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Siderophore production: A unique quality of pathogenic *Klebsiella pneumonia* to survive in low iron concentration

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Abstract: *Klebsiella pneumonia* is the second most causing agent of urinary tract bacterial infection among human beings. Its ability to grow and to produce siderophores is dependent on the iron content and the type of carbon sources in the culture medium. This study has aimed to find out the unique properties of siderophore production by *Klebsiella* species in fluctuating iron concentrations. The study was carried out on 252 urine samples collected from UTI patients at Doon (PG) Paramedical College and Teaching Hospital, Dehradun, India. All urine samples were tested microbiologically using standard procedure and biochemical panel tests were performed to identify and to isolate *Klebsiella pneumonia* from colonies obtained on differential media plate used for urine culture and the result was interpreted according to National Committee for Clinical Laboratory Standards (NCCLS) guide line to confirm the *Klebsiella pneumonia*. Then CAS siderophore detection method was applied to quantify the siderophore production in various iron concentration supplied to culture medium. A total of 47 patients out of 252 UTI patients were found to be infected by *Klebsiella pneumonia*. Women were more prone to be UTI infected by *Klebsiella pneumonia* in comparison to men (14.89% men, 85.1% women). Most of the UTI patients infected by *Klebsiella pneumonia* were in the age group of 50-79 years (53.19%) which was followed by 20-49 years (23.40%) and 80-99 years (14.89%) respectively. Least people having age below 19 years old were prone to be urinary tract infected by *Klebsiella pneumonia*. In the same way, siderophore production was found to be increased significantly when there was no iron in the culture medium and its production was decreased gradually with increase in iron concentration up to 200mg/L in the culture medium. Siderophore is an important metabolite product for pathogenic *Klebsiella pneumonia* to survive and cause pathogenicity in low iron concentration medium.

Keywords: *Klebsiella pneumonia*; siderophores; UTI infection; iron concentration

1. Introduction

A siderophore is a low molecular weight (500-1000 Daltons), high affinity ferric iron-chelating compound secreted by microorganisms (Pal and Gokarn, 2010). It is the strongest soluble Fe³⁺ binding agents known yet (Hung et al., 2012). It is one of the determinants of virulence of a pathogen because of having high affinity to

bind with host iron binding protein. It acts to seize and to solubilize the iron. In the same way, iron is also very important mineral for survival of microorganism to run the internal metabolic environment like electron transport chain, a cofactor of enzymes of intermediary metabolism (Ali and Vidhale, 2013). Because of the strongest binding affinity to Fe^{3+} ions, medical sciences have attractive interest on siderophores for metal chelation therapy, iron poisoning and Thalassemia treatment (Challis, 2005; Ali and Vidhale, 2013).

Klebsiella pneumonia (Friedlander's bacillus) is clinically the most important member of the enterobacteriaceae. It is a gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic and rod shaped bacterium naturally found as normal flora of the mouth, skin, and intestines of human (Hadzic *et al.*, 2012). It is distinguished from *Klebsiella oxytoca* by indole testing and growing on both melezitose and 3-hydroxybutyrate (Hadzic *et al.*, 2012). It also occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. Members of the genus *Klebsiella* typically express two types of antigens on their cell surface. The first is 'O' antigen having nine varieties which is a component of the lipopolysaccharide (LPS). The second is K antigen that is a capsular polysaccharide with more than 80 varieties. Both antigens show pathogenicity and are novel tools for sero-grouping of *Klebsiella* species (Barry and Challis, 2009).

