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INCIDENCE OF *KLEBSIELLA PNEUMONIAE* SUBSP *PNEUMONIAE* IN FOOD AND ENVIRONMENTAL SAMPLES ISOLATED FROM MYSORE CITY, INDIA AND ITS ANTIBIOGRAM

LITTY BABU, HARISHCHANDRA SHRIPATHY MURALI AND HARSH VARDHAN BATRA*

Defence Food Research Laboratory, Mysore, Karnataka, India

ABSTRACT

Foodborne illnesses are of great concern and serious global threat. There are both human and economical losses worldwide due to consumption of contaminated food and water. *K. pneumoniae*, a Gram-negative rod shaped bacteria belonging to the family Enterobacteriaceae is responsible for foodborne infections all over the world. Therefore, the aim of our study is to evaluate the incidence of *Klebsiella pneumoniae* from food and environmental samples in Mysore region. A total of 82 samples were screened for the presence of possible contamination by *K. pneumoniae* subsp *pneumoniae*. Among these twenty three (28%) strains were confirmed as *K. pneumoniae* subsp *pneumoniae* by MALDI-TOF. The present study revealed a high percentage (53%) of incidence of *Klebsiella* species from the samples. All the *K. pneumoniae* subsp *pneumoniae* were susceptible to all the antibiotics (26) excluding few strains. However, all the tested strains are resistant to ampicillin and all these strains were capable of forming biofilms.

KEYWORDS: Foodborne pathogen, *Klebsiella pneumoniae,* MALDI-TOF, PCR

HARSH VARDHAN BATRA
Defence Food Research Laboratory, Mysore, Karnataka, India

INTRODUCTION

Foodborne pathogens primarily belong to Enterobacteriaceae which are considered as the common intestinal flora of human beings and animals. They are mainly transmitted through fecal-oral route, the reason which renders them among the most important pathogens studied in food microbiology. Members of this family are known to cause diarrhea, vomiting, and gastrointestinal infections. One of the foodborne pathogen is *Klebsiella*, which is ubiquitous in nature. They are common inhabitants of sewage, soil, respiratory and intestinal tracts of human beings and animals. *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the most important species of this genus which are significant in both community acquired (foodborne) and hospital acquired infections[1]. *K. pneumoniae* is an opportunistic pathogen causing both human and animal diseases. In humans the etiology of the diseases are septicemia, bacteremia, endophthalmitis, meningitis, liver abscess, soft tissue infections, intra-abdominal infections, diarrhea, urinary and respiratory tract infections[2,3]. The morbidity and mortality are high especially among people with severe underlying complications and immunocompromised people. The importance attributed to *K. pneumoniae* is because of the emergence of drug resistant strains isolated from food and their spread as nosocomial foodborne pathogen[4]. It is a neglected food pathogen in India and there is little data available on the isolation and surveillance of *K. pneumoniae* from food and environment. There are several reports on the occurrence of *K. pneumoniae* in various street foods such as fruit juices, hamburgers, raw milk and ready to eat vegetables[5,6,7]. There are numerous methods for the detection of food pathogens beginning with the “gold standard” which is the culture based techniques followed by biochemical profiling for identification. These techniques are time consuming and provide ambiguous and variable results. The other techniques for identification comprise molecular methods such as monoplex PCR, multiplex PCR and RT-PCR[8]. Immunoassays like ELISA are also used for detection of food pathogens[9]. The MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry) is an advanced diagnostic tool currently being used for the identification of microorganisms. MALDI-TOF has several advantages over the other techniques. It is robust, reliable, highly accurate and cost effective which can be used on a wide range of microorganisms and user friendly. The species *K. pneumoniae* encompasses three subspecies namely, *K. pneumoniae* subsp *pneumoniae* (K. pneumoniae▪), *K. pneumoniae* subsp *ozaenae* (K. ozaenae▪) and *K. pneumoniae* subsp *rhinoscleromatis* (K. rhinoscleromatis▪) among which K. pneumoniae being the most common (▪Taxonomic style according to Le Minor[10]). These subspecies are difficult to distinguish from each other by other methods. This can be achieved by the MALDI Biotyper which can easily identify at the subspecies level and hence it can be applied on the isolated strains from various sources. The aim of the study was to determine the incidence of *K. pneumoniae* from cakes, fish, soil and water samples to assess the quality of these sources. Besides isolation, antibiotic susceptibility testing was used to study the resistance pattern of these non-clinical isolates.

