



Performance of selective media for *E. coli* O157, using non-sorbitol fermenting strains and physiological characters of shiga-toxin producing species isolated in Côte d'Ivoire

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ABSTRACT

Performance of three selective media, temperature and pH effects on growth of *E. coli* O157:H7 isolated in Ivory Coast were investigated. Thirty-eight (38) non-sorbitol fermenting *E. coli* were characterized by serotyping, detection of shiga-toxin gene (PCR) and plating on SMAC, VR-MUG and BCMtm media for Performance evaluation. Temperature and pH effects on *E. coli* O157:H7 were measured in varied culture conditions. All culture media were sensitive (100%) for the growth of *E. coli* O157. Their specificities were 97.3, 94.7 and 50.7% respectively for VR-MUG, BCMtm and SMAC media. Predictive value for VR-MUG medium was 66%, whereas that for SMAC medium was only 5.4%. Serotype O157:H7 *E. coli* population grew slightly at 42 °C and didn't grow under acid conditions (pH 4.5, 3.5). The performance of the culture media evaluated and the physiological data obtained for *E. coli* O157:H7 during this study would contribute to improve both isolation and identification methods of the pathogens.

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INTRODUCTION

Shiga-toxin producing *Escherichia coli* (STEC), especially serotype O157:H7 is a rare variety of *E. coli* which has emerged as an important pathogen since 1982 (Riley et al.,

1983; Wells et al., 1983). These pathogroups are frequently associated with outbreaks of illness. The number of infections caused by STEC has increased significantly since the first reported outbreak and strains are highly

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spread in the world (Mead and Griffin, 1998). It's now well recognized, serotype O157:H7, the prototype of enterohemorrhagic *E. coli* (EHEC) group, produces shiga-toxins that cause severe damage to the intestinal epithelial cells. Attachment and effacement (AE) and enterohemolysin, encoded by *eaeA* and *ehly* genes are the other virulence factors expressed by *E. coli* O157:H7. Illness is characterized by severe abdominal pain, diarrhea which is initially watery but becomes grossly bloody and occasional occurrence of vomiting and fever (Scheutz et al., 2001). Infection progresses to acute kidney failure, hemolytic uremic syndrome (SHU) in less than 10-15% of the cases (Schimmer et al., 2008; Serna and Boedeker, 2008). Children under the age of five and the elderly are the most susceptible (Kaper et al., 2004). Dairy cattle have been identified as the main reservoir and raw milk and milk products, meat and vegetables, principle foods vehicles of STEC (CDC, 2008).

Isolation and detection methods used for *E. coli* O157:H7 are based on specific biochemical reactions. Serotype *E. coli* O157:H7 is characterized by inability to ferment sorbitol in 24 h and lack of β -glucuronidase (Neaves, et al., 1994). Many media like sorbitol MacConkey (SMAC), MUG, HC-medium, and chromogenic media have been developed (Vernozy-Rozand and Montet, 2001). Shiga-toxin detection by using molecular methods (PCR or immunological methods) is an average standard procedure to confirm the presence of STEC in food. Numbers of them prior need enrichment or isolation on plating media as the first step for the assay. Thus, it is necessary to improve sensitivity and specificity of plating media for easy detection of *E. coli* O157:H7 in food or clinical specimens.

The purpose of this study is to evaluate the efficacy of three selective media for *E. coli* O157: H7, using non-sorbitol fermenting strains of *E. coli* and to determine the effects

of pH and temperature on the growth of shiga-toxin producing species isolated in Ivory Coast.

MATERIALS AND METHODS

Control strains and plating media

Thirty eight (38) strains of non-sorbitol fermenting *E. coli* and isolates that belong to previous study (Dadie et al., 2000) were used. Seventeen (17) of them were isolated from milk and milk products and twenty one (21) from stool specimens of human diarrheal patients. The following *E. coli* control strains were used: one Stx1 and Stx2 toxin-producing *E. coli* from BRNO University of Czech Republic; two strains, Stx1 and Stx2 toxins producing *E. coli* from Pasteur Institute, Paris (France). Serotypes non-O157:H7 *E. coli* and sorbitol fermenting *E. coli* were also used as controls. The plating efficiencies were determined using sorbitol MacConkey (SMAC) (Oxoid, Hamp-shire, England), 4-methyl-umbelliferyl- β -D-glucuronide (VR-MUG) (Oxoid, Hamp- shire England), and a chromogenic plating medium, BCMtm (Biosynth, Naperville, IL, USA).

Serotyping

The O and H antigens of *E. coli* strains were detected by standard agglutination as described by Orskov and Orskov (1992). Murex kit (Diagnostics, Inc., Norcross, GA, USA) was used as confirmation to detect serotype O157:H7.

PCRs

Olsvik and Strockbine (1993) technique has been used. A total of 50 μ l PCR master mix was constituted, containing 37.75 μ l sterile water, 5 μ l of 10X PCR buffer (Tris-HCL 100 mM, pH 8.3, KCL 500 mM, MgCl₂ 15 mM, gelatin 0.1%), 5 μ l of 10X dNTPs, 0.5 μ l of 200 μ M each primer, 0.25 μ l of 1U Taq polymerase (Bioprobe, Montreuil sous Bois, France) and 5 μ l of template DNA. The following primers sequences purchased from

genset (Singapore biotechn. ltd) were used: primers stx1 5'- CAG TTA ATG TGG TGG CGA AG-3' and stx3-5'-CAC AGA CTG CGT CAG TGA GG-3' to amplify an 894 bp fragment of *stx1* gene and primers Stx2- 5'-CT TCG GTA TCC TAT TCC CGG-3' and Stx2-5'- CGC TGC AGC TGT ATT ACT TTC-3' to amplify a 475 bp fragment of *stