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DETERMINATION OF PREVALENCE AND ANTIBACTERIAL ACTIVITY OF ESBL (EXTENDED SPECTRUM BETA-LACTAMASES) PRODUCING *KLEBSIELLA* SPECIES ISOLATED FROM RAW MILK OF DOON VALLEY IN INDIA

S. CHAUHAN[1], U. FAROOQ[1*], V. SINGH[2] AND AJAY KUMAR[3]

1 Dept. of Microbiology, faculty of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Solan (H.P.) India
2 Dept. of Microbiology, Himachal Institute of Life Sciences, Paonta Sahib (H.P.) India
3 Dept. of Microbiology, Indian Institute of Education, Ghanahatti, Shimla (H.P.) India

ABSTRACT

Milk is a key contributor to improving nutrition and food security. The virtues of milk as a food have long been recognized by human beings. Milk is however a good medium for bacterial growth and an efficient vehicle for bacterial infection when consumed without boiling or pasteurization. The aim of the present study was to evaluvate the presence of ESBL producing *Klebsiella* species present in raw milk of Doon valley in India. The study was carried out from July 2007 to July 2008. A total of 100 samples of raw milk were collected from Doon valley. These samples were cultured and the isolated organisms were identified by standard bacteriological methods. A total of 27 samples were found to be positive for *Klebsiella* species. Further ESBL phenotypic screening was performed. All 27 isolates found ESBL producing. Isolated *Klebsiella spp* showed 96.29% susceptibility to imipenem followed by ciprofloxacin (62.96%), piperacillin/tazobactam combination (51.85%) and ciftazidime (18.51%). This study reveals that *Klebsiella* isolated from raw milk in Doon valley produce ESBL in large proportion. The imipenem antibiotic which was found highly sensitive to ESBL producing *klebsiella* isolates might be a drug of choice.

KEYWORDS: ESBL, antibiotic resistance, *Klebsiella pneumonia,* raw milk.

U. FAROOQ
Dept. of Microbiology, faculty of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Solan (H.P.) India

Corresponding author

INTRODUCTION

Raw milk often contains microorganisms which may cause food borne diseases. Pasteurization of raw milk is a method to increase the safety of milk and milk products by destroying the bacterial pathogens common to raw milk, excluding spore-forming bacteria and possibly *Mycobacterium paratuberculosis.* But most of the people especially in small villages, believe that raw milk is safe to drink and continue to drink raw milk, due to which milk borne diseases are still observed in different parts of the world. Also, due to improper pasteurization or by recontamination, outbreaks of milk born diseases can occur[1,2]. Therefore, raw milk culture, pasteurized milk and natural sour raw milk are usually analyzed for the presence of different species of bacteria such as *E.coli*, *Klebsiella spp*, *Salmonella* species, *Shigella* species etc. In this study we have analyzed raw milk to check the prevalence of *Klebsiella spp*. *Klebsiella* species are important opportunistic nosocomial pathogens causing a variety of infections including pneumonia, bacteremia, thrombophlebitis, cholecystitis diarrhea, upper respiratory tract infection, wound infection, urinary tract infection (UTI) osteomyelitis and meningitis. *K. pneumonia* and *K. oxytoca* are the two members of this genus responsible for most human infections. The clinical isolates of *Klebsiella pneumonia* and *K. oxytoca* were reported to develop an increasing prevalence of the ESBL (extended spectrum beta lactamase) which is pathogenic. Resistance to beta lactams has been reported among members of Enterobacteriaceae family associated with ESBL[3]. Beta lactams belong to the family of antibiotics which is characterized by a beta lactam ring. Penicillins, cephalosporins, clavams and carbapenems are the members of this family. The integrity of beta lactam ring is necessary for the activity which results in activation of a set of transpeptidase that catalyzes the final cross linking reaction of peptidoglycan synthesis. The resistance to beta lactams in clinical isolate is due to the hydrolysis of the antibiotic by beta-lactamase. Thus, it is necessary to identify the prevalence of *Klebsiella* species to check the resistance of beta lactam based antibiotics. In the present study, the prevalence of *Klebsiella* species in raw milk was checked and the resistances of different antibiotics for *Klebsiella* species in context of ESBL production were analyzed in Doon valley in India.

MATERIALS AND METHODS

Bacterial Isolates

A total of 100 samples of raw milk were collected from different diaries situated in Doon valley regions. The sample received were diluted and inoculated on Nutrient agar and MacConkey agar. After 24 hours of aerobic incubation at 37°C, the isolates were identified on the basis of colony morphology and biochemical reactions.