MATERIALS AND METHODS

Supplements, primers and reagents

Dehydrated media, supplements and antibiotic disks were procured from HiMedia laboratories (Mumbai, India). The primers used were synthesized at Sigma (Bengaluru, India). *Taq* DNA polymerase was purchased from Sigma (St. Louis, MO) and dNTPs were purchased from MBI Fermentas (Hanover, MD).

Isolation

The samples were collected from various places, markets and bakeries in Mysore city. All the samples were collected in sterile pouches and containers. A total of 82 samples were screened for the incidence of *Klebsiella*, particularly, K. pneumoniae. The food and environmental sources consisted of 16 cakes (C), 8 fish (F), 30 soil (S) and 28 water samples

(W) (tap and pond water). 10g/10mL of the sample was homogenized and added to sterile 90 mL Brain Heart Infusion broth and incubated overnight at 37 °C. One mL of this homogenate was plated and purified on Modified MacConkey agar, supplemented with 50 mg per liter carbenicillin. The pure cultures were also stored at -80 °C in 15% glycerol. Standard cultures were procured from MicroBiologics (St Cloud, MN).

Genomic DNA extraction and PCR identification

DNA was extracted from purified cultures using Hi-media bacterial genomic DNA extraction kit as per the manufacturer's instructions. The quality of extracted DNA was checked by agarose gel electrophoresis and was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Bengaluru, India). All the cultures purified were subjected to monoplex PCR for species identification. The gene targeted, primers and conditions followed were as described previously[11]. Two reference strains ATCC 13883 and ATCC 10031 were included as positive controls. The colonies were chosen based on their colony morphology.

MALDI-TOF

A total 104 colonies were selected and subjected to MALDI-TOF for further identification. MALDI-TOF mass spectra measurements were carried out using Bruker Daltonik MALDI Biotyper Ultraflex III instrument (Bruker Daltonik, Bremen, Germany) operated in the linear positive ion mode using FlexControl 3.0 software. The principle, procedure and interpretation have been previously described[12].

Antibiotic susceptibility

Twenty seven antibiotics were used for determining the resistance and sensitivity pattern. The testing was carried on Mueller-Hinton agar by Kirby Bauer disk diffusion method following the guidelines laid out by CLSI.

SDS-PAGE for profiling

The cultures were grown overnight in Brain Heart Infusion broth and incubated overnight at 37 °C. One milliliter of each culture was pelleted and suspended in 200 µL of Laemmli's lysis buffer. The supernatant was run in 12% SDS-PAGE and the gel was stained with Coomassie brilliant blue for band visualization.

Biofilm assay

The isolates were checked for their ability to form biofilm as described previously[13] with minor modifications. Briefly, 100µL of each isolate containing 10^4 cells was inoculated into a 96-well tissue culture plate (Nunc). Wells with growth medium alone served as negative controls. After 24 h of incubation at 37 °C, the planktonic bacteria were removed and biofilm formed was measured by staining with 1% aqueous solution of crystal violet for 15 min. The excess of stain was removed by gentle washing and bound dye was released from the stained cells using 95% ethanol followed by measurement at 595 nm was determined in Infinite M200 Pro multimode microtiter plate reader (Tecan, Austria).

RESULTS

Isolation

Microbial growth could be obtained from most of the samples. Initial screening was based on their morphological characteristics and Gram's staining. Of the 82 samples totally screened, 41 isolates were presumptively identified as *Klebsiella* spp by biochemical profiling. Though all the strains isolated from 8 fish and 30 soil samples were presumptive positive for *Klebsiella* spp, not all were PCR positive. Strains from 35 samples were confirmed as *Klebsiella* spp by PCR, which majorly comprised cake samples and water samples.