Klebsiella species ranks second to *E coli* for urinary tract infections in older persons. It is also a common pathogen of nosocomial infection for dilated, ventilator-dependent patient; surgical wound infections; and biliary tract infection (Baral *et al.*, 2012). The major pathogenic effect of *Klebsiella pneumonia* in human being is a serious life threatening disease typically known as bronchopneumonia and bronchitis (Hadzic *et al.*, 2012). These patients have an increased tendency to develop lung abscess, cavitations, emphysema, and pleural adhesions defects. The mortality rate can be nearly 100% for persons with alcoholism and bacteremia (Dielubanza, 2011). The clear mechanism for the cause of alcoholism of spreading of *Klebsiella pneumonia* is not known but typically these bacteria can gain access after a person aspirates colonizing oropharyngeal microbes into the lower respiratory tract. *Klebsiella* infections are mostly seen in people with a weakened immune system. Most often illness affects middle-aged and older men with debilitating diseases (Pallett and Hand, 2010). Persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary diseases (COPD), glucocorticoid therapy, renal failure, and certain occupational exposures (such as paper mill workers) have prone to develop *Klebsiella* infection (Collee *et al.*, 2006; Pallett and Hand, 2010; Dielubanza, 2011).

There are four iron uptake protein systems such as enterobactin, aerobactin, ferrichrome and coprogen available in *Klebsiella pneumoniae* (Perry and San-Clemente, 1979; Jaya and Sanjay, 1995). All of these systems are under the control of fur repressor gene, and need a specific outer membrane receptor protein and various other membrane proteins. In addition to these specific ferric ion transport systems, there is also a system for transportation of ferrous ion to grow under anaerobic conditions which is specified by the Feo gene and is regulated by the fur repressor protein (Flores-Mireles *et al.* 2015). The colicin I receptor protein encoded by the cir gene is another outer membrane protein synthesized by *Klebsiella pneumonia* in response to iron limitation. Once the iron-siderophore complexes are entered into the cytosol, iron is released from this complex by breakdown of the ligand itself. In case of enterobactin, an esterase specific to ferric ion hydrolyze the complex to release iron (Jaya and Sanjay, 1995; Ali and Vidhale, 2013). Similarly, in certain case of iron-siderophore complex, ferric iron is reduced to the ferrous state which has little affinity for siderophore binding. Therefore, these studies were carried out to detect the activity of siderophore production in iron limited condition so that we can evaluate the pathogenicity of *Klebsiella pneumonia* (Esteves *et al.* 1995; Jaya and Sanjay, 1995; Ali and Vidhale, 2013; Flores-Mireles *et al.* 2015).

2. Materials and Methods

The present study has been carried out on 252 patients of UTI in the department of Medical Laboratory Science at Doon (PG) Paramedical College and Teaching Hospital, Dehradun, India.

2.1. Ethical considerations

The study was approved by the Institutional ethical committee on human research, Doon PG Paramedical College and Teaching Hospital, Dehradun India.

2.2. Sample collection

For isolation of pathogenic *Klebsiella* strains, 10 mL urine sample was collected in a sterilized container from the patient suffering from urinary tract infection. The mid-stream urine was collected after carefully cleaning the genitalia to avoid contamination.

2.3. Urine culture

Freshly collected urine samples were transferred to sterile tube and centrifuged at 2000rpm for 10 min to get bacterial pellet. After centrifugation, a loopful inoculum was taken and streaked on the sterilized MacConkey agar, blood agar and EMB medium. The plates were incubated at 37°C for 24-48hrs. Pure cultures of *Klebsiella pneumoniae* species were obtained following successive selection of colonies on MacConkey agar, blood Agar and EMB agar media (Harley and Prescott, 2002; Collee *et al.*, 2006).

2.3.1. MacConkey agar medium

This medium containing bacto-peptone as a source of carbon, nitrogen, vitamins and minerals; neutral red as pH indicator; and bile salts and crystal violet for inhibition of gram positive bacteria showed red colonies with red-purple halos by glucose fermenting bacteria. And *Klebsiella* species showed pink mucoid colonies. (Harley and Prescott, 2002; Collee *et al.*, 2006).

2.3.2. Blood agar medium

A sterile freshly prepared sterile blood agar plate per urine sample was used to study the microbial activity. Here 0.1mL of sample/swab spread on blood agar plate followed by incubation at 28±1°C for 24-48hrs were shown mixed culture colonies (Harley and Prescott, 2002; Collee *et al.*, 2006).

