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Emergence of extended spectrum beta-lactamase (ESBLs) producing *Proteus* in raw milk of Doon Valley

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Abstract: The present study deals with the determination of extended spectrum of ß-lactamase **(**ESBL) producing *Proteus* in raw milk of Doon Valley. ESBL producing isolates were screened by double disc approximation test using commercially available ß-lactam (Piperacillin) and ß-lactam/ß-lactamase inhibitor combination (Piperacillin/ Tazobactam). All isolates of *Proteus sp.* were reported to resistant against Methicillin and were sensitive to Piperacillin, Cephotaxime, Ceftazidime, and Cefoperazone. This indicates that all the isolates were able to produce ß-lactamase in low and higher amount. This amount of ß-lactamase is inactivated by Tazobactam (ß-lactamase inhibitor) and the zone of inhibition with Piperacillin/Tazobactam combination was greater as compared to Piperacillin alone. There was a significant difference (>4 mm) in zone of inhibition was reported with Piperacillin and in combination of Piperacillin and Tazobactam. Hence, the overall emergence of ß-lactamase producing *Proteus sp.* in raw milk of Dehradun city was 100%, which is an alarming situation for public health and needs serious concern.

Keywords: Enterobacteriaceae, Extended spectrum beta lactamase (ESBL), *Proteus*, Raw milk

INTRODUCTION

Milk is almost sterile from healthy udder but subsequent milking and post milking operation contaminate the milk. Most common food poisoning microbes associated with milk and its products include *Staphylococcus aureus, Escherichia coli, Salmonella species (Typhoid and paratyphoid), Shigella dysenteriae, Proteus species, Vibrio cholera. Proteus* is a gram negative, facultative anaerobic organism with swarming motility and urease activity. *Proteus* is also found in multiple environmental habitats, like food, including milk and milk products and in hospitals. Infection primarily occurs from these reservoirs. *Proteus mirabilis* has been reported for 90% of all '*Proteus*' infections. *Proteus* are responsible for serious infections i.e. wound infections, bacteremia, septicemia, systemic inflammatory response syndrome (SIRS), struvite stones, urethritis, prostatitis, and pneumonias, mostly in hospitalized patients. ESBL refers to ß-lactamase enzymes produced mainly by gram negative bacteria that confer resistance to ß-lactam antibiotics (Sanders *et. al*., 1996) by attacking the amide bond in the ß-lactam ring of Penicillin or Cephalosporin rendering them antibacterially inactive. Widespread use of 3rd generation Cephalosporin and Aztreonam is believed to be the major cause of mutation in these enzymes that has laid to emergence of ESBLs because of their greatly extended substrate range; these enzymes were called Extended Spectrum ß-lactamase (Thomson, 2001). Now days the third generation Cephalosporins is being used to treat the infection of *Proteus* like Ceftriaxone, Cefoperazone, Cephlexin, Ceftazidime. Among the ß-lactam Penicillin, Piperacillin is also used. They are used with ß-lactamase inhibitor like Piperacillin/Tazobactam to suppress the ß-Lactamase activity of bacteria. The production of these ß-lactamase enzymes are either chromosomally mediated or plasmid mediated (Brown and Izundu, 2004).The detection of ESBL production by Enterobacteriaceae is of great importance because horizontal transfer of ESBL producing organisms is a frequent occurrence but can be limited by use of contact isolation and serious infection with ESBL producing organism should not be treated with Cephalosporin. The present study deals with the determination of ESBL producing *Proteus* in raw milk of Doon Valley.

MATERIALS AND METHODS

Bacteria strains and media: One hundred strains of *Proteus* were isolated from raw milk of Doon valley. Raw milk samples were collected from different geographical locations from dairy shops and individual household. Among them, *Proteus vulgaris* 76%, *Proteus mirabilis* 19%, *Proteus morganii* 5%, were reported**.** For their isolation 1 ml of milk sample was enriched in 10 ml of Buffer peptone water aseptically and incubated at 37°C for 24 hrs. Inoculum from the enrichment broth was streaked on Hektoen Enteric Agar (HEA) and MacConkey

