repeated pipetting. Add 30micro/lit of 10%SDS and 3micro/lit of 20mg/ml proteinaseK to give a final

concentration of 100mg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 1hr at 37c.

Add 100micro/lit of 5M Nacl and mix thoroughly. Add 80micro/lit of CTAB/Nacl solution. Mix thoroughly and incubate 10min at 65c.

Add an approximately equal volume (0.7to0.8) of chloroform/isoamyl alcohol, mix thoroughly, and spin 4to5min in a microcentrifuge.

Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.

Transfer the supernatant to a fresh tube. Add 0.6 volume isopropanol to precipitate nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point it is possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a micropipette that has been heat-sealed and bent in a Bunsen flame. Alternatively, the precipitate can be pelleted by spinning briefly at room temperature.

Wash the DNA with 70% ethanol to remove residual CTAB and respin 5min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer. Redisslove the pellet in 100ml TE buffer.

QUANTIFICATION OF DNA:

Isolated DNA was quantified by measuring absorbance at 260 and at 280nm. Ratio of absorbance 260/280 was used to determine the quality of isolated DNA. The concentration was calculated using the following formula.

Concentration of the DNA = OD260 X 50 X dilution factor = mg of DNA /ml

POLYMERASE CHAIN REACTION (PCR):

COMPONENTS of PCR:

DNA template: That contains the DNA region (target) to be amplified.

Deoxynucleoside Triphosphates (dNTPs):

Also very commonly and erroneously called deoxynucleotide triphosphates Primers- These are complementary to the DNA at 5’ or 3' ends. DNA polymerase, such as Taq polymerase or another DNA only and erroneously called deoxynucleotide triphosphates, thebuilding blocks from which the DNA polymerases synthesizes a new DNA strand.

Buffer Solution: Providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent Cations:

Magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis.

STEPS IN PCR:

Initialisation: This step consists of heating the reaction to a temperature of 94-96°C, which is held for 1-9 minutes.

Denaturation:

The reaction mixture was heated at 94-98°C for 20-30 seconds causes melting of DNA template and primers by disrupting hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing:

Temperature is lowered to 50-65°C for 20-40 seconds for annealing of primers to SS-DNA template. Annealing temperature is about 3-5°C below the Tm of primers used. Stable DNA-DNA hydrogen bonds are only formed when primer sequence closely matches template sequence. Polymerase binds to primer-template hybrid and begins DNA synthesis.

Extension:

Temperature at this step depends on DNA polymerase used; Taq polymerase has the optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used.

Final Elongation:

This single step is performed at 70-74°C temperature for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final Hold:

This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

PCR is commonly carried out in a reaction volume of 20-150 µl in small reaction tubes in a thermal cycler for about 20 t0 40 cycles.

OPTIMISATION OF PCR:

Initially, there was some variation from test to test when the same PCR program was used. Solving this reproducibility problem required adjustments of PCR components.

Amount of Primer:

Initially equimolar primer concentrations of each can be used in multiplex PCR, if there was uneven amplification, with some products barely visible even after the reaction was optimized for cycling conditions, changing the proportions of various primers in the

reaction, with an increase in amount of primers for "weak" amplicons and a decrease in strong"amplicons amount.

MgCl2 Concentration:

A recommended MgCl2 concentration in a standard PCR is 1.5 mM at dNTP concentrations of around 200 mM each. If the amplification was not complete for all the primers varying concentrations of MgCl2 ranging from 1.5mM to 3mM concentrations.

Amount of Template:

Template DNA concentrations for Normal PCR is 10 ng to 50 ng. For a multiplex PCR depending on the intensity of the amplicons template concentrations can vary between 50 ng to 200ng.

Amount of Taq DNA Polymerase:

Different concentrations of Taq DNA polymerase can be used, optimum concentration per reaction is 0.3 mL or 1U/25 mL reaction volume. Too much enzyme, possibly because of high glycerol concentration in stock solution, can result in an unbalanced amplification.

Use of Adjuvants:

Various authors recommend DMSO and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific roducts) of PCR, when used in concentrations varying between 5%–10% (vol/vol).

Polymerase chain reaction was performed using primers that were designed from the specific gene of the Shigella Sps Primers were commercially obtained from Bio-serve India pvt, Ltd. All the primers designed were 20 base pairs in length with GC content in the range of 40-80%. Tm was calculated according to the formula. The annealing temperature for each pair of PCR primers was optimized experimentally.

$Tm(C) = 2(A+T) + 4(G+C) \pm 5$

PCR amplification was carried out with following reaction parameters.

S.NO	COMPONENTS	VOLUME
1	DNTPs	200µM
2	PCR reaction buffer	1X
3	Magnesium chloride	1.5
4	Primer (forward)	2.5 mM
5	Primer (reverse)	10 pmoles
6	Template DNA	100ng
7	Taq DNA polymerase	1U

The final reaction volume was 15µl.