2.3.3. EMB Agar medium

The freshly prepared sterile EMB agar plate was used for differential colonies characteristic study. *Klebsiella* species showed large, pink with blue centered colonies (+++) after 24-48hours incubation at 35°C (Harley and Prescott, 2002; Collee *et al.*, 2006).

2.4. Gram staining

Gram staining was deployed to differentiate bacteria, whether they are Gram-negative or positive. The Gram-positive bacteria appeared violet while the Gram-negative bacteria as pink colour (Harley and Prescott, 2002; Collee *et al.*, 2006).

2.5. Biochemical panel tests

2.5.1. Urea hydrolysis

This test is based on the principle that some bacteria degrade the urea present in the medium into ammonia and CO₂. A loopful inoculum was taken and streaked on the sterilized urea agar medium. The change in color into pink showed positive after incubation at 37°C for 24-48hours (Collee *et al.*, 2006)

2.5.2. Acid production in different sugar source (C-source)

Freshly prepared sterile tube containing inverted Durham tube, different fermentation broth medium supplemented with different carbon sources (like lactose, maltose, mannitol, fructose, sucrose, trehalose, xylose, cellobiose, raffinose and mannose) and phenol red as an indicator; were inoculated with log phase culture of microbes; and incubated at 37°C for 24-72 hours. The change of color from red to yellow showed microbes produced acid by sugar fermentation (Harley and Prescott, 2002).

2.5.3. Methyl red (MR) and Voges-Proskauer (VP) tests

These tests were used to differentiate two major types of facultative anaerobic bacteria that produce the large amount of acid and neutral product acetone as end products. *Klebsiella* species showed MR negative and VP positive. Both these tests were performed simultaneously because they were physiologically related and were performed on the same medium MR-VP Broth. But the reagents used for the detection were different for both tests. Methyl red, which was the indicator for the MR test, gave red color as a positive indicator while for Voges-Proskauer test the indicator was VP reagent -I (Naphthol solution) and VP-reagent-II (40% potassium hydroxide) (Harley and Prescott, 2002).

2.5.4. Indole production test

This test was done to identify indole forming bacteria from tryptophan as a nutrient source. Here bacterial culture, inoculated in freshly prepared and sterile tryptone broth, followed by incubation at 37°C for 48 hours,

was treated with 0.5mL of the Kovac's reagent. A red colour band was appeared at the junction of medium and reagent in case of Indole positive bacteria (Harley and Prescott, 2002).

2.5.5. Citrate utilization test

Klebsiella pneumonia utilizes citrate as carbon source. Citrate is an intermediate product of Kreb's Cycle which oxidises Pyruvate to Carbon-dioxide. Bacterial colonies, inoculated to the slants of Simmon's citrate agar medium by stabbing to the base, and thereafter streaking to the surface; showed the alkaline media after 24-48 hours incubation at 37⁰ C (Harley and Prescott, 2002).

2.6. Siderophore production

Siderophores production is identified for their unique iron-chelating properties, by a highly sensitive method developed by Schwyn and Neilands. A strong ligand (siderophore) is added to a highly colored iron dye complex. The formation of the iron ligand is accompanied by the release of free dye. The free dye has a distinctly different color change. Since the dye used is Chrome Azurol S (CAS) and detection methods used is fluorescence visualization under UV light after spraying with 1% ferric chloride solution (Vishnu and Madhusudan, 2013). The CAS siderophore detection method can be used as a spray or within agar medium. Here a thin layer of CAS reagent in 0.7% agar was spread over the colonies produced over Mannitol agar after incubation at 37±1⁰C for 24 hours and plates were re-incubated at 37⁰C for 24 hours. The change in color from blue to orange is confirmed siderophore production by particular microbes (Louden *et al.*, 2011; Vishnu and Madhusudan, 2013).

