Efficacy of Two Commercial Systems for Identification of Clinical and Environmental *Escherichia coli*

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Abstract

The aim of this study was to test the efficacy of API 20E and Fluorocult® LMX broth in identifying a collection of 200 *E. coli* isolates. A total of 100 isolates originated from clinical samples (UTI) and 100 isolates from environmental water receiving faecal contamination. Randomly selected isolates that were identified by API 20E and Fluorocult® LMX broth were further identified by PCR targeting a fragment of the *E. coli* 16S rRNA gene. The results showed that overall 95% and 100% of the clinical and environmental isolates respectively were identified with various degrees of accuracy as *E. coli* by API 20E. However, only 86% of the clinical isolates and 32% of environmental isolates were identified with high level of discrimination (90% and above). Identification by Fluorocult® LMX broth successfully identified 90% and 96% of the clinical and environmental isolates respectively as *E. coli*. Further identification by PCR showed that 70% (n = 20) and 55% (n = 20) of the isolates that were previously identified by the two commercial systems were successfully identified by PCR. Identification of *E. coli* isolates of clinical and environmental origins by rapid commercial systems should be interpreted with care, PCR might be used to further confirm the result of rapid identification systems.

Keywords: API 20E, *Escherichia coli*, Fluorocult® LMX broth, PCR

1. Introduction

Escherichia coli is a common inhabitant of the intestinal tract of humans and warm blooded animals (Ashbolt et al., 2004). Generally, *E. coli* is regarded as a commensal in the intestinal tract, yet there are a number of strains that cause diarrheal diseases through contaminated drinking water and food (Chen & Frankel, 2005; Bettelheim, 2007), commensal or non-pathogenic strains of *E. coli* can cause a variety of infections in humans such as urinary tract infection (UTI), bacteremia and meningitis (Janda & Abbott, 2006). Given the exclusive faecal origin of *E. coli*, their presence in the environment, particularly in water is a strong indication of faecal contamination and represents a risk of disease (Leclerc et al., 2001). Thus *E. coli* was suggested to be the best biological faecal indicator and has been recommended by World Health Organization for the microbiological assessment of safe drinking water supplies (Edberg et al., 2000; Tallon et al., 2005).

Escherichia coli is exogenous to aquatic environments, and it is expected that the bacterium would not be able to grow in water due to unfavourable environmental conditions. However, a number of studies have shown that *E. coli* can persists for prolonged periods in various types of aquatic environments, surviving the hostile environmental abiotic and biotic factors (Davies & Evison, 1991; Alkan et al., 1995; Janakiraman & Leff, 1999; Whitman et al., 2004; Wcisto & Chróst, 2000; Wanjugi & Harwood, 2013), being able to survive in water, *E. coli* may undergo physiological and morphological changes probably as a survival strategy. These changes may, in part, enable the bacteria to enter the debatable viable-but-nonculturable (VBNC) stage, which make these bacterial cells undetectable by conventional culture media, yet still viable and may retain their virulence factors and pathogenicity (Xu et al., 1982; Barer & Harwood, 1999; Pommepuy et al., 1996; Oliver, 2000; Pinto et al., 2011).

Different commercial miniaturized systems were developed to identify presumptive *E. coli* isolates of clinical and environmental origins. These identification systems include the widely used API 20E (bioMerieux, France) and the Fluorocult® LMX Broth (Merck, Germany). The API 20E is one of the original miniaturized systems, still in wide spread use for the identification of members of the *Enterobacteriaceae* since the 1970s. This system is based on 20 different biochemical tests such as the production of indole; citrate utilization; voges-proskauer

reaction; carbohydrate fermentation and other tests (Smith et al., 1972). Fluorocult® LMX broth is a modified lauryl sulfate tryptic broth for simultaneous detection of coliforms and *E. coli*. This selective enrichment broth contains the chromogenic substrate 5-bromo-4-chloro-3 indolyl-β-D-galactopyranoside, cleaved by coliforms' β-D-galactosidase, and the fluorogenic substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG), which is highly specific for *E. coli* β-D-glucuronidase (Ossmer, 1993).