RESOLUTION OF DNA FRAGMENTS ON AGAROSE GELS:

MATERIAL:

Electrophoresis buffer (1X TAE)
Ethidium bromide solution 0.5 mg/ml
Electrophoresis-grade agarose
6 X loading buffer
DNA molecular weight markers
Horizontal gel electrophoresis apparatus
Gel casting platform
Gel combs (slot formers)
DC power supply

GEL PREPARATION:

Prepare an adequate volume of electrophoresis buffer fill the electrophoresis tank and prepare the gel.

Add 0.25g of the Agarose in 25 ml of 1XTAE to prepare 1%gel and dissolve it by heating it at 70°C in a micro-wave, cooled and add 1ml of the Ethidium bromide.

Pour it onto the gel-castner which has comb for wells creation and remove comb after gel solidification and Place the gel into gel loading tank and fill it with the 1 X TAE till the gel submerged into the buffer.

Load the samples mixed with gel loading dye which has bromophenol blue (Front dye) and Xylene cyanol (Tracking dye) and the Reference size standard (to know the size) in the gel and run at 75 volts till the time the bromophenol blue reaches 3/4th of the gel.

Visualize the DNA by placing on a UV light source and can be photographed directly by gel documentation unit.

PURIFICATION OF PCR PRODUCT BY GEL ELUTION:

Spin Column Method:

Principle:

The Ethidium bromide stained fragment of interest is excised from the agarose gel.

The agarose gel slice is dissolved in a chaotropic solution with the addition of heat and then placed into a spin column where the DNA comes into contact with a glass fiber membrane and DNA binds to the membrane. Salts, organics and other contaminating molecules are washed away, and the purified DNA is then eluted with either Elution Buffer or Molecular Biology Grade Water.

Assay Procedure:

1. The separated DNA was cut with a new sterilized blade and then transformed in to a sterile 1.5ml tube.
2. Added 750ul of binding buffer (i.e three times the weight of the gel piece).
3. The eppendorf tube was then kept in a water bath that was preset at 60°C, for about 10 minutes.

4. The entire mix was then slowly transferred to the column placed in 2.0ml collection tube and was spin at 10000rpm for 2 minutes at room temperature.
5. Discarded the eluted solution and then added 750ul of wash buffer to the column and centrifuged at 10000rpm for 2 minutes at room temperature.
6. Centrifugation step was repeated to remove excess isopropanol from the sample.
7. The column was then transferred to a fresh collection tube and added 50ul of autoclaved distilled water. Spin at 10000rpm for 2 minutes at room temperature.
8. Again added 10ul of autoclaved distilled water and centrifuged at 10000rpm.
9. Transferred the eluted product in to clean 1.5ml eppendorf tube and preserved at -20°C.

Dna Sequencing: DNA sequencing was performed with forward primer of the 16s rRNA gene to know the first 600bp sequencing by Sanger's DNA sequencing method performed at EUROFINS sequencing company.

Blast Analysis: The result obtained on sequencing was subjected for blast analysis for the identification of microorganism.

RESULTS

BIOCHEMICAL TEST RESULTS

1) Catalase Test:
Interpretation:

Bubbles	Positive
No Bubbles	Negative

Results: The unknown bacterial culture is Catalase Positive.

2) Triple Sugar Iron Test:
Interpretation:

Results(slant|butt)	symbol	Interpretation
Red|Yellow	K|A	Glucose fermentation only
Yellow|Yellow	A|A	Glucose or Lactose or Sucrose fermentation
Red|Red	K|K	No fermentation
No change|Nochange	NC|NC	No fermentation

3) Oxidase Test:
Interpretation:

Colorless	Oxidase –ve
Blue or Purple Color	Oxidase +ve

Result: The unknown Bacterial Culture is Oxidase Positive.

4) Indole Production Test:
Interpretation:

Red Color	Indole +ve
Yellow Color	Indole –ve
Orange Color	Indole variable

Result: The unknown Bacterial Culture is Indole negative.

5) Methyl Red Test:
Interpretation:

Red Color	MR +ve
Yellow Color	MR –ve
Orange Color	MR variable

Result: The subjected Bacterial Culture is Methyl Red negative.

6) Urease Test:
Interpretation:

Red Color Slant	Urease +ve
Yellow Color Slant	Urease -ve

Result: The unknown Bacterial Culture is Urease negative.

7) Gelatin Hydrolysis Test:
Interpretation:

Liquid	Positive
Semi Solid / Solid	Negative

Result: Medium Liquefied even after refrigeration. So the unknown Bacterial Culture is Positive.

8) Simmon's Citrate Agar Test:
Interpretation:

Blue Color	Citrate +ve
Green Color	Citrate -ve

Result: The subjected unknown Bacterial Culture is Citrate Positive.

9) Glucose fermentation Test:
Result: The subjected unknown Bacterial Culture is Negative to Glucose fermentation.

RESULT OF MOLECULAR ANALYSIS:

DNA Isolation- As we have chosen two bacterial strains for analysis we isolated DNA from those samples the isolated DNA has shown in the picture.

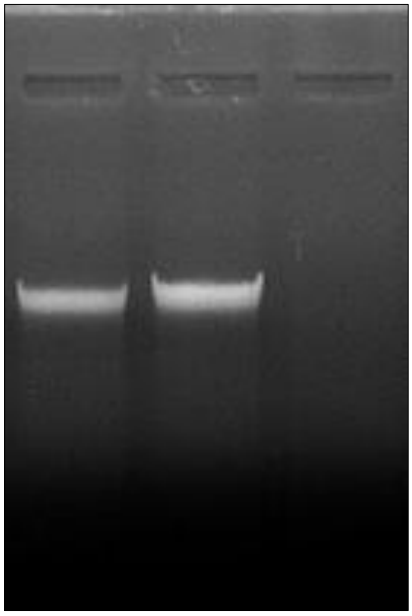


Figure: DNA isolated from the cultures

PCR result – PCR reaction was standardized at the following conditions

Thermocycling Conditions:

Stage 01		**Stage 02**		**Stage 03**		**Stage 04**	
Hold (Initial Denaturation)		Three Temperature cycle (Repeat 40 times)		OneTemperature		Hold	
Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
95°C	2 min	95°C	1min	72°C	5min	4°C	∞
		56°C	1min				
		72°C	1.30min				

Amplified Product Was Shown in the Bellow Figure

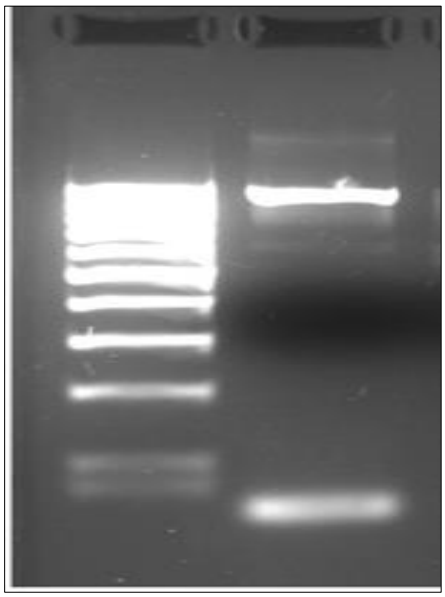


Figure: Lane 1 100 bp ladder, Lane 2 1500 bp PCR amplified product

0213_362_005_P6_RDF-C03.ab1

CGGGGGGGCCGCCTATAACGTCGACGA
TGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTG
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TCTGCCTGGTAGTGGGGGATAACGTTCGGAAA
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GATGAGCCTAGGTCGGATTAGCTAGTTGGTGA
GGTAATGGCTCACCAAGGCGACGATCCGTAAC
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TGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGAAAGCC
TGATCCAGCCATGCCGCGTGTGTGAAGAAGGT
CTTCGGATTGTAAAGCACTTTAAGTTGGGAGGA
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TTACCGACAGAATAAGCACCGGCTAACTCTGTG
CCAGCAGCCGCGGTAATACAGAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCGCG
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AGCAGGTGGAGCATGTGGTTTATTCGAGCACG
CGAGACCTACCAGGCCTGACATCAATGACTTTC
TAAGATAGATGGTGCCTGCGGGACTTGAGACA
GTGCTGCATGGCTGACGATCGCTCCTGTCCTGG
CATGTCGGTAGGTACGGTACGACCGCAACCAT
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CGGCCAGAGAGTGTGTAAGGGCTCTATCGGTC
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AGGTTGCGCCCGCGGAGGAATCTCACCCGAGG
TGCATTACTCATAAGATCATCATCAGACTCACG
CCGAGCGATCGAC

Pseudomonas fluorescens strain 4.9.3 16S ribosomal RNA gene, partial sequence Sequence ID: gi|399137073|gb|JX127246.1|Length: 1445Number of Matches: 1Related Information Range 1: 24 to 1102GenBankGraphics Next Match Previous Match First Match

Alignment statistics for match #1					
Score	**Expect**	**Identities**	**Gaps**	**Strand**	**Frame**
1665 bits (1846)	0.0()	1032/1080(96%)	22/1080(2%)	Plus/Plus	

Features:

Query 27
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Query 1031 GTCGG--TAGGTACGGT-ACGACCGCAA-CCATGTCCCTACTTACCATCA-GTTATGGTG 1085
|| || || || | || |||| ||||| || |||| || ||||| || ||||||||
Sbjct 1043 GTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT GTCCTTAGTTACCAGCACGTTATGGTG 1102

DISCUSSION & CONCLUSION

The microorganism identified after sequencing is Pseudomonas fluorescens. Remaining 17 cultures are yet to sequence. The organisms identified in so far already reported as Pseudomonas sps. To identify any recent Pseudomonas sps we need to complete the sequencing of the remaining samples.

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