2.6.1. Quantitative estimation of siderophore production

Hydroxamate type of siderophore measurement is done quantitatively. It is based on the measurement of activity of hydroxamic acid released during reaction between siderophore and Gibson & Magrath reagent. Isolates/procures of Klebsiella pneumonia grown at 28⁰C for 24 hours on Tryptic soy agar medium was followed by transfer one bacterial colony on Tryptic soy broth and incubate as above. After that, the culture obtained after 24 hours of growth were centrifuged at 7,000rpm for 15minutes at 4⁰C. In 0.5mL culture supernatant, 0.5mL of 6M H₂SO₄ solution, 1mL of sulphanic acid (1:1w/v, prepared in 30% acetic acid) and 0.5mL of iodine solution (1.3%, prepared in 30% acetic acid) were added. Excess of iodine was removed by the addition of 2% Na₂ASO₄ (w/v) solution. Then measure absorbance was measured at 630nm by using a spectrophotometer after 1ml of alfa -aphthalylamine addition into it, followed by incubation for 30 minutes (Louden *et al.*, 2011).

2.6.2. Qualitative detection of siderophore production

Active bacterial culture of Klebsiella pneumonia was inoculated on three spots of freshly prepared solid, sterile CAS medium Petri-dishes (Chrom-Azurol S dye; PIPES Buffer pH-6.8 solution; substrate solution containing glucose, mannitol, MgSO₄.7H₂O, CaCl₂, MnSO₄.H₂O, H₃PO₄, CuSO₄.5H₂O, ZnSO₄.7H₂O and Na₂MoO₄.2H₂O) and siderophore production marked by orange halo against blue background was observed after incubation at 28⁰C for 48hours (Vishnu and Madhusudan, 2013).

2.6.3. Liquid assay for siderophore production

The change in color of the blue Dyechrome Azurol sulphonate assay solution to purple-orange indicates the presence of siderophore. Samples (i.e culture supernatant taken after growing the cultures at 37⁰C for 24hours under static condition) showed lower readings as siderophore removes the iron from the dye complex (As). The values of the siderophore excreted were determined using the formula which gives percentage siderophore unit (Vishnu and Madhusudan, 2013).

$$\% \text{ of Siderophore Produced: } \left(\frac{Ar-As}{Ar} \times 100 \right)$$

2.7. Data Analysis

The data collected was entered in Microsoft Excel and checked for any inconsistency. The value of p<0.05 was taken as significant. All the analysis was carried out by using SPSS 15.1 version.

3. Results

The study was carried out among the 252 patients attending Outpatient and Inpatient Department of Doon (PG) Paramedical College & Hospital, Dehradun, India from 1st October 2010 to 31st March 2011. And differential microbiological culture and biochemical tests were carried out to identify the *Klebsiella* species from urine sample in the department of Medical Laboratory Technology (MLT) under circumference of WHO guideline. Reagents used were from Tulip Company Private Limited, India. We collected the sample for our research from concerned patients with their knowing vision to participate in this study.

3.1. Cultural characteristics of urine pathogenic bacteria indicate the presence of *Klebsiella pneumonia*

Pink mucoid bacterial colonies were obtained from urine sample on MacConkey Agar plate due to capsular material produced by bacteria after 24-48 hours incubation at 37^o C (Figure1). In the same way, bacterial colonies obtained from urine sample on EMB Agar plate after incubation for 24-48 hours at 35^oC showed large, pink with blue center colonies due to *Klebsiella pneumonia*.

3.2. Confirmation of *Klebsiella pneumonia* by biochemical panel test

As shown in the Table 1 and Figure 1, these bacteria obtained on culture plate of EMB agar are gas forming, acid producing, VP-positive, MR- negative, citrate-positive, urease-positive, malonate -positive, and lysine decarboxylase test -positive. So these findings confirm that these bacterial colonies are of *Klebsiella pneumonia*.