The current study aims to evaluate the efficacy of the API 20E and Fluorocult® LMX broth in identifying a collection of *E. coli* isolates that were recovered from environmental water and clinical specimens. Further confirmation was performed by PCR amplifying a target fragment of the *E. coli* 16S rRNA gene.

2. Materials and Methods

2.1 Bacterial Isolates

A collection of 200 *E. coli* isolates were used to evaluate the efficacy of two widely used commercial identification systems; the API 20E and the Fluorocult® LMX broth. A total of 100 *E. coli* isolates were obtained from clinical laboratories mainly derived from urine samples of patients suffering a urinary tract infection (UTI), these isolates will be referred to as clinical *E. coli* (CEC). Another 100 *E. coli* isolates were recovered from fresh water receiving faecal contamination and rain-related run-off, these isolates will be referred to as environmental *E. coli* (EEC). Detection of environmental *E. coli* isolates was carried out as described by Abulreesh et al. (2004). Briefly, water samples were collected into sterile polypropylene bottles. A volume of 100 ml subsample was assayed by colony counts on 0.45 μm membrane filters. Membrane filters were incubated on eosin methylene blue agar (EMB) (Oxoid, Basingstoke, UK), plates were initially incubated at 30 °C for 4 hours, followed by 14 hours at 44 °C. Randomly six typical *E. coli* colonies (colonies with 2-3 diameter, exhibiting a greenish metallic sheen by reflected light and dark purple centers by transmitted light), were picked up from each plate (total 100 colonies) and used for further tests. The *E. coli* K-12 strain was used as a positive control for all identification methods including PCR.

2.2 Identification of E. coli by API 20E and Fluorocult® LMX Broth

Before the start of identification tests, all 200 isolates were purified on EMB agar plates to ensure that there is no mixed culture that could influence the reactions of the identification systems. A bacterial suspension approximating a 0.5 McFarland standard was prepared from each of the 200 purified isolates and was used for inoculation of the API 20E strips (bioMerieux, France), incubation was at 37 °C for 24 hours, after incubation the addition of reagents and interpretation of reactions were done according to manufacturers' directions. The 20 biochemical test reactions were converted into an octal profile number. Each profile number was then decoded by using the Analytical Profile Index for the completion of identification.

All 200 previously purified isolates were identified by culture in Fluorocult® LMX broth (Merck, Germany), aliquots of 1.0 ml of the broth were aseptically transferred into a U-bottom sterile polystyrene well cluster (Coaster, Coring Inc., NY, USA). Each well was inoculated with a single purified *E. coli* colony using an inoculation loop. Incubation of the wells was at 37 °C for 24 hours. The formation of blue-green colour (β-D-galactosidase) together with the observation of fluorescence (β-D-glucuronidase) under UV transillumination (366 nm) confirmed that an isolate was *E. coli* (Ossmer 1993).

2.3 Identification by PCR Detecting Fragment of the E. coli 16S rRNA Gene

Identification by PCR was carried out on a randomly selected 40 isolates (20 clinical and 20 environmental), some of these isolates were identified as *E. coli* by API 20E and Fluorocult® LMX broth, and other isolates were not identified by these two systems. The primers used for PCR assay targeted fragment of *E. coli* 16S rRNA gene, the primers RW01 ('5-AACTGGAGGAAGGTGGGGAT-3') and DG74 ('5-AGGAGGTGATCCAAGCA-3') (Invitrogen, Paisley, UK), is expected to give a PCR product of 371 bp as described by (Tsai & Olson, 1992). Bacterial rRNA was extracted by suspending a loopful of *E. coli* colonies in 100 μl of sterile, pure water and boiling for 5 minutes. The suspension was then centrifuged for 5 minutes at 1260 g and 10 μl of the supernatent were used as target DNA. The PCR reaction mixture (50 μl total volume) consisted of the following: 25 μl of 2 x PCR master mix (ABgene, Surry, UK) contains the following: 75 mmol l^{-1} Tris-HCL; 20 mmol l^{-1} $(NH_4)_2SO_4$; 20 mmol l^{-1} $MgCl_2$; 0.01% (v/v) Tween® 20; 0.2 mmol l^{-1} each of dATP, aGTP, dCTP and dTTP; 1.25 units of Theroprime Plus DNA Polymerase; 0.5 μl of each primer (0.25 μmol l^{-1}); 10 μl bacterial rRNA extract and 14 μl sterile pure water. The PCR program consisted of initial denaturation at 95 °C for 2 minutes, followed by 40 PCR cycles (each cycle is a 1.5 minutes at 95 °C for denaturation and 1 minute at 62 °C for annealing and extension) and a 7 minutes final extension at 62 °C (Tsai & Olson, 1992). The PCR products were analyzed by electrophoresis on a 2.0% agarose gel (BioLine, London, UK), and made visible by ethidium bromide (1.0 μg