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was determined by Disc Diffusion method of Kirby Bauer on Muller-Hinton agar as described by the Clinical and Laboratory Standard Institute (CLSI)[4]. The antibiotic discs used (Hi-Media) were Ciprofloxacin (Cf) (5 µg), Imipenem (I) (10 µg), Ceftazidime (Ca) (30 µg), Pipracillin/Tazobactam (Pt) (100/10 µg), Pipracillin (Pc) (100 µg) and Ampicillin (A) (10 µg).

Detection of extended spectrum beta lactamases (ESBL)

ESBL production was detected by Double Disc Diffusion Method by placing a Piperacillin disc alone towards the disc containing Piperacillin/ Tazobactum, showing a figure of 8 impressions were considered as ESBL producers. In the phenotypic confirmatory test, cefatazidime and cefotaxime alone and in combination with clavulanic acid was performed for the production of ESBL. The ceftazidime (30 µg)

and ceftazidime-clavulanic acid (30 µg + 10 µg) discs were placed on the agar. Similarly Cefotaxime (30 µg) and cefotaxime-clavulanic acid (30µg + 10 µg) discs were also placed. After incubating overnight at 37^{o}C, a difference of ≥ 5 mm between the zone of inhibition of a single disc and in combination with clavulanic acid was interpreted as positive for ESBL production. *Escherichia coli* ATCC 25922 and *K. pneumonia* ATCC 700603 were used as control strains.

RESULTS

The prevalence of *Klebsiella spp* and antibiotic activity against *Klebsiella* has been studied in raw milk. A total of 27 samples out of 100 samples were found to be positive for *Klebsiella* species. Finally *Klebsiella* was identified on the basis of motility and biochemical tests (Table no.1).

Table . 1

Showing the characteristics of Klebsiella spp

Test	Characteristics of *Klebsiella* spp.
Gram reaction	Gram negative rods
Motility	–
Indole	–
Methyl red	–
Voges Proskauer	+
Citrate	+
TSI test	A/A, with Gas production

Symbols: (+) = Positive, (-) = Negative, A/A = Acidic/Acidic

All these isolates were then examined for antibiotic sensitivity/resistance on Muller Hilton agar by using Kirby-Baur disc diffusion method against the beta-lactamase producing bacteria (Table No.2).

Table 2

Showing the zone of inhibition of Beta-lactam antibiotics against Klebsiella spp

S.No.	Isolate No.	Beta - Lactam Antibiotics (Diameter Zone of Inhibition in mm)					
		A	Pc	Pt	Cf	Ca	I
1	K2	10(R)	15(R)	24(S)	27(S)	19(S)	31(S)
2	K3	13(R)	14(R)	21(S)	15(R)	13(R)	15(I)
3	K7	11(R)	17(R)	19(I)	13(R)	12(R)	23(S)
4	K9	10(R)	16(R)	24(S)	29(S)	11(R)	25(S)
5	K13	10(R)	12(R)	21(S)	31(S)	14(R)	29(S)
6	K14	12(R)	16(R)	26(S)	30(S)	12(R)	35(S)
7	K25	11(R)	13(R)	23(S)	15(R)	13(R)	27(S)
8	K28	13(R)	11(R)	17(R)	14(R)	10(R)	28(S)
9	K29	10(R)	08(R)	15(R)	12(R)	14(R)	23(S)
10	K31	10(R)	14(R)	27(S)	22(S)	12(R)	24(S)

11	K32	09(R)	11(R)	22(S)	23(S)	11(R)	32(S)
12	K35	11(R)	15(R)	28(S)	14(R)	11(R)	26(S)
13	K36	11(R)	12(R)	23(S)	13(R)	12(R)	23(S)
14	K38	13(R)	16(R)	24(S)	14(R)	14(R)	23(S)
15	K40	12(R)	12(R)	16(R)	12(R)	19(S)	33(S)
16	K42	10(R)	15(R)	31(S)	26(S)	13(R)	28(S)
17	K49	09(R)	09(R)	17(R)	22(S)	14(R)	30(S)
18	K57	11(R)	17(R)	29(S)	21(S)	11(R)	26(S)
19	K61	10(R)	14(R)	27(S)	22(S)	14(R)	33(S)
20	K62	10(R)	16(R)	23(S)	26(S)	12(R)	35(S)
21	K65	12(R)	09(R)	16(R)	14(R)	11(R)	30(S)
22	K75	11(R)	11(R)	17(R)	24(S)	18(S)	38(S)
23	K79	10(R)	15(R)	28(S)	23(S)	14(R)	33(S)
24	K86	10(R)	12(R)	17(R)	21(S)	12(R)	32(S)
25	K88	10(R)	14(R)	17(R)	26(S)	13(R)	30(S)
26	K90	12(R)	08(R)	16(R)	21(S)	18(S)	34(S)
27	K98	12(R)	11(R)	15(R)	23(S)	12(R)	32(S)

Symbols: (S) = Susceptible, (R) = Resistant, (I) = Intermediate.