3.3. Distribution of *Klebsiella pneumonia* in UTI patients

Table 2 shows that only 18.65% of urinary tract infections are due to *Klebsiella pneumonia*, whereas maximum people suffering from UTI infection (81.35%) are *Klebsiella* negative. In the same way, female is more prone to UTI infection by *Klebsiella pneumonia* in comparison to male (Table 3). Male comprises only 14.89% whereas 85.1% UTI is seen by female patients. And the majority of patients in this study are female 85.1% (40) in comparison to the male 14.89% (Figure 2). Similarly, Table 4 and Figure 3 show that most of the patients infected by *Klebsiella pneumonia* in urinary tract is in the age group of 50-79 years (53.19%) followed by 20-49years (23.40%), 80-99years (14.89%) respectively.

3.4. Siderophore production is highly affected by presence of iron in bacterial culture medium

Influence of iron and medium content on siderophores production by *Klebsiella pneumonia* are shown in Table 5. When there was no iron in the culture medium, siderophore production was high whereas its production was decreased gradually with continuous elevation of iron concentration up to 200mg/L in the medium (Figure 4). Siderophore production is the greatest (26.98%) at iron concentration 0mg/L, whereas its production is the least (2.99%) at elevated iron concentration (200mg/L).

4. Discussion

This cross sectional, analytical study was carried out in the Department of Medical Laboratory Science at Doon (P.G.) Paramedical College and Hospital, Dehradun, India. A total of 252 urine samples were collected from the urinary tract infected patients attended at outpatient and inpatient ward of the hospital. Among them, 47 patients were found to have UTI infection caused by *Klebsiella pneumonia* confirmed by studying gram staining and biochemical panel tests after obtaining the significant colonies on Mac Conkey agar, blood agar and EMB agar media. EMB agar medium was used as differential media for specially *Klebsiella pneumonia* isolation. And this organism showed large, pink with blue center colonies after 24-28 hours of incubation at 35^oC. After confirmation of *Klebsiella pneumonia* colonies on culture plate, siderophore production was studied in various iron supplementary condition.

Our study shows that female is more prone to urinary tract infection caused by *Klebsiella pneumonia* in comparison to male. Similar findings have been reported by Baral *et al.*, 2012; Bhatt *et al.*, 2012; Prakash *et al.*, 2013.

