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A novel approach for identification of members of Enterobacteriaceae isolated from clinical samples

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Abstract
A simple algorithm is proposed for the identification of Enterobacteriaceae which routinely necessitates numerous biochemical tests and a prolonged time span [may extend upto 96 hours].This simple scheme is economical and time saving, reducing the turnaround time for identification of the pathogenic organisms to 24 hours, so that the final reports can be given to the clinician within the 3 day time frame. The basic tests used are catalase, oxidase, indole, citrate, urease, and phenylalanine. With this scheme, 12 genera and 23 species can be identified using these limited biochemicals which are within the reach of most of the microbiology laboratories having limited resources.

Keywords: Enterobacteriaceae; lactose fermenters; lactose non-fermenters.

Introduction

Gram-negative bacilli belonging to the family Enterobacteriaceae are the most frequently encountered bacterial isolates recovered from clinical specimens. The members of Enterobacteriaceae may be incriminated in virtually any type of infectious disease and recovered from any specimen received in the laboratory. Immunocompromised or debilitated patients are highly susceptible to hospital-acquired infections, either after colonization with environmental strains or following invasive procedures, such as catheterization, bronchoscopy, colposcopy or surgical biopsies (Winn et al, 2006). Enterobacteriaceae may account for 80% of clinically significant isolates of Gram-negative bacilli and 50% of clinically significant bacteria in clinical microbiology. They account for nearly 50% of septicemia cases, more than 70% of UTI, a significant percentage of intestinal infections (Murray et al, 2003) and some important pathogens like *Klebsiella pneumoniae* are involved in hospital acquired infections (Shukla et al, 2004).

In a study on critically ill patients in a Brazilian Hospital, Enterobacteriaceae accounted for 24.6% of blood stream infection, 48% cases of UTI and 14% of Tracheal aspirates (Carvallo and Fillo, 2008). Some members of the Enterobacteriaceae family like *Serratia* and *Citrobacter* are emerging as significant pathogens (Rizvi et al, 2009) but are usually missed by the routine identification methods employed in most of the laboratories.

Identification Scheme

We propose a scheme for the identification and differentiation of commonly encountered genera and species of Enterobacteriaceae. The basic aim of this simple algorithm is to identify the common pathogenic species within a turnaround time of 24 hours with the help of biochemicals, which are employed routinely in the microbiology laboratories of resource poor countries like India.

Definitive identification of the members of the Enterobacteriaceae may require a battery of biochemical tests. There is a need for greater vigilance for the identification of pathogens, which are usually missed in the routine microbiology laboratories. Considerable time and possible misidentification can be avoided if a few preliminary observations are made to ensure that the organism being tested belongs to this group.

With few exceptions, all members of the Enterobacteriaceae demonstrate the following characteristics:

- Grow on simple media like MacConkey.
- Ferment glucose.
- Cytochrome oxidase is negative.
- Catalase is positive.
- Nitrates are reduced to nitrites (Collee et al, 2006).

The family Enterobacteriaceae is divided into two major groups on the basis of lactose fermentation:

1. Lactose fermenters
2. Lactose nonfermenters

Important pathogens among the lactose fermenters include:

Escherichia coli

Klebsiella pneumoniae
Citrobacter freundii [late lactose fermenter]
Citrobacter koseri [late lactose fermenter]
Other Citrobacter species [late lactose fermenter]
Enterobacter aerogenes
Enterobacter cloacae

Among the lactose non-fermenters, important pathogens are:

Citrobacter freundii
Citrobacter koseri
Other *Citrobacter* species
Edwardsiella tarda
Hafnia
Esherichia coli [inactive]
Morganella morganii
Proteus mirabilis
Proteus penneri
Proteus vulgaris
Providencia rettgeri
Providencia stuartii
Salmonella species
Shigella species
Serratia marscecens

1. Lactose Fermenters

The lactose fermenters are divided into two groups on the basis of indole test:

A) Indole positive group of lactose fermenters.
B) Indole negative group of lactose fermenters.