PCR and MALDI-TOF

MALDI-TOF was able to identify a total of 71 organisms. The strains subjected to PCR revealed that 30 strains were positive for haemolysin gene of *K. pneumoniae* giving an amplicon at an expected size of 400bp (Fig 1). The two standard strains were used as positive

control for PCR and MALDI-TOF. The isolates were confirmed by MALDI-TOF which correlated with the PCR results. MALDI-TOF was able to identify the subspecies of *K. pneumoniae*. K. ozaenae and K. rhinoscleromatis were identified among the isolated strains. A total of 34 strains belonging to *Klebsiella* species were identified using MALDI-TOF which comprised of *K. pneumoniae* and *K. oxytoca*.

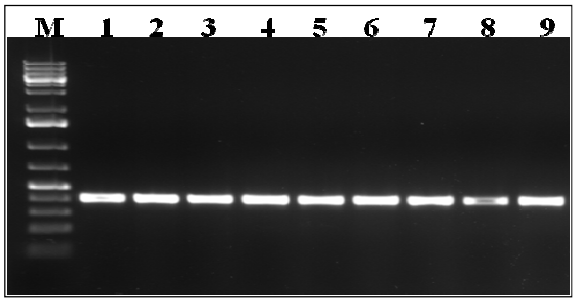
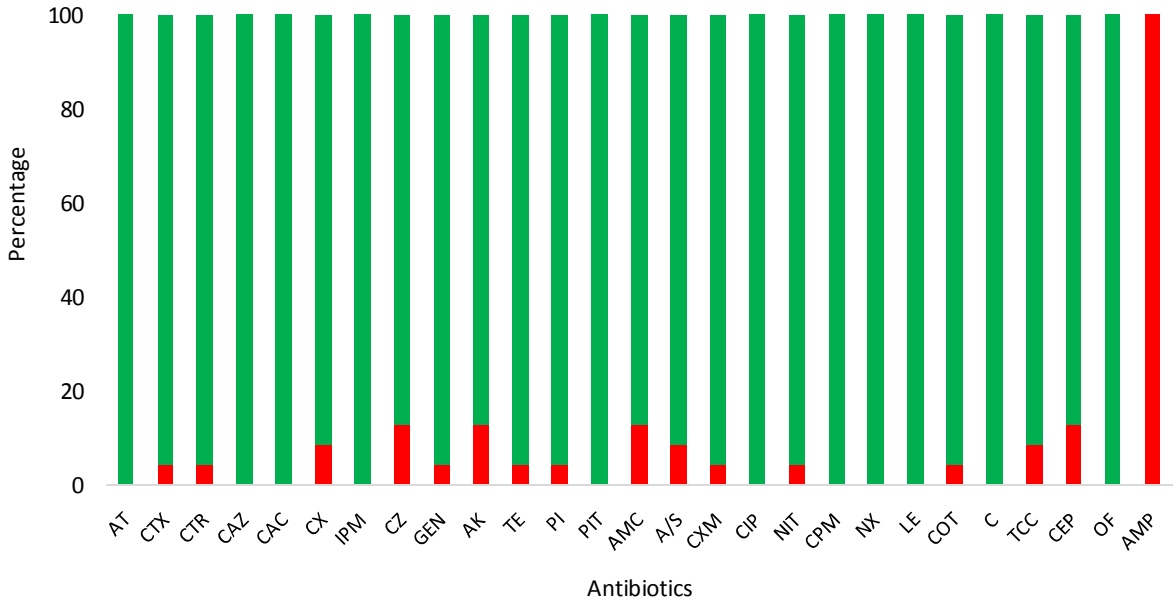


Figure 1

Agarose gel (1 %) showing amplified monoplex PCR products. Lanes: M 100 bp DNA marker (Fermentas), L1 & L2: Klebsiella pneumoniae ATCC 13883 and ATCC 10031 respectively, L3-L9: Isolated Klebsiella pneumoniae strains

Antibiotic susceptibility

The pattern of antibiotic resistance exhibited is shown in Fig 2. Isolates from cake (C7) and soil (S5) were resistant to Cefoxitin. Two strains from cakes (C6 and C11) were resistant to amikacin. There was absolute resistance (100%) to ampicillin by all K. pneumoniae strains isolated. Three isolates namely F2, S3 and S5 were the only 3 isolates which were multidrug resistant. Apart from 6 strains, all the other strains were susceptible to the entire 26 antibiotics tested.