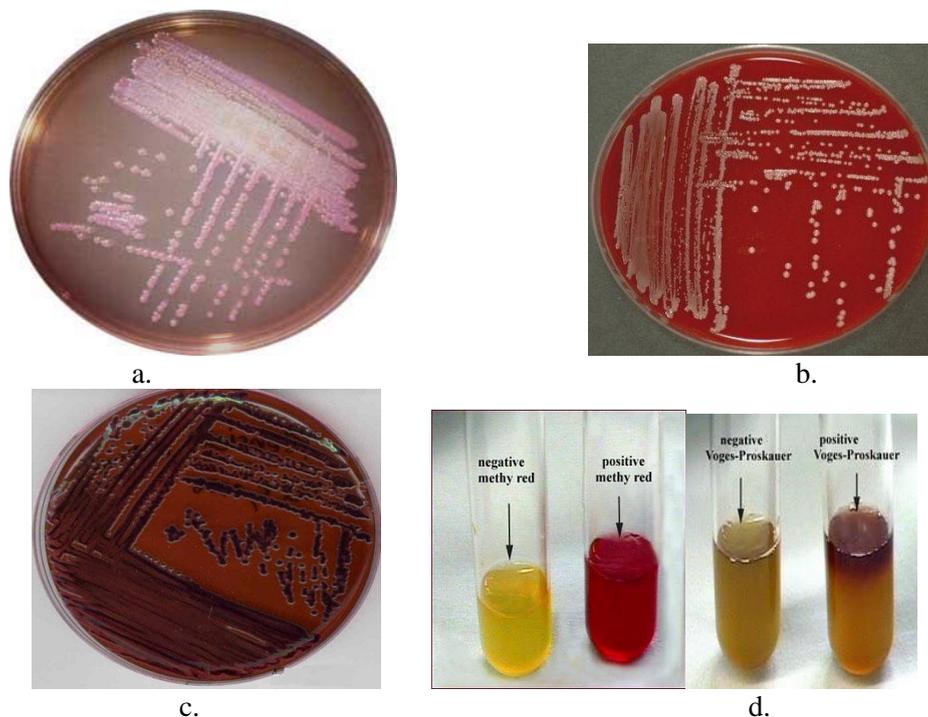


Figure 1. Bacterial growth obtained from urine culture in different culture media plates and tubes. a, bacterial colonies obtained from urine sample on MacConkey Agar plate. b, Bacterial colonies obtained from urine sample on Blood Agar plate. c, Bacterial growth colonies obtained from test urine samples on EMB Agar plate. d, *Klebsiella* species showing MR negative and VP positive.

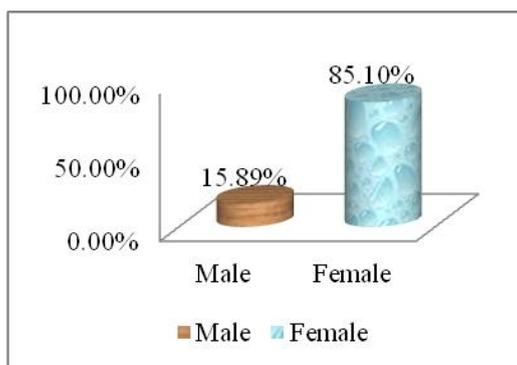


Figure 2. Sex percentage distribution of UTI infection by sex.

Table 1. Various biochemical tests for *Klebsiella pneumonia*.

Biochemical tests	Result
Gas & acid formation from sugar	Positive
Urease test	Positive
Citrate test	Positive
MR test	Negative
VP test	Positive
Lysine decarboxylase test	Positive
Malonate	Positive

Table 2. Number of isolated UTI cases in patients.

Sample	Number of isolated <i>K. pneumonia</i> sample
Positive	47 (18.65%)
Negative	205 (81.35 %)
Total	252 (100%)

Table 3. Sex-wise distribution of *Klebsiella pneumonia* in UTI patients.

Sex	Number of <i>Klebsiella pneumonia</i> infected UTI patients
Male	7 (14.89%)
Female	40 (85.10%)

Table 4. Age-wise distribution of *Klebsiella pneumonia* in UTI patients.

Age (in year)	Number of patients
0-19	4 (8.51%)
20-49	11 (23.40%)
50-79	25 (53.19%)
80-99	7 (14.89%)

Table 5. Influence of iron and medium content on siderophores production by *Klebsiella pneumonia*.

Fe3+ added (mg/L)	Growth (O.D. at 630 nm)	Percentage of siderophore
0	0.633	26.989
5	0.635	26.758
10	0.636	26.643
50	0.645	25.605
100	0.758	12.572
150	0.746	11.880
200	0.841	2.998

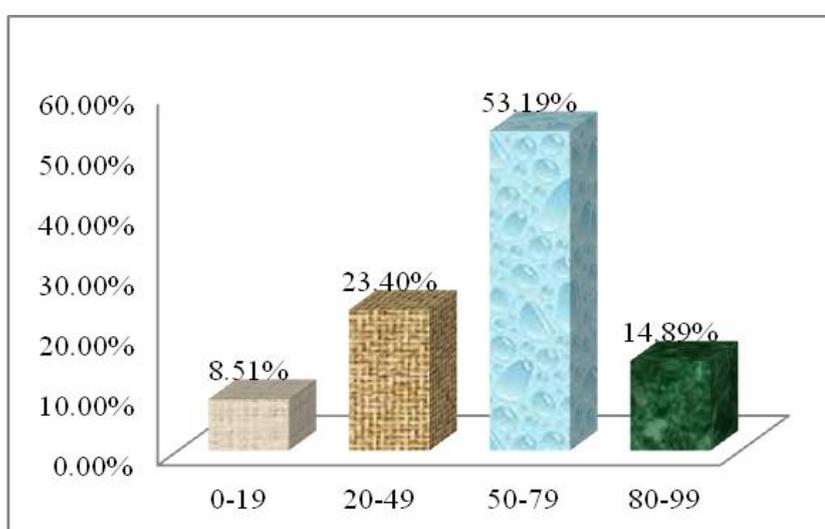


Figure 3. Age-wise distribution of UTI infection by *Klebsiella pneumonia*.