A) Indole positive group includes:

Esherischia coli
Citrobacter species [other than *Citrobacter freundii*]
Klebsiella oxytoca

These can be further differentiated on the basis of their ability to grow on Simmons citrate medium (C), urease (U) and indole test (I) [+ indicates more than equal to 90% positive, - indicates less than equal to 10% negative, +/- indicates more than 50% positive and -/+ indicates less than 50% positive].

Escherichia coli	I+U-C-
Citrobacter koseri	I+U+***/-***C+
Klebsiella oxytoca	I+U+C+

Urease positive strains of *Citrobacter koseri* can be differentiated from *Klebsiella oxytoca* on the basis of a simple test like motility. *Citrobacter* are motile whereas *K. oxytoca* are non-motile, if still in doubt capsular staining can be resorted to or methyl red (MR) test can be used but it will take 48 hours for confirmation [*C. koseri* - MR positive, *K. oxytoca* - MR negative].

B) Indole negative group includes:

Citrobacter freundii
Klebsiella pneumoniae
Enterobacter species

Citrobacter freundii can be differentiated from others by H_2S production in Kligler iron agar (KI). *Klebsiella* and *Enterobacter* are differentiated by growth in Simmons citrate medium and urease test.

Citrobacter freundii	I-/+U-/+C+/- H_2S+
Enterobacter aerogenes	I-U-C+ A/A with gas
Enterobacter cloacae	I-U+/-C+ A/A with gas
Klebsiella pneumonia	I-U+C+ A/A with gas

Urease positive strains of *E.cloacae* are differentiated from *Klebsiella* with the help of motility. When necessary urease negative strains of *E.cloacae* can be differentiated from *E.aerogenes* on the basis of lysine and ornithine decarboxylase tests. *E.aerogenes* will decarboxylate lysine and *E.cloacae* will decarboxylate arginine. For the identification of various lactose fermenters refer to Flowchart 1.

2. Lactose Nonfermenters

Lactose nonfermenters are divided into two major groups on the basis of their ability to deaminate phenylalanine to phenylpyruvic acid i.e. PPA test.

A) PPA positive group of lactose nonfermenters.
B) PPA negative group of lactose nonfermenters.

A) PPA positive group:

Proteus species
Providencia species
Morganella morganii

They all belong to tribe Proteae. These are further divided into two groups on the basis of their ability to produce H_2S.

H_2S producing species of tribe Proteae are:

Proteus mirabilis
Proteus vulgaris
Proteus penneri [30% strains produce H_2S] (Winn et al, 2006)
Morganella morganii [20% strains produce H_2S] (Winn et al, 2006)

These are further differentiated with the help of indole, citrate and urease test.

Proteus mirabilis	I-U+C+/-H_2S+
Proteus vulgaris	I+U+C-/+H_2S+
Proteus penneri	I-U+C-H_2S+
Morganella morganii	I+U+C-H_2S+

P.mirabilis and *P,penneri* are indole negative whereas *P.vulgaris* and *M.morganii* are indole positive. *P.mirabilis* and *P,penneri* are differentiated by utilization of citrate. Similarly, *P.vulgaris* and *M.morganii* are also differentiated by citrate test. None of the above-mentioned species ferments mannitol.

H_2S nonproducing strains of tribe Proteae are:

Providencia rettgeri
Providencia stuartii
Proteus penneri [70% strains do not produce H_2S] (Winn et al, 2006)
Morganella morganii [80% strains do not produce H_2S]

P.rettgeri can be easily differentiated from others on the basis of mannitol fermentation. Indole, citrate, urease and KI prove useful for identification of other species.