We observed that the most effective antibiotics were imipenem having 96.29% susceptibility, followed by ciprofloxacin having 62.96% and ceftazidime having 18.51% susceptibility. Further, piperacillin+tazobactam in combination showing 51.85 % susceptibility while piperacilin alone found 100% resistant against ESBL producing *Klebsiella spp*. All the 27 isolates were resistant to ampicillin (100%) along with piperacillin. These were followed by ciftazidime having 81.48%, piperacillin+tazobactam having 59.26% and ciprofloxacin having 37.04 % resistance to *Klebsiella* isolates (Fig. A-C).

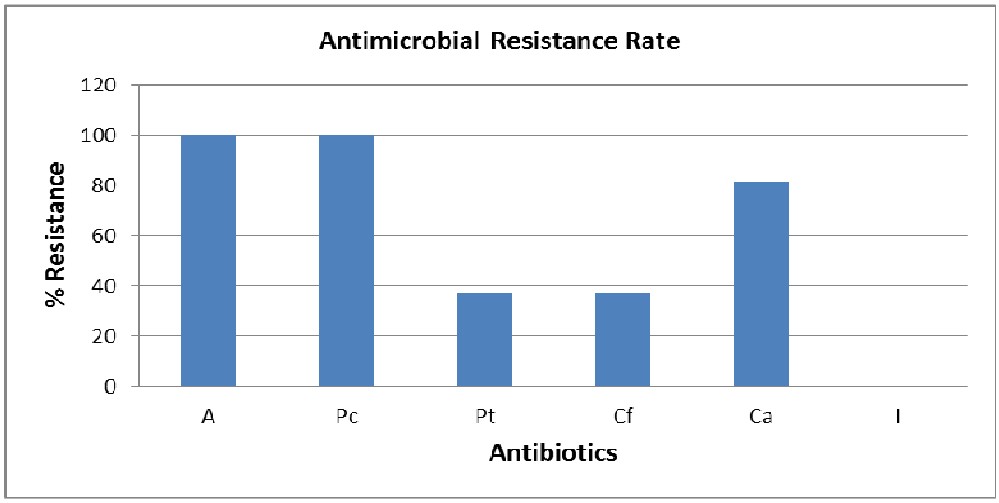

Figure A.
Bar diagram depicts antimicrobial resistant rates among the isolated Klebsiella species

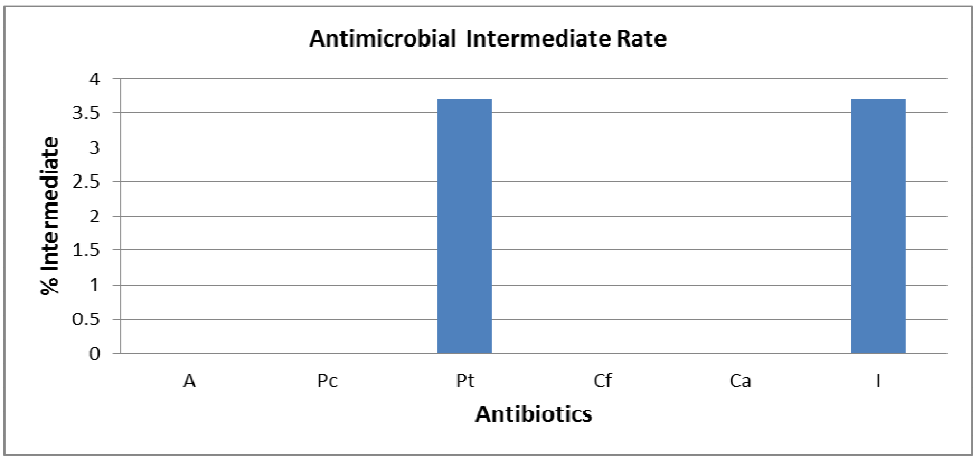

Figure B

Bar diagram depicts antimicrobial intermediate rate among the isolated Klebsiella species.

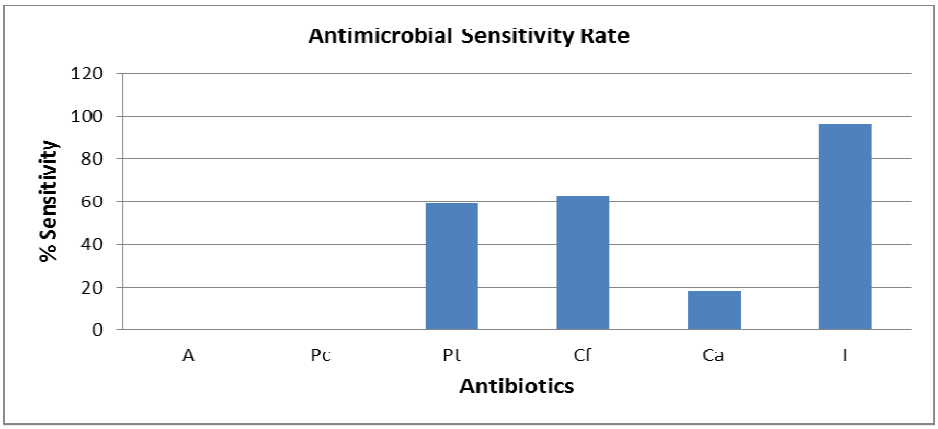

Figure C

Bar diagram depicts antimicrobial sensitivity rate among the isolated Klebsiella species

Further ESBL phenotypic screening by double disc diffusion method test was performed by using commercially available beta lactam (Piperacillin) and beta lactam/beta lactamase inhibitor combination (piperacillin+Tazobactam), against isolates. The zone showing a figure of eight impression indicated the presence of ESBL. Further ESBL confirmation has been done by cefatazidime and cefotaxime alone and in combination with clavulanic acid. A difference of ≥ 5 mm was observed confirming the presence of ESBL in all 27 isolates of *Klebsiella*. This indicates that all the isolates were able to produce beta lacamase in high amount.

DISCUSSIONS

Klebsiella is potent pathogen caused infection to the human beings and inhabited in the intestine of man. It is transmiiteed through contaminated food and water. The presence of *Klebsiella* has been reported from raw milk[5,6]. In this study we have planned to detect *Klebsiella* from raw milk collected from different diaries situated in Doon valley. We have observed that the *Klebsiella* species was frequently present in raw milk among all the enteropathogens and is responsible for milk borne infection in human beings, if consumed without proper boiling or pasteurization[1]. The

emergence of resistance to antimicrobial agents is a global public health problem, particularly in pathogens causing nosocomial infections. Antimicrobial resistance results high degree of morbidity and mortality. Production of Extended spectrum beta-lactamase (ESBL) is an important mechanism in Gram negative bacilli that confer resistance against broad-spectrum β-lactam antibiotics, normally having activity against Gram negative bacilli. Over the past 2 decades, ESBL producing gram-negative bacilli have emerged as a major problem worldwide[7].

The incidence of ESBL-producing isolates varies according to countries, regions or even hospitals. Despite of numerous report of nosocomial outbreaks, the prevalence of ESBL producing bacteria in most hospitals remain unknown. Important ESBL producing Gram negative bacilli include *Klebsiella pneumoniae*, *E.coli*, *Proteus mirabilis*, *Enterobacter sp., Citrobacter freundii, Pseudomonas aeruginosa, Acinetobacter* and *Stenotrophomonas maltophila. Klebsiella pneumoniae* and *E.coli* were found to be most frequently associated with ESBL-production in the study[8]. In India, a study from Coimbatore, 41% isolates of *Klebsiella pneumonia* and 40% of *E. coli* were reported as ESBL producers[9]. In a study carried out in All India Institute of Medical Sciences, New Delhi, 678 isolates were tested, of which 68% were ESBL producers among all Gram negative bacilli[10]. Singhal et al[11] reported 73% isolates of *Klebsiella pneumonia* and 62% of *E. coli* were ESBL producers[11]. In the present study, the prevalence of ESBL producing *Klebsiella* species was found to be 27% in raw milk. Most of the isolates were obtained from raw milk have shown antibiotic resistance.

Carbapenems are the drugs of choice for many infections caused by gram positive and gram negative bacteria. Carbapenem was found to be the most effective antibiotic, as 96.29 % isolates were susceptible to Imipenem derivative of Carbapenems, which is in accordance with the study carried out by Al-Zahrani and Akhtar from Saudi Arabia[12]. Penicillin is an important bactericidal which inhibits the synthesis of bacterial cell wall. *Klebsiella* has developed 100% resistance against penicillin. In the present study, the drug sensitivity assay was also performed for Penicillin, 100% isolates of *Klebsiella* were observed resistant to the both derivatives of Penicillin *i.e.* Piperacillin and Ampicillin respectively. The present study is in accordance with previous studies reported from Orrett in 2005[13]. However, increase sensitivity was shown by piperacillin+tazobactam (59.26 % susceptibility) as has been reported earlier[14]. In the present study, 81.48% isolates of *Klebsiella* were found resistant to the ciftazidime.We have observed that the carbapenem is the drug of choice for the ESBL producing organisms. Penicillin has also shows good susceptibility with beta-lactam-beta-lactamase inhibitor combination (piperacillin+tazobactam). However, the situation may vary from region to region; therefore local patterns of susceptibility should be used to determine the drug of choice. Also from our study it reveals that milk could be a source of ESBL producing *Klebsiella* species, which could cause serious infection to human being if consumed without proper boiling or pasteurization.

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