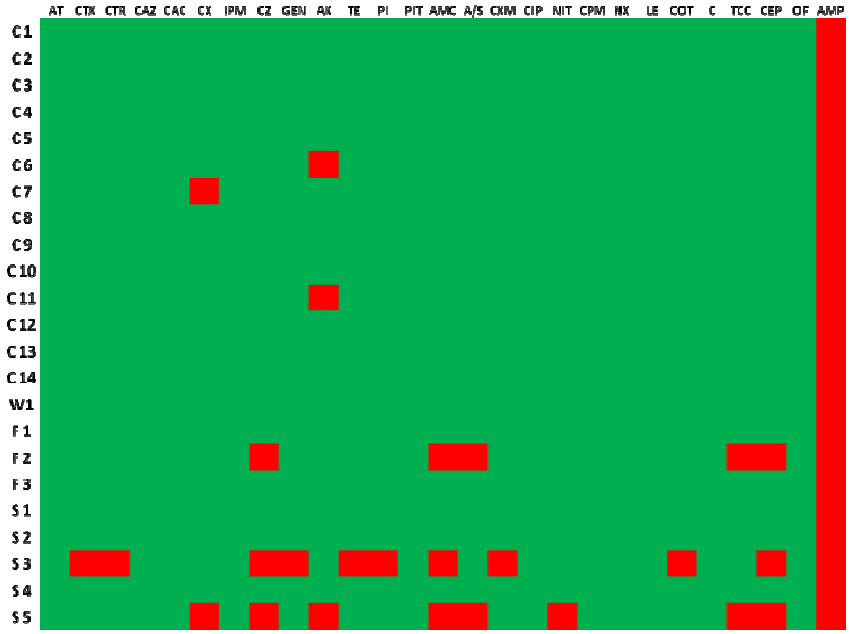


Figure 2

Antibiotic resistance exhibited by the isolates. AMP-Ampicillin (10 µg), AT-Aztreonam (30 µg), CTX-Cefotaxime (30 µg), CTR-Ceftriaxone (30 µg), CAZ-Ceftazidime (30 µg), CAC-Ceftazidime/clavulanic acid (30/10 µg), CX-Cefoxitin (30 µg), IPM-Imipenem (10 µg), CZ-Cefazolin (30 µg), GEN-Gentamicin (10 µg), AK-Amikacin (30 µg), AMC-Amoxycillin/clavulanic acid (20/10 µg), A/S-Ampcillin/sulbactam (10/10 µg), PIT-Piperacillin/tazobactam (100/10 µg), TCC-Ticarcillin/clavulanic acid (75/10 µg), CXM-Cefuroxime (30 µg), CPM-Cefepime (30 µg), PI-Piperacillin (100 µg), COT-Cotrimoxazole (1.25/23.75 µg), TE-Tetracycline (30 µg), C-Chloramphenicol (30 µg), CIP-Ciprofloxacin (5 µg), NIT-Nitrofurantoin (300 µg), NX-Norfloxacin (10 µg), LE-Levofloxacin (5 µg), CEP-Cephalothin (30 µg), OF-Ofloxacin (5µg).

Protein profile analysis

Analysis of SDS-PAGE of total protein showed about 26 different bands, in each of the 23 K. pneumoniae strains, ranging in size from 10 kDa to 100.0 kDa. The 23 isolates depicted the same protein profile (sizes and number of electrophoretic bands) and could not be clustered into groups. Protein analysis was not efficient to differentiate K. pneumoniae strains.

Biofilm assay

All the strains were capable of forming biofilms after 24 h of incubation. The degree of biofilm formation of strains was estimated based on the OD_{595} and were categorized as highly positive (OD_{595} ≥1), moderately positive (0.1≤ OD_{595} <1) and negative (OD_{595} <0.1). All the strains could be categorized as highly positive biofilm formers except for two cake samples (C3 and C10) which were categorized as moderately positive in comparison to the standard strains.

DISCUSSION

K. pneumoniae is being studied extensively during the recent time for its incidence, multidrug resistance, and production of ESBL. The biochemical reactions and virulence of *Klebsiella* strains isolated from the environment have been proven to be similar to that of clinical strains isolated from humans. The environmental isolates also have been proven as pathogenic as the clinical isolates. Though the clinical significance of *Klebsiella* strains isolated from the food and the environment is unclear, these habitats are thought to be potential reservoirs for the growth and spread of these pathogens which may colonize animals and humans[14]. In this study the incidence of *K. pneumoniae* in various samples were studied. We isolated 23 K. pneumoniae strains from cakes, fish, soil, tap and pond waters. Except for soil and fish, the remaining samples were foods that are consumed directly without any

further processing. In the samples screened, majority of the isolates encountered belonged to *K. pneumoniae*. Among the subspecies, K. pneumoniae (23 isolates) was identified in large numbers, the results which are in line with other studies. K. ozaenae and K. rhinoscleromatis are of clinical importance known to be the causal organisms for ozena and rhinoscleroma respectively[15]. A report of horizontal gene transfer between *Shigella* and K. ozaenae has possibly become the reason for the latter becoming an enteropathogen causing bloody diarrhea[16]. These strains which are strictly associated with humans but not commensals were also encountered in the food samples which clearly indicate the dissemination of pathogen from food handlers. Only a single pond water sample was encountered with *K. pneumoniae* contamination. The tap water samples were free from *K. pneumoniae* incidence because of probable treatments. The major concern with regard to *K. pneumoniae* is the emergence of epidemic clones. In India, multidrug resistant and ESBL *K. pneumoniae* has been increasing at an alarming rate. In this study, absolute resistance was observed only against ampicillin. On clinical basis for screening of multidrug resistant strains all *K. pneumoniae* have been proven to be intrinsically resistant to ampicillin and the same was inferred from our study. Interestingly only three isolates were resistant to more than two antibiotics and all of the other strains isolated were susceptible to all the antibiotics. The results of antibiogram contradicted our expectations of finding a large number of multidrug resistant strains. However, this in itself is not a criteria for determining the virulence nature of these strains. In addition, a wide range of other foodborne pathogens were also isolated which included various species of *Citrobacter*, *Enterobacter*, *Escherichia*, *Vibrio* and *Yersinia*. Apart from these, species of *Pseudomonas* and *Burkholderia cenocepacia* were also encountered. Apart from *K. pneumoniae*, *K. oxytoca* and *K. variicola* which are of clinical importance were also isolated from the above mentioned sources. Earlier classification had placed *Raoultella ornithinolytica*, *Raoultella planticola* and *Raoultella terrigena* in the genus *Klebsiella* which has now been separated. These strains were also among the isolates identified by MALDI-TOF. The possible primary cause for high incidence of *Klebsiella* in the present study might be that the food samples were unprocessed. The other probable reason is the food handlers. The potential health risks due to the consumption of improperly cooked food harboring pathogens should not be underestimated. Transmission or cross contamination of pathogens during processing, processed or cooked and ready to eat foods still pose a great threat for foodborne illnesses and intoxications. Hence, a stringent, continuous and regular monitoring of such foods is necessary to prevent both human and economical losses. Although not many MDR strains were encountered, the virulence potential of these strains cannot be underestimated. The capacity to detect genetic diversity in different strains is especially important for the surveillance of outbreaks. Efforts on these studies can help further to understand the spreading of this bacterium.

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