# Application of CHROMagar Orientation in the identification and differentiation of *Proteeae* from other *Enterobacteriaceae*

J. Merlino

*Department of Microbiology and Infectious Diseases, Concord Repatriation General Hospital, Concord*

**ABSTRACT: This study describes a new chromogenic plate medium, CHROMagar Orientation, that may be used for the identification and differentiation of common members of the *Proteeae* tribe from other *Enterobacteriaceae*. Of 617 different strains of *Proteeae* tested on this plate media, 507 gave a clear–brown diffused melanin–like pigmented colony colour. Species in this group included *Proteus mirabilis* (n = 449), *Proteus vulgaris* biogroup 3 (n = 18), *Proteus penneri* (n = 6), *Providencia stuartii* (n = 29) *Providencia rettgeri* (n = 4) and *Providencia alcalifaciens* (n = 1). By contrast *Morganella morganii* (n = 87) isolates tested produced a clear transparent colour with slight brownish background. It was difficult to distinguish these isolates by colour alone from the above mentioned species. Of 23 strains of *Proteus vulgaris* biogroup 2 tested, all produced a distinctive blue–green colony colour with a brown diffused background. *Proteus vulgaris* biogroup 2 isolates could be visually distinguished from other *Proteeae* and enterobacteria without further biochemical testing. By combining this colony colour characteristic with a proposed reduced sequence of identification directly on the chromogenic media, all members of the *Proteeae* could be identified to a species level and correlated well with other multi–test systems.**

*Keywords:* CHROMagar Orientation, chromogenic plate media, *Proteeae*, *Enterobacteriaceae*, identification, differentiation.

## Introduction

Members of the *Proteeae* are well recognised as important nosocomial pathogens (1–5). They are found in soil, water and faecal contaminated materials, they are also isolated from blood, urine, stools, bronchial exudates, ulcers, abdominal wounds and other clinical sites (6–10). They are frequent colonising flora in hospitalised patients. However, they are also one of the most common species of bacteria isolated from cases of nosocomial urinary tract infections and usually associated with long–term urinary catheterisation (6–8,10–11). *Proteus mirabilis* is one of the major aetiologic causes of community–aquired urinary tract infections. Some of the *Proteeae* members are also resistant to certain antibiotics commonly used in hospital practice, this makes their identification important when isolated from clinically significant sites (2–4,7,12,13).

This study describes the routine application of CHROMagar Orientation, a new chromogenic plate medium, in combination with a few simple biochemical tests that can be used for the identification and differentiation of the most frequently encountered *Proteeae* in routine laboratories. Such a pattern of investigation could be used together with molecular techniques by hospital laboratories when investigating episodes of cross infection and the spread of these organisms within hospital environments. Small clinical laboratories may also find ways to use our scheme to expedite identifications of isolates as an alternative to the setting up of expensive multi–test systems.

*Address for correspondence:*
Mr J. Merlino
Department of Microbiology and Infectious Diseases
Concord Repatriation General Hospital
Hospital Road
Concord NSW 2139

## Materials and methods

*Bacterial strains*

Bacterial strains isolated by routine means from all body sites and catheter specimens were included in this study. Majority of the bacterial strains tested were recovered randomly from urine specimens, however blood culture and wound isolates were also included. One strain of *P. alcalifaciens* was isolated from faeces. All bacterial isolates were identified in the Department of Microbiology and Infectious Diseases, Concord Repatriation General Hospital, Sydney.

Reference organisms: *Enterobacter cloacae* ATCC13047; *Escherichia coli* ATCC35218; and *Proteus vulgaris* ATCC6380; were used to quality control the media and assess colour stability.