ml^{-1}) staining and UV transillumination.

2.4 Statistical Analysis

Chi-square test (χ^2) was used to determine the accuracy of API 20E and Fluorocult LMX in identifying all *E. coli* isolates from clinical and environmental sources. The null hypothesis that was tested by Chi-squared statistics reads: Both API 20E and Flurocult LMX broth can accurately identify all *E. coli* isolates regardless of their origin.

3. Results

This study examined the efficacy of the API 20E and the Fluorocult® LMX broth for the identification of a collection of 200 *E. coli* isolates from clinical and environmental origins. Overall 95% of clinical *E. coli* were successfully identified by API 20E with various levels of discrimination (Table 1). All 100 environmental isolates were successfully identified as *E. coli* by API 20E (100%) with various degrees of discrimination (Table 2).

Table 1. Identification of 100 clinical isolates of *E. coli* by API 20E

Level of discrimination	Number of isolates identified	Isolate reference number
Not identified as *E. coli*	5	CEC 1, CEC 12, CEC 22, CEC 27, CEC 35
Low discrimination	8	CEC 3, CEC 5, CEC 26, CEC 40, ECE 77-79, CEC 99
Acceptable discrimination	1	CEC 31
Good discrimination	68	CEC 2, CEC 7-10, CEC 16, CEC 18, CEC 20, CEC 21, CEC 23, CEC 25,CEC 28, CEC 29, CEC 32-34, CEC 37, CEC 38, CEC 41-48, CEC 50, CEC 51, CEC 53, CEC 54, CEC 56-59, CEC 61-76, CEC 81-97, CEC 100
Very good discrimination	12	ECE 6, ECE 13, ECE 14, ECE 17, ECE 24, ECE 30, ECE 36. ECE 39, ECE 49, ECE 52, ECE 55, ECE60
Excellent discrimination	6	CEC 4, CEC 11, CEC 15, CEC 19, CEC 80, CEC 98
Total number of isolates	100	

Table 2. Identification of 100 environmental *E. coli* isolates by API 20E

Level of discrimination	Number of isolates identified	Isolate reference number
Not identified as *E. coli*	0	
Low discrimination	66	EEC 2, EEC 3, EEC 5, EEC 6, EEC 9, EEC 11, EEC 12, EEC 15-17, EEC 19-25, EEC 28, EEC 30-42, EEC 44, EEC 45, EEC 44, EEC 47, EEC 48, EEC 52-55, EEC 58, EEC 59, EEC 61-69, EEC 72-76, EEC 81, EEC 83, EEC 84, EEC 87, EEC 89, EEC 91, EEC 96-100
Acceptable discrimination	2	EEC 43, EEC 70,
Good discrimination	18	EEC 1, EEC 4, EEC 7, EEC 8, EEC 26, EEC 29, EEC 46, EEC 49, EEC 56, EEC 57, EEC 77-80, EEC 90, EEC 93-95
Very good discrimination	10	EEC 10, EEC 13, EEC 14, EEC 18, EEC 50, EEC 51, EEC 60, EEC 82, EEC 85, EEC 86
Excellent discrimination	4	EEC 27, EEC 71, EEC 88, EEC 92
Total number of isolates	100	

Given the importance of accurate identification of these isolates, it can be seen from Table 3 that 86 isolates of the clinical *E. coli* were identified by API 20E with high levels of discrimination (90% and above), in contrast, 68 isolates of the environmental *E. coli* isolates were identified with low levels of discrimination (75% and below). Statistical significant difference ($P < 0.01$, χ^2) showed that API 20E accurately identified more clinical *E. coli* isolates that those of environmental origin (Table 3).