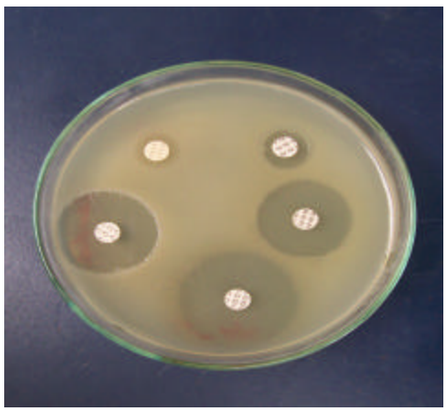

Fig. 1. *Showing Kirby- Bauer disc diffusion test.*

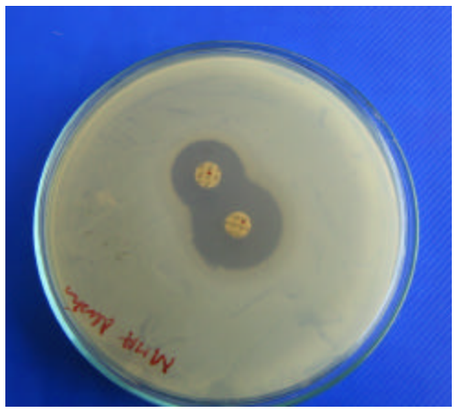

Fig.2. *Showing double disc diffusion.*

Agar and incubated at 37°C for 24hrs. The cultures were identified on the basis of their morphological, and biochemical characteristics. Isolated strains were maintained in nutrient agar slants at 4^0C and subcultred at regular interval of one month.

Antibiogram of Isolates: All the strains isolated in the present study were examined for their resistance pattern by disc diffusion method (Baur *et al.*, 1966) using seven standard antibiotic discs Cefoperazone (75 mcg), Methicillin (5 mcg), Cephotaxime (30 mcg), Ceftazidine (30 mcg), Carbenicillin (100 mcg), Piperacillin (100mcg), Piperacillin/Tazobactam (100 mcg) of HiMedia, India were used. Eighteen hours growth corresponding to 0.5 McFarland was spread on Mueller Hinton Agar (Hi-Media), using a sterile swab and antibiotic discs were placed on it by sterile forceps. After incubation at 37°C for 24 hours the zone of inhibition (Fig.1) for each antibiotic was measured.

Double Disc Diffusion test: All the isolates of *Proteus* were tested for ß-Lactamase production by the Double Disc Diffusion test on the Muller Hilton Agar plates and inoculated with the standard inoculum (Corresponding to 0.5 McFarland tube). Antibiotic discs of Piperacillin and Piperacillin/tazobactam were placed at a distance of 15 mm. The plates were incubated aerobically at 37^0C. Enhancement of zone of inhibition of discs of Piperacillin alone towards the disc containing Piperacillin/tazobactam, showing a figure of eight impression was considered as ESBL producer (Fig. 2).

RESULTS AND DISCUSSION

The double disc approximation test is a simple and most convenient method to detect ESBLs, a phenotypic confirmatory test as recommended by NCCLS is mended to confirm the presence of ESBLs. The test was done using commercially available ß-lactam (Piperacillin) and ß-lactam / ß-lactamase inhibitor combination (Piperacillin/ Tazobactam) against isolates. All 21 isolates of *Proteus sp*. were reported to resistant against Methicillin and were sensitive to 3rd generation antibiotics (Piperacillin, Cephotaxime, Ceftazidime, and Cefoperazone). There was a significant difference in zone of inhibition with Piperacillin alone and in combination with Piperacillin and Tazobactam (Figs. 3 and 4). This indicates that all the isolates were able to produce ß-lactamase in low amount. This amount of ß-lactamase is inactivated by Tazobactam (ß-lactam inhibitor) and the diameter of zone of inhibition with Piperacillin/Tazobactam combination was greater as compared to Piperacillin alone. Hence, the overall emergence of ß-lactamase producing *Proteus sp*. in milk and milk products of Dehradun city is 100%. Mohonty *et al.* (2005) studied comparative *in vitro* activity of ß-lactam/ ß-lactamase inhibitor combination against gram-negative bacteria and concluded 91.37% *Proteus sp*. sensitive to Piperacillin/Tazobactam combination. This study highlights that detection of ESBLs strain of *Proteus* in raw milk and milk products becomes necessary, as strains with susceptible zone *in vitro* may not respond *in vivo* to the antibiotic leading to treatment failure. The rapid and irrepressible increase in resistance of pathogenic bacteria (especially ESBL's against ß-lactam antibiotics) that has been observed over the last two decades is widely considered to be one of the major problems in human infections. The development and spread of ESBL in raw milk is most likely caused by the overuse of antibiotics to treat cattle disease like mastitis (Adesiyun *et al.*, 1995). The data generated from such studies provide physicians in these countries as important scale and scientific community as a whole and understanding of resistance rate on a global scale. In addition, it also helps in formulating effective guideline in therapy and appropriate antibiotics policy for the hospital and prevents further development and spread of resistant strain. Proper infection control practices and barriers are essential to prevent spreading and break of ESBL producing bacteria. The present study showed that all isolates of *Proteus sp*. were found resistant to Methicillin and were sensitive to 3rd