Providencia rettgeri	I+C+U+K/A
No Gas, Mannitol+	
Providencia stuartii	I+C+U-/+K/A
No Gas, Mannitol-	
Proteus penneri	I-C-U+K/A
With Gas, Mannitol-	
Morganella morganii	I+C-U+K/A
With Gas, Mannitol-	

B) PPA negative group of lactose nonfermenters includes:

Citrobacter species
Edwardsiella tarda
Escherichia coli [inactive]
Hafnia
Salmonella species
Shigella species
Serratia marscecens

These can be differentiated easily with the help of the biochemicals used routinely in the microbiology laboratories. They are divided into two groups:

i) Mannitol fermenting.
ii) Mannitol nonfermenting.

i) Mannitol fermenting group includes:

Citrobacter species
Escherichia coli [inactive]
Hafnia
Salmonella species
Shigella species [other than *S.dysenteriae*]
Serratia marscecens

These are categorized on their ability to produce H_2S.

H_2S producing species are:

C.freundii
S.typhi
S.paratyphi B
S.typhimurium

Citrobacter freundii produces abundant H_2S and is ONPG positive. *Salmonella* species are ONPG negative. *S.typhi* produces a streak of H_2S, is anaerogenic and do not grow in Simmons citrate medium. *S.paratyphi B* produces H_2S, is aerogenic and is citrate positive. *S.typhimurium* produces H_2S, is aerogenic and is citrate positive. It is differentiated from *S.paratyphi* B by lysine decarboxylase. *S.typhimurium* decarboxylates lysine whereas *S.typhimurium* does not.

Mannitol fermenters which do not produce H_2S include:

Citrobacter species [other than *C.freundii*]
Esherichia coli [inactive]
Hafnia
Salmonella paratyphi A
Serratia marscecens
Shigella species

These can be differentiated by the indole, citrate, urease, KI and with the help of simple tests like motility.

C.koseri	I+U-C+K/A
With Gas, Motile	
C. species (other than *C.freundii and C.koseri)*	
I-U+C+K/A With Gas, Motile	
Esherichia coli [inactive]	I+/-U-C-K/A
No Gas, Nonmotile	

Hafnia I-U-C-K/A
With Gas, Motile
Salmonella paratyphi A I-U-C-K/A
With Gas, Motile
Serratia marscecens I-U-C+K/A
No Gas [55%], With Gas [45%] (Winn et al, 2006), Motile
Shigella species I-U-C-KI-K/A
No Gas, Nonmotile

Any *Salmonella* species isolated should be confirmed by serotyping. This also helps in differentiating *S.paratyphi A* from *Hafnia*. However, if required, decarboxylation of arginine can also help in their identification. *Hafnia* will decarboxylate arginine while *S.paratyphi* will not. Similarly, serotyping done for confirmation of *Shigella* species will rule out inactive *E.coli*.

ii) Mannitol nonfermenting group includes:

Edwardsiella tarda
Shigella dysenteriae

In routine clinical microbiology laboratories, *Shigella* and *Edwardsiella* are isolated from different body sites i.e., *Shigella* usually from stool specimens and *Edwardsiella* may be isolated from a variety of extraintestinal sites including liver abscess and infections related to trauma in aquatic environment (Collee et al, 2006). So routinely, their differentiation is not required. However, if necessary, for example in pure cultures, they can be differentiated by indole test and H_2S production in KI. *Edwardsiella* is always indole positive and produces H_2S whereas *Shigella* is indole negative and it does not produce H_2S. For the identification of various lactose nonfermenters refer to Flowchart 2.

Diagnostic laboratories deal with the commonly encountered organisms prevalent in the hospital and the community. The biochemical tests for the identification of the common bacterial pathogens are usually in the form of checkerboard matrices. It is difficult for the laboratory workers to choose the relevant tests, which help in identification of the bacterial pathogens. The commercial identification system API-20E (Biomerieux) determines 21 characteristics of Enterobacteriaceae within 24 hours and has become the reference method for the well-equipped laboratories (Winn et al, 2006). However, the cost is beyond the scope of many laboratories especially in the developing nations like India.