Table 3. Levels of identification for 200 clinical and environmental *E. coli* isolates by API 20E

Level of identification	Clinical *E. coli* (n = 100)	Environmental *E. coli* (n = 100)	P
High level of discrimination (90% and above)	86	32	< 0.01
Low level of discrimination (72% and below)	9	68	
Not identified as *E. coli*	5	0	

P = the probability that API 20E should accurately identify all *E. coli* isolates regardless of their origin (χ^2).

The result of identification of the 200 *E. coli* isolates by Fluorocult® LMX broth is summarized in Table 4. Of the 100 clinical isolates examined, only 90 isolates were identified as *E. coli*, while 96 out of 100 environmental isolates were identified as *E. coli*. No statistical significant difference ($P > 0.5$) was found in the accuracy of identification of *E. coli* isolates from clinical and environmental sources as tested by Chi-squared statistics (Table 4).

Table 4. Identification of 200 *E. coli* isolates by Fluorocult® LMX Broth

Isolates origin	N/P	Isolates that were not identified
Clinical *E. coli*	100/90	CEC 12, CEC 18, CEC 22, CEC 27, CEC 35, CEC 58, CEC 66, CEC 72, CEC 90, CEC 92
Environmental *E. coli*	100/96	EEC 38, EEC 40, EEC 70, EEC 71
P	NS	

N = Total number of isolates tested;

P = Total number of isolates identified as *E. coli*;

P = the probability that Fluorocult LMX broth should accurately identify all *E. coli* isolates regardless of their origin (χ^2);

NS = not significant > 0.5.

It can be noted that of the 10 clinical isolates that were not identified by Fluorocult® LMX broth, four isolates were also not identified as *E. coli* by API 20E (CEC 12, 22, 27, 35), while six of these 10 isolates were identified as *E. coli* by API 20E with good level of discrimination (CEC 18, 58, 66, 72, 90, 92) (Table 5). As far as the environmental isolates are concerns, all of the four isolates that were not identified by Fluorocult® LMX broth, they were identified by API 20E with various degrees of discrimination as follows: one isolate was identified with excellent level of discrimination (EEC 71), one isolate was identified with acceptable level of discrimination (EEC 70) and two isolates with low level of discrimination (EEC 38 and 40) (Table 5).

Table 5. API 20E identification of clinical and environmental *E. coli* isolates that gave negative reaction with Fluorocult® LMX Broth

Isolate reference number	API 20E Identification	Level of discrimination
CEC 12	*Enterobacter agglomerans*	74.75
	Kliebsiella oxytoca	23.2% low discrimination
CEC 18	*E. coli*	96.5% good
CEC22	*Klebsiella oxytoca*	97.4% doubtful
CEC 27	*Citrobacter freundii*	99.9% excellent
CEC35	*Citrobacter freundii*	99.9% excellent
CEC58	*E. coli*	96.5% good
CEC66	*E. coli*	96.5% good
CEC 72	*E. coli*	96.5% good
CEC 90	*E. coli*	96.5% good
CEC 92	*E. coli*	96.5% good
EEC 28	*E. coli*	72% low
	Yersinia aldovae	25%
EEC 40	*E. coli*	72% low
	Yersinia aldovae	25%
EEC 70	*E. coli*	77% acceptable
EEC 71	*E. coli*	99.9% excellent

A total of 40 (20 clinical and 20 environmental) isolate were randomly selected and further identified by PCR targeting a fragment of the *E. coli* 16S rRNA gene. On the whole, PCR successfully identified 14 (70%) out of the 20 clinical isolates that were examined, of which, 13 isolates identified as *E. coli* by API 20E, Fluorocult® LMX broth and PCR, and three isolates (CEC 12, 41 and 35) were not identified as *E. coli* by all three methods (Table 6). A total of four isolates (CEC 2, 9, 20 and 41) were successfully identified by API 20E and Fluorocult® LMX broth, however they gave no PCR products with primers RW01 and DG74 (Table 6).

Table 6. Identification of randomly selected clinical *E. coli* isolates by PCR

Isolate reference number	Identification by API 20E	Identification by Fluorocult® LMX broth	Identification by PCR
CEC 2	good discrimination	+	-
CEC 9	good discrimination	+	-
CEC 11	excellent discrimination	+	+
CEC 12	not identified as *E. coli*	-	-
CEC 15	Excellent discrimination	+	+
CEC 18	good discrimination	-	+
CEC 20	good discrimination	+	-
CEC 28	good discrimination	+	+
CEC 34	good discrimination	+	+
CEC 35	not identified as *E. coli*	-	-
CEC 39	Very good discrimination	+	+
CEC 41	Good discrimination	+	-
CEC 44	Good discrimination	+	+
CEC 49	Very good discrimination	+	+
CEC 55	Very good discrimination	+	+
CEC 58	Good discrimination	+	+
CEC 62	Good discrimination	+	+
CEC 69	Good discrimination	+	+
CEC 75	Good discrimination	+	+
CEC 88	Good discrimination	+	+

Identification of environmental isolates by PCR showed that only 11 isolates out of 20 (55%) produced the expected PCR products. These isolates were also successfully identified by API 20E and Fluorocult® LMX broth (Table 7). Three environmental isolates (EEC 38, 40 and 70) were not identified by PCR and by Fluorocult® LMX broth as well. Five isolates (EEC 5, 12, 41, 42, 43) were not identified by PCR although they were successfully identified by API 20E and Fluorocult® LMX broth (Table 7).

Table 7. Identification of randomly selected environmental *E. coli* isolates by PCR

Isolate reference number	Identification by API 20E	Identification by Fluorocult® LMX broth	Identification by PCR
EEC 3	Low discrimination	+	+
EEC 5	Low discrimination	+	-
EEC 12	Low discrimination	+	-
EEC 18	Very good discrimination	+	+
EEC 25	Low discrimination	+	+
EEC 38	Low discrimination	-	-
EEC 40	Low discrimination	-	-
EEC 41	Low discrimination	+	-
EEC 42	Low discrimination	+	-
EEC 43	Acceptable discrimination	+	-
EEC 44	Low discrimination	+	+
EEC 45	Low discrimination	+	+
EEC 46	Good discrimination	+	+
EEC 47	Low discrimination	+	+
EEC 50	Very good discrimination	+	+
EEC 56	Good discrimination	+	+
EEC 66	Low discrimination	+	+
EEC 70	Acceptable discrimination	-	-
EEC 88	Excellent discrimination	+	+
EEC 92	Excellent discrimination	+	+

4. Discussion

The rapid commercial identification systems were developed to provide fast and accurate identification of bacteria in clinical settings and when investigating health hazards associated with faecal contamination in water and food. The API 20E is a standardized miniaturized system that was developed by Analytab Products Inc. in the 1970s and still in widespread use for the identification of *Enterobacteriaceae* and some other gram negative rods. Published data suggests that *E. coli* isolates are usually identified with high degree of accuracy (96%) by most rapid commercial identification systems, including the API 20E (Janda & Abbott, 2006). Various studies evaluated the efficacy of API 20E system using a collection of clinically originated isolates with typical and atypical strains, and reported an accurate rate of identification between 93-96% (Washington et al., 1971; Smith et al., 1972; O'Hara et al., 1992). The results reported in this study showed that 95% and 100% of the clinical and the environmental *E. coli* respectively were identified by API 20E system with various level of accuracy (Tables 1 and 2). However, in contrast with other studies, 86% of the clinical *E. coli* and 32% of the environmental isolates were accurately identified (Table 3). This low accuracy is probably due to incubation for 24 h only. O'Hara et al. (1992) noted that after 24 h the accuracy of API 20E identification was 87.7%, however after 48 h incubation, they noted an increase to 96.3% accuracy.

In the current study, 86% of the clinical *E. coli* isolates were identified with high level of discrimination, while only 32% of the environmental isolates were identified with the same degree of discrimination ($P < 0.01$, χ^2) (Table 3). This is probably because most of *E. coli* isolated from clinical specimens (e.g. UTI) are tend to be biochemically typical (Janda & Abbott, 2006), in contrast, environmental *E. coli* isolates may exhibit atypical biochemical characteristic due to physiological changes required for better survival in aquatic habitats, it was particularly found that some proteins are repressed while other are induced under stressful conditions (Roszak & Colwell, 1987; Smith et al., 1994; Jordan et al., 1999). The results of this study may suggest that API 20E may produce more accurate identification with clinical but not environmental *E. coli* isolates.

Fluorogenic substrates that detect the activities of β-D-glucuronidase and β-D-galactosidase, two enzymes specific for *E. coli* and coliforms respectively were proposed by Feng and Hartman (1982). These substrates were employed in culture media for direct detection and/or confirmation of *E. coli* and coliforms in environmental samples, particularly investigating faecal contamination in water and food (Blood & Curtis, 1995; Manafi, 1996). Successful detection and/or confirmation of *E. coli* isolates from various water samples employing fluorogenic substartes is well documented (O'Toole & Chang, 1999; Prats et al., 2008). The results reported in this study using Fluorocult® LMX broth showed 90% and 96% accurate identification of *E. coli* isolates from clinical and environmental sources respectively, with no statistical significant difference ($P > 0.5$, χ^2) (Table 4), these results consistent with results reported elsewhere from investigation on water and food (Suwansonthichai & Rengpipat, 2003; Abulreesh et al., 2004; Nikaeen et al., 2009). Of the 200 *E. coli* isolates examined in this study, 14 isolates were not identified by Fluorocult® LMX broth, four of these isolates were also not identified as *E. coli* by API 20E (Table 5). However, the other 10 isolates were identified as *E. coli* by API 20E, these isolates probably β-D-glucuronidase negative, either they do not have the gene (Chang et al., 1989; Manafi, 1996) or they possess but cannot express the gene (Bej et al., 1991; Feng et al., 1991).

In this study, PCR was used to confirm the accuracy of API 20E and Fluorocult® LMX broth by randomly selecting different isolates that were identified as *E. coli* with various degrees of accuracy, and other isolates that were not identified as *E. coli* by both commercial systems. The primers used (RW01 and DG74) were targeting a fragment of the *E. coli* 16S rRNA gene (Tsai & Olson, 1992). The results showed that PCR further confirm the identification of 70% (n = 14) of the clinical isolates (Table 6) and also 55% (n = 11) of the environmental isolates (Table 7). Few isolates (total 5 out of 40) were only identified by API 20E, but not by Fluorocult® LMX broth and PCR (Tables 6 and 7), this might be incorrectly identification by API 20E as *E. coli*, since three of these five isolates rendered low discrimination (75% and below), incorrect identification of *Enterobacteriaecae* by API 20E has been reported due to aberrant reactions by API and/or atypical strains (Washington et al., 1971; Smith et al., 1972; O'Hara et al., 1992). Also *E. coli* strains in particular are extremely phenotypically diverse and groups of biochemically distinct strains exists (Janda & Abbott, 2006). It can be noted from the results of the PCR that 9 isolates of the 40 examined (4 clinical and 5 environmental isolates) were not identified by PCR despite being identified by API 20E and gave positive reaction with Fluorocult® LMX broth (Tables 6 and 7). Incorrect identification by API 20E is a possible explanation, also it has been reported that there are some genera of the *Enterobacteriaceae* (e.g. *Shigella*, *Klebsiella* and *Citrobacter*) may produce a positive fluorescence response with fluorogenic substrates (Manafi, 1996; APHA, 1998; O'Toole & Chang, 1999). In general the results reported in this study with PCR reaffirm that PCR provide sensitivity in the identification of *E. coli* when compared with biochemical and enzyme-substrate methods (Bej et al., 1991; Feng et al., 1991; Rompré et al., 2002).

In conclusion, identification of *E. coli* isolates of clinical and environmental origins by rapid commercial systems should be interpreted with care, API 20E was found to produce more accurate identification with isolates of clinical but not environmental origin, while Fluorocult LMX broth produced high level of accurate identification of *E. coli* isolates regardless of their origin. Perhaps a combination of two methods might be appropriate. However in routine laboratories this might be laborious, and therefore PCR might be used to further confirm the result of rapid identification systems.

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