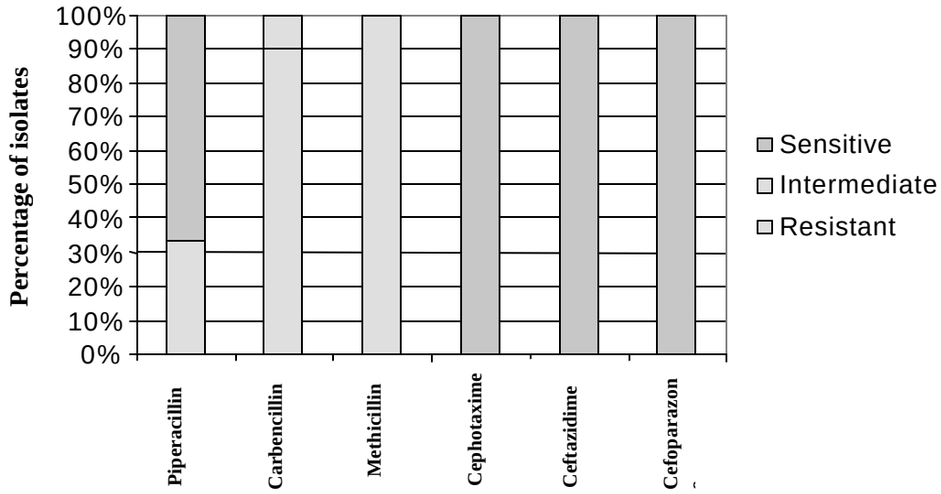

Fig. 3. *Antibiogram of Proteus sp. by Kirby-Bauer disc diffusion method.*

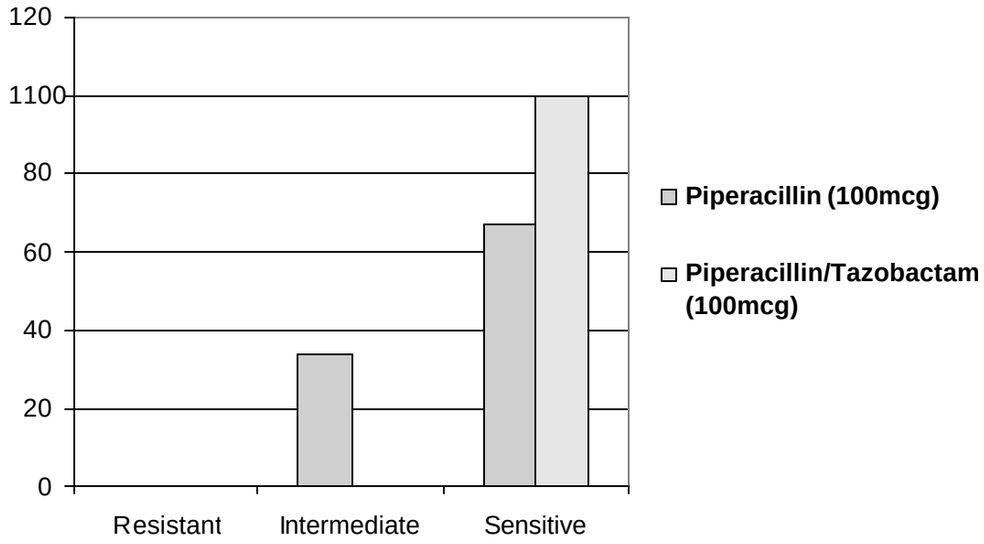

Fig. 4. *Antibiogram of Proteus sp. by using Double disc diffusion method.*

generation antibiotics. The prevalence of ß-lactamases producing *Proteus* sp. showing alarming rise in near future may become serious due to treatment failure.

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