Therefore, flow diagrams are designed to reduce the tedium of reading the checkerboard matrices and to facilitate the likely bacterial identification by tracing a series of positive and negative branch points in a dichotomous algorithm. In addition to saving the cost of setting up commercial test panels, these dichotomous schemes coupled with a few inexpensive biochemical tests and observations offer an additional advantage over automated systems in that they require thought, reasoning and knowledge on the part of the microbiologists, who are otherwise reduced to passive observers.

Conclusion

With the help of this algorithm that we have evaluated for the lactose fermenters and nonfermenters using a limited battery of biochemicals which are very cost effective and within the parameters of the developing countries, 12 genera and 23 species of Enterobacteriaceae can be identified within 24 hours thus saving approximately two to three days which are lost just for the identification of the pathogenic organisms when the standard tests like MR, VP, and the Decarboxylases are used. However, it is necessary to perform the other standardized biochemical tests and serotyping for certain pathogens which should be considered as a second step after resorting to the primary format detailed in this scheme or for the research purposes.

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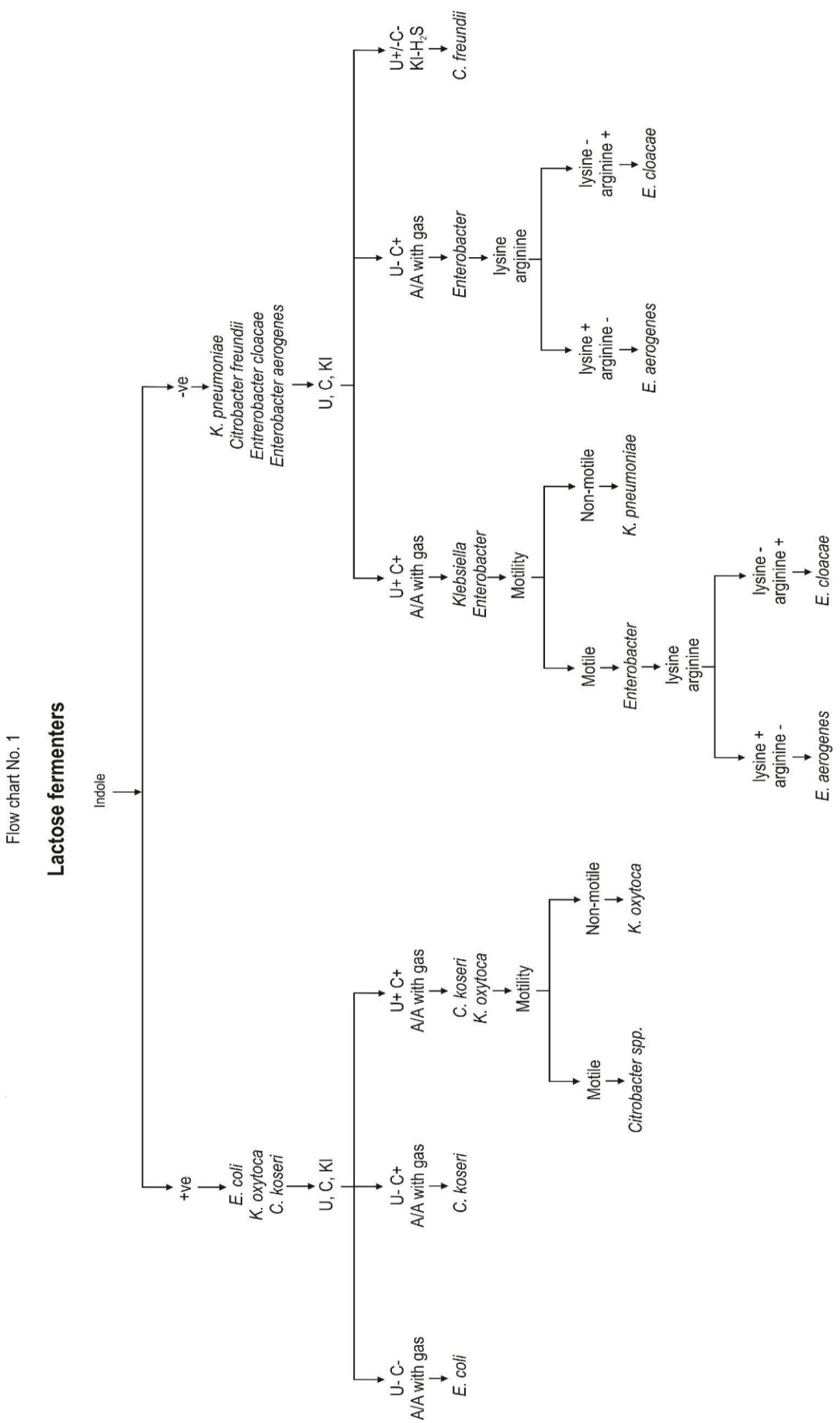
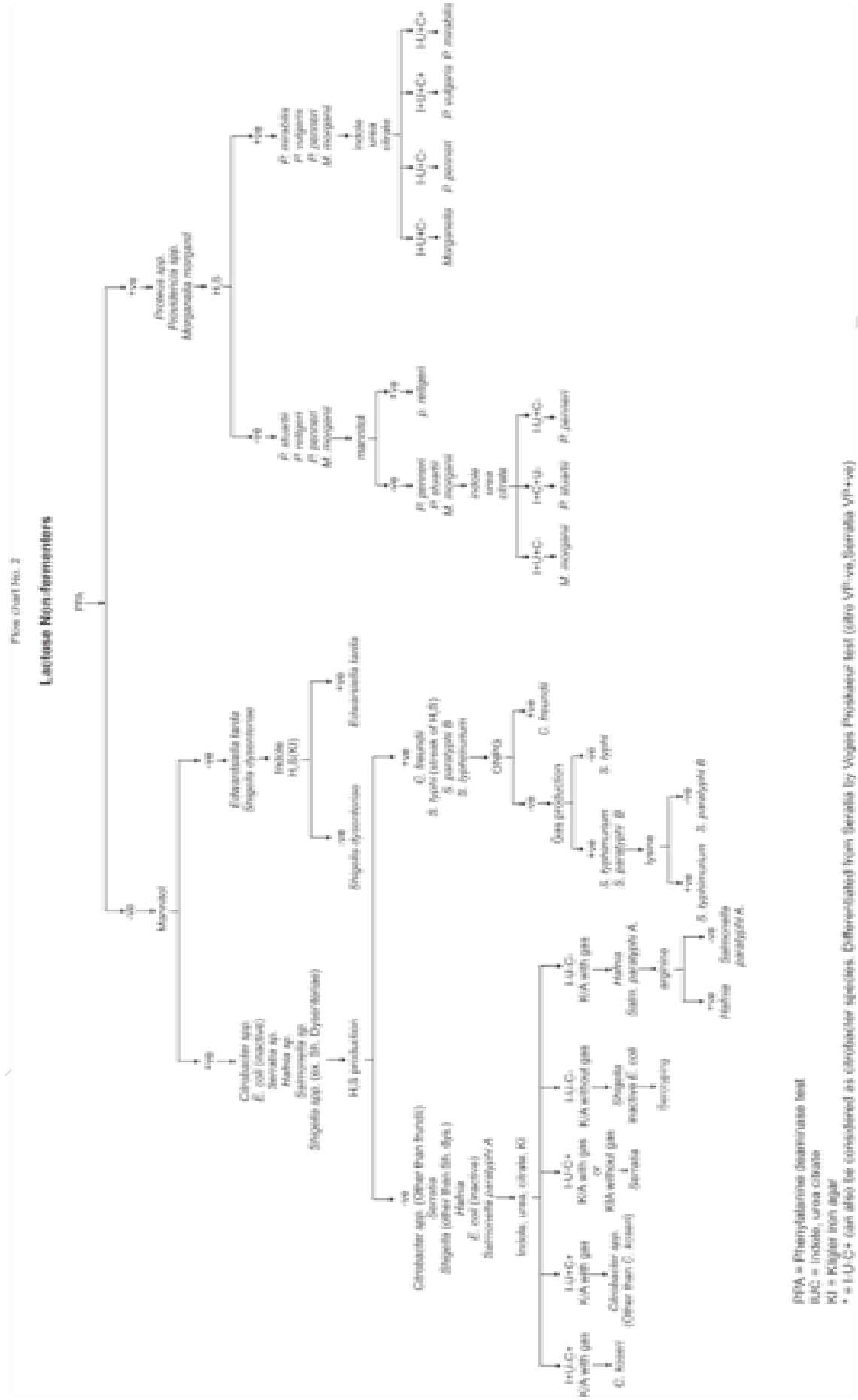