*Culture media*

CHROMagar Orientation was obtained as powdered medium from CHROMagar Company, Paris, France

through DUTEC Diagnostics, Sydney. The medium contained peptone, meat, and yeast extract (16 g per liter); agar (15 g per liter); and in addition 'chromogenic mix', pH 7. The medium was dispensed into petri dishes (17 – 20 mL into 85 mm dishes). The chromogenic agar was prepared, incubated, and plates read according to the manufacturer's instructions with minor modifications when reading, as shown in our previous study (14). MacConkey agar (CM7, Oxoid, Basingstoke, England) and 5 per cent horse blood agar (Oxoid base) were also used to subculture and test for viability. Mast ID (Mast International Ltd, Merseyside, U.K.) replicator medium was prepared, incubated and read according to manufacturer's instructions.

*Bacterial identification and comparative test procedures*

All isolates were first identified by Mast ID systems using a multipoint inoculation or replicator technique (14–15). The biochemical test reactions used at Concord Hospital were identical to those appearing on the API 20E strip, namely o–nitrophenyl B–d–galactopyranoside (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate (CIT), hydrogen sulphide production (H2S), urease (UREA), phenyl pyruvic acid production (PPA), indole (IND), Voges–Proskauer (VP), gelatinase (GEL), and the fermentation of glucose (GLU), mannitol (MAN), inositol, (INOS), sorbitol (SORB), rhamnose (RHAM), sucrose (SUC), melibiose (MEL), amygdalin (AMYG) and arabinose (ARAB). With the addition of DNase reaction in the Mast ID system. The API 20E data base was used to identify the organisms from the biochemical profile obtained. All organisms demonstrated a described colour on CHROMagar Orientation and a normalised probability of 95 to 99 per cent identification. Organisms showing a lower probability were further identified by API 20E (bioMerieux, France). Results obtained were further analysed by computer from data base API 20E Version 3.1. Discrepant tests were supplemented by further manual tests such as adonitol and salicin (16,17). *Proteus vulgaris* species were categorised into biogroups based on the esculin and salicin reactions as described by Farmer *et al.* (16). *Proteus vulgaris* biogroup 2 is esculin positive and salicin postive. While *Proteus vulgaris* biogroup 3 is esculin negative and salicin positive.

Spot tests: Indole spot tests were performed as described by Vracko and Sherris (18). Confirmatory direct testing was performed by washing pure cultures with sterile saline and a small amount of suspension placed in a test tube and Kovacs' reagent added (DMABA, p–dimethylaminobenzaldehyde – positive results reddish–purple to red colour) or DMACA (p–Dimethylamino–cinnamaldehyde – positive results blue to green colour).

Enzymatic tablet tests: Double Tests tablet (Rosco Diagnostica, Taastrup, Denmark) for ornithine decarboxylase and indole reactions were used. A dense suspension (at least McFarland No. 2) of the strain to be tested in 0.25 mL of saline in a small tube, was prepared. One diagnostic tablet and 3 drops of paraffin oil were added and the tube was covered. Incubated at 35 to 37°C for 3 to 4 hours. A positive reaction was a blue or violet colour. A negative reaction was a yellow, green or grey colour. After reading the ornithine decarboxylase test, 3 drops of Kovacs' reagent was added and shaken gently and read after 3 minutes. Only the colour of the surface layer was looked at. A positive reaction was a red or pink colour on the surface layer. A negative reaction was a yellow colour. For specific sugar fermentation tablet tests (mannitol, inositol), a suspension as described above (at least McFarland No. 2) of the strain that was to be tested was prepared, one sugar diagnostic tablet (Rosco Diagnostica, Taastrup, Denmark) was added. This was incubated at 35 to 37°C for 4 hours. A positive reaction was yellow to yellow–orange. A negative result was red to orange–red.

*Multipoint inoculation technique*

This technique has been previously described (14). All isolates were emulsified in phosphate buffered saline (pH 7.4) (BR 14a, Dulbecco A tablets, Oxoid), and the suspension was standardised to contain $10^8$ CFU/ml. Mast multipoint inoculator was used to inoculate the bacterial suspension onto the agar. In this case, Mast multipoint inoculator (Mast sytems) was used to apply a maximum of 36 organisms with an approximate inoculum of $10^4$ CFU each to a CHROMagar Orientation plate and MacConkey plate (Oxoid, CM7) to check organism viability. Inoculated plates were incubated in air at 35 to 37°C for 16 to 24 hours in the dark prior to reading as instructed by the manufacturer.

*Interpretation of results*

Interpretation of colour on CHROMagar Orientation was as described in our previous study (14). Colour was matched to the profile identification systems, and later a data base of consistency of single test reactions to colour was analysed. The aim was to obtain an accurate and precise identification for an organism using CHROMagar Orientation with a diminished number of manual single tests. It compared well with other multi–test commercial kits routinely used. To facilitate the practicability of the use of this medium in routine laboratories a series of flow charts based on our data was created (Figure 1), very similar to the practical work flow devised by Edberg and Trepeta (19).

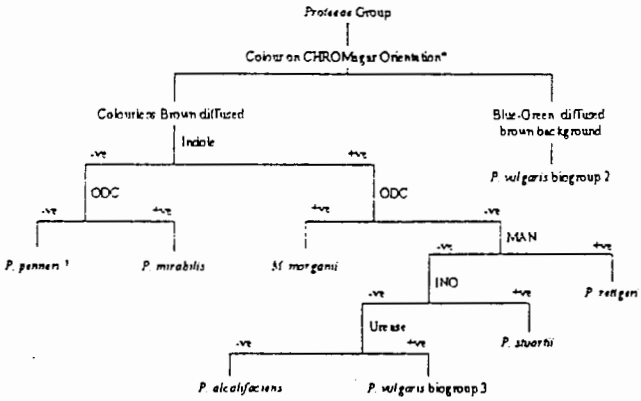


Figure 1. Algorithm for identification of common *Proteea* members using CHROMagar Orientation and a few simple tests. *See Table 1 for explanation of abbreviated tests; † Usually resistant to chloramphenicol.

## Results

All 617 members of the *Proteeae* tribe surveyed in this study grew on CHROMagar Orientation after 24 hours incubation, at 35 to 37°C, and were all differentiated by colour and colony morphology from other common *Enterobacteriaceae*.

*Proteus mirabilis* (n = 449), *Proteus penneri* (n = 6), *Proteus vulgaris* biogroup 3 (n = 18), *Providencia stuartii* (n = 29), *Providencia rettgeri* (n = 4) and *Providencia alcalifaciens* (n = 1) all produced clear–brown diffused colonies on CHROMagar Orientation, *Proteus vulgaris* biogroup 2 (n = 23) produced a blue–green colony colour with a diffused brown background. Of the *Morganella morganii* isolates tested (n = 87), they all produced a clear transparent colour with a slight brownish background.

TABLE 1
Members of *Proteeae* strains tested on CHROMagar Orientation with key reactions

| Species | Number of strains tested tested n = 617 | Colour on CHROMagar Orientation* | Key biochemical tests† | | | |
|---|---|---|---|---|---|---|
| | | | IND | ODC | MAN | INO |
| *Proteus spp.* | | | | | | |
| *P. mirabilis* | 449 | C–B diffused | 0¶ | 449 | 0 | 0 |
| *P. vulgaris* bioG2 | 23 | B–G diffused | 23 | 0 | 0 | 0 |
| *P. vulgaris* bioG3‡ | 18 | C–B diffused | 18 | 0 | 0 | 0 |
| *P. penneri* | 6 | C–B diffused | 0 | 0 | 0 | 0 |
| *Morganella spp.* | | | | | | |
| *M. morganii* | 87 | CT–B | 87 | 87 | 0 | 0 |
| *Providencia spp.* | | | | | | |
| *P. stuartii* | 29 | C–B diffused | 29 | 0 | 0 | 29 |
| *P. alcalifaciens* | 1 | C–B diffused | 1 | 0 | 0 | 0 |
| *P. rettgeri* | 4 | C–B diffused | 4 | 0 | 4 | 4 |

* C–B – clear brown diffused; CT–B – clear transparent brown diffused; B–G – blue green.
† Key reactions: IND – indole spot test; ODC – ornithine decarboxylase; MAN – mannitol; INO – inositol.
‡ *Providencia alcalifaciens* is urease negative but has similar reactions. *P. alcalifaciens* may be also confirmed by a positive adonitol reaction.
¶ Negative reaction.

By using CHROMagar Orientation coloured characteristics with the described algorithm in Figure 1, each member of the *Proteeae* tribe tested was identified accurately to the species level. This algorithm incorporated 4 simple biochemical test reactions, IND (spot indole), ODC (ornithine decarboxylase), MAN (mannitol fermentation), and INO (inositol fermentation). The identification scheme proposed was compared to the multi-test Mast ID system and gave an accurate (100%) correlation account of all organisms tested.

When in mixed culture, members of the *Proteeae* tribe could be easily distinguished from other *Enterobacteriaceae* on CHROMagar Orientation by colour and colony morphology as described in our previous study (14). Direct plating of urine (n = 10) specimens which yielded pure or mixed cultures of members of the *Proteeae* tribe on CHROMagar Orientation also confirmed that there was no interference in colour or pigment production for organisms by either mucus or cellular materials.

## Discussion

Results of this study show that CHROMagar Orientation was a good chromogenic agar in the identification and differentiation of the most common members of the *Proteeae* tribe from other *Enterobacteriaceae*. Other species of *Enterobacteriaceae* appeared different in colour on this agar, as described in our previous published study and shown on Table 2. The characteristic clear-brown colour pigment produced by *Proteus mirabilis*, *Proteus penneri*, *Proteus vulgaris* biogroup 3, *Providencia stuartii*, *Providencia rettgeri* and *Providencia alcalifaciens* was exclusively produced by these species of organisms (14,20,21). *Proteus vulgaris* biogroup 2 however, produced its own distinctive blue-green colour with a brown diffused background. All of the 23 different strains tested in this study produced this distinctive colour and could be visually identified without further biochemical testing. To our knowledge this is the first time that this species has been described on this new chromogenic plate media and demostrated to exhibit this coloured characteristic. In the past this organism has been confused with both *Proteus mirabilis*, and *Proteus penneri* (10,16,22).

TABLE 2
Colour appearance of other common members of *Enterobacteriaceae* on CHROMagar Orientation after 24 hours incubation

| Species | Colony colour on CHROMagar Orientation (14) |
|---|---|
| *Escherichia coli* | Pink-red* |
| *Klebsiella spp.* | Metallic blue with or without pink halo |
| *Enterobacter spp.* | Metallic blue with or without pink halo |
| *Citrobacter diversus* | Light bluish green with strong purple halo |
| *Citrobacter freundii* | Metallic blue with strong purple pink halo or violet purple interior with pinkish border |
| *Serratia marcescens* | Light aqua blue – may darken to navy blue with pigment production† |
| *Serratia liquifaciens* | Light aqua blue |

* *O*nitrophenyl-β-D-galactopyranoiside (ONPG) positive strains. ONPG negative strain colourless.
† After exposure to room temperature and light for 60 minutes.

All of the *Morganella morganii* strains tested produced a clear transparent colour with a brownish appearance, and this together with the spot indole test and a positive ODC reaction confirmed their identification.

*Providencia alcalifaciens*, a less frequently encountered member of this tribe, was differentiated from *Proteus vulgaris* biogroup 3 by being INO negative and urease negative (16,17).

In previous studies different authors (16,17) advocated the use of additional biochemical test reactions such as D-arabitol, adonitol, salicin, esculin and trehalose to differentiate members of the *Proteeae*. In this study there was no need to use these tests in conjunction with CHROMagar Orientation.

By combining the colour characteristic on CHROMagar Orientation with four additional biochemical tests (Table 1), and by following the proposed algorithm (Figure 1), all of the 612 different strains in this study were accurately identified and matched with the Mast ID multi-test system results. Some of the biochemical tests performed on the chromogenic medium were enzymatic,

these are available as 4 hour tests or less (indole spot test) and final results were obtained within 24 hours (18,23).

One of the distinct advantages of this medium was that it inhibited the swarming of isolates on the surface of the medium, a characteristic exhibited by certain members of the *Proteeae* group, allowing easy visualisation of other growth on the plate medium.

Other than a desire for accuracy, there are other reasons for obtaining the complete identification of clinical isolates in a laboratory (11,20,21). Infection epidemiology and control may be improved by accurate identification and reporting of isolates. The *Proteeae* tribe are well recognised nosocomial pathogens and some are resistant to antibiotics in hospital practice (1–4).

*Morganella morganii*, *Providencia stuartii*, *Providencia rettgeri* and *Providencia alcalifaciens* have Amp C β–lactamases with inducible expression of enzymes (12,13,24). *Proteus vulgaris* and *Proteus penneri* isolates have Class I chromosomal β–lactamases (Bush *et al.*, group 2e) and are sometimes referred to as cefuroximase types. Ampicillin, amoxycillin, and first generation cephalosporins are likely strong inducers for *P. vulgaris* and *P. penneri* enzymes, hence their MICs for inducible and depressed strains are high. Ureido– and carboxy–penicillins, and third generation cephalosporins are likely weak inducers and, therefore, remain initially active against inducible strains. In *Proteus mirabilis*, the most commonly encountered member of the *Proteeae* tribe in this survey, chromosomal β–lactamase expression is negligible. Knowledge of the pathogenic organism is important in the empiric selection of appropriate antibiotic therapy (2,12,13,24).

## Conclusion

CHROMagar Orientation has the added advantage over MacConkey agar and other previously described selective differential plate media, in that it allows accurate differentiation of the *Proteeae* tribe while at the same time differentiating and identifying other *Enterobacteriaceae* that may be present in culture. The clear brown colour and diffused melanin–like pigment was produced distinctively by all members of this group with the exception of *Proteus vulgaris* biogroup 2, which could be distinguished by its own individual colour. By combining this medium characteristics with the described algorithm in Figure 1, all of the common members of this group of organisms encountered in this study could be easily and accurately identified to the species level.


## Acknowledgement

I wish to thank the staff of the Department of Microbiology and Infectious Diseases at Concord Repatriation General Hospital for their technical assistance. The skilled support of Mohammad Siddique for the media preparation was greatly appreciated. I also thank Dr. Ross Bradbury, Dr. Thomas Gottlieb, Dr. Gabrielle O'Kane, and Mr. Glenn Funnell for the critical review of the article.


## References


1. Burke JP, Ingall D, Klein JO, Gezon HM, Finland M. *Proteus mirabilis* infections in a hospital nursery traced to a human carrier. *N Eng J Med* 1971; 284: 115–121.
2. Chow AW, Taylor PR, Yoshikawa TT, Guze LB. A nosocomial outbreak of infections due to multiply resistant *Proteus mirabilis*: role of intestinal colonization as a major reservoir. *J Infect Dis* 1979; 139: 621–627.
3. Kocka FE, Srinivasan S, Mowjood M, Kantor HS. Nosocomial multiply resistant *Providencia stuartii*: a long–term outbreak with multiple biotypes and serotypes at one hospital. *J Clin Microbiol* 1980; 11: 167–169. (Erratum 12:295.)
4. Tucci V, Isenberg HD. Hospital clusters epidemic with *Morganella morganii*. *J Clin Microbiol* 1981; 14: 563–566.
5. Williams EW, Hawkey PM, Penner LJ, Senior BW, Barton LJ. Serious nosocomial infection caused by *Morganella morganii* and *Proteus mirabilis* in a cardiac surgery unit. *J Clin Microbiol* 1983; 18: 5–9.
6. Breitenbucher RB. Bacterial changes in the urine samples of patients with long–term indwelling catheters. *Arch Intern Med* 1984; 144: 1585–1588.
7. Clayton CL, Chawla JC, Stickler DJ. Some observations on urinary tract infections in patients undergoing long–term bladder catheterization. *J Hosp Infect* 1982; 3: 3 9–47.
8. Hawkey PM, Penner JL, Potten MR, Stephens M, Barton LJ, Speller DCE. Prospective survey of fecal, urinary tract, and environmental colonization by *Providencia stuartii* in two geriatric wards. *J Clin Microbiol* 1982; 16: 422–426.
9. Hawkey PM, Potten MR, Stephens M. The use of preenrichment for the isolation of small numbers of gentamicin–resistant *Providencia stuartii* from faeces. *J Hosp Infect* 1982; 3: 369–374.
10. Muller HE. Occurence and pathogenic role of Morganella–Proteus–Providencia group bacteria in human feces. *J Clin Microbiol* 1986; 23: 404–405.
11. Damron DJ, Warren JW, Chippenadle GR, Tenney JH. Do clinical laboratories report complete bacteriology in urine from patients with long–term catheter? *J Clin Microbiol* 1986; 24: 400–404.
12. Bush K. Characterization of β–Lactamases. *Antimicrob Agents Chemother* 1989; 33: 259–276.
13. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for ,B–Lactamases and its correlation with molecular structures. *Antimicrob Agents Chemother* 1995; 39: 1211–1233.
14. Merlino J, Siarakas S, Robertson GJ, Funnell GR, Gottlieb T, Bradbury R. Evaluation of CHROMagar Orientation for differentation and presumptive identification of gram–negative bacilli and Enterococcus species. *J Clin Microbiol* 1996; 34: 1788–1793.
15. Funnell GR, Parkinson DL, Bradbury R. Biochemical identification of enterobacteriaceae by multipoint inoculation using Mast ID media. *Aust J Med Lab Sci* 1986; 7:75–77.
16. Farmer JJ III, Davis BR, Hickman–Brenner FW, McWhorter A, Huntley–Carter GP, Asbury MA, Riddle C, Wathen–Grady HG, Elias C, Fanning GR, Steigerwalt AG, O'Hara CM, Morris GK, Smith PB, Brenner DJ. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J Clin Microbiol* 1985; 21: 46–76.
17. Kelly MT, Brenner DJ, Farmer III JJ. In: Lennette EH, Balows A, Hausler WJr, Truant JP, 4th ed. Manual of Clinical Microbiology. Washington, D.C: American Society for Microbiology, 1985; 263–277.
18. Vracko R, Sherris JC. Indole spot–test in bacteriology. *Am J Clin Path* 1963; 39: 429–432.
19. Edberg SC, Trepeta RW. Rapid and economical identification and antimicrobial susceptibility test methodology for urinary tract pathogens. *J Clin Microbiol* 1983; 18: 1287–1291.
20. Hawkey PM, McCormick A, Simpson RA. Selective and differential medium for the primary isolation of members ofthe *Proteeae*. *J Clin Microbiol* 1986; 23: 600–603.
21. Xilinas ME, Papavassiliou J, Legakis NJ. Selective medium for growth of *Proteus*. *J Clin Microbiol* 1975; 2: 459–460.
22. Hickman FW, Steigerwalt AG, Farmer III JJ, Brenner DJ. Identification of *Proteus penneri* sp. nov., formerly known as *Proteus vulgaris* indole neg. or as *Proteus vulgaris* biogroup 1. *J Clin Microbiol* 1982; 15: 1097–1102.
23. Casals JB. Rapid chromogenic enzymatic tests for screening and identification of bacterial strains. 1st European Congress of Clinical Microbiology, 1983; paper 23, Bologna (Italy).
24. Livermore DM. β–Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev* 1995: 8: 557–584.