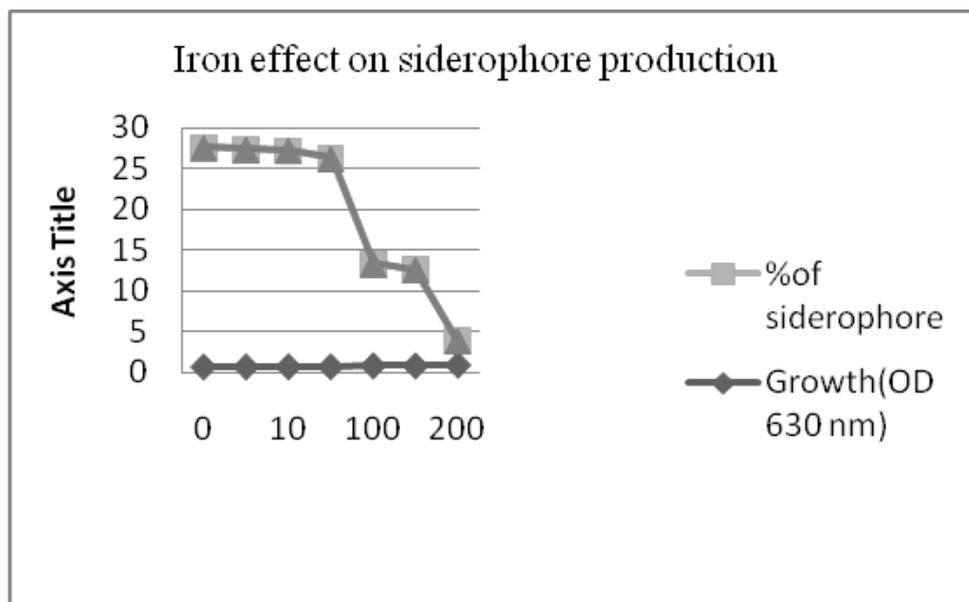


Figure 4. Iron effect on siderophore production.

Our finding also expressed that most of the patients infected by *Klebsiella pneumonia* in urinary tract is in the age group of 50-79 years (53.19%) followed by 20-49 years (23.40%), and 80-99 years (14.89%) respectively. The least people with age below 19 years were infected by *Klebsiella pneumonia* in urinary tract. The similar findings have been reported in Nepal by Baral *et al.*, 2012 and Bhatt *et al.*, 2012. This result has also supported by the finding of Mahesh *et al.*, 2010. In their studies, they showed that people with 0-19 years having urinary tract infection were 2.4%. Similarly, the people with 20-49 years age suffering from urinary tract infection were 9%; followed by 50-59 years by 18.4%, and 70-79 years by 20.4 %.

In the same way, we found there is suppression in synthesis of siderophore by *Klebsiella pneumonia* when iron concentration is increased in bacterial culture medium. The similar finding was obtained by Perry and San Clemente, 1979 and Jay and Sanjay, 1995.

5. Conclusions

This study shows that *Klebsiella pneumonia* isolated from 47 out of 252 UTI sample had produced maximum 37.6% and minimum 2.99% of siderophore. With increase in concentration of iron supplement in the culture medium, siderophore production by *Klebsella pneumonia* was gradually decreased. Hence our study concludes that siderophore is an important metabolite product for pathogenic *Klebsiella pneumonia* to survive in low iron concentration medium. And siderophore is also necessary for these bacteria to cause pathogenicity so that active chelation and removal of iron from iron-binding proteins like lactoferrin and transferrin take place to meet their iron demand.

Conflict of interest

None to declare.

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