Flow chart No. 1
Lactose fermenters
Indole
+ve
E. coli
K. oxytoca
C. koseri
U, C, KI
U- C+
A/A with gas
C. koseri
U+ C+
A/A with gas
C. koseri
K. oxytoca
Motility
Motile
Citrobacter spp.
Non-motile
K. oxytoca
U- C-
A/A with gas
E. coli
-ve
K. pneumoniae
Citrobacter freundii
Entrerobacter cloacae
Enterobacter aerogenes
U, C, KI
U+ C+
A/A with gas
Klebsiella
Enterobacter
Motility
Motile
Enterobacter
lysine
arginine
lysine +
arginine -
E. aerogenes
lysine -
arginine +
E. cloacae
Non-motile
K. pneumoniae
U- C+
A/A with gas
Enterobacter
lysine
arginine
lysine +
arginine -
E. aerogenes
lysine -
arginine +
E. cloacae
U+/-C-
KI-H2S
C. freundii
U,C,KI = urea, citrate, kligler iron


Flow chart No. 2
Lactose Non-fermenters
PFA
-ve
Manitol
+ve
Proteus spp.
Providencia spp.
Morganella morgani
H2S
-ve
Edwardsiella tarda
Shigella dysenteriae
Indole
H2S(KI)
+ve
Edwardsiella tarda
-ve
Shigella dysenteriae
+ve
Citrobacter spp.
E. coli (inactive)
Serratia sp.
Hafnia sp.
Salmonella sp.
Shigella spp. (ex. Sh. Dysenteriae)
H2S production
+ve
Citrobacter spp. (Other than Koseri)
Serratia
Shigella (other than Sh. dys.)
Hafnia
E. coli (inactive)
Salmonella paratyphi A
Indole, urea, citrate, KI
I+U+C+
KIA with gas
Citrobacter spp.
(Other than C. Koseri)
I+U+C+
KIA with gas
or
KIA without gas
Serratia
I+U-C-
KIA without gas
Shigella
inactive E. coli
Salmonella
I+U-C+
KIA with gas
C. koseri
+ve
C. freundi
S. typhi (strain of H2S)
S. paratyphi B
S. typhimurium
ONPG
-ve
Gas production
+ve
S. typhimurium
S. paratyphi B
-ve
C. freundi
S. typhi
Hafnia
Salm. paratyphi A
arginine
+ve
Hafnia
-ve
S. typhimurium
Salmonella paratyphi A
S. paratyphi B
+ve
Indole
-ve
Edwardsiella tarda
+ve
P. mirabilis
P. vulgaris
P. penneri
M. morgani
Indole
urea
citrate
I+U+C+
P. vulgaris
I+U+C-
P. penneri
I+U-C+
Morganella
P. mirabilis
-ve
P. mirabilis
P. vulgaris
P. penneri
M. morgani
malonate
+ve
P. vulgaris
-ve
P. penneri
P. mirabilis
M. morgani
Indole
urea
citrate
I+U+C-
P. penneri
I+U+C+
P. mirabilis
I+U-C+
M. morgani
PFA = Phenylalanine deaminase test
IUC = Indole, urea citrate
KI = Kligler iron agar
* = I+U-C+ can also be considered as citrobacter species. Differentiated from Serratia by Voges Proskauer test (citro VP+ve,Serratia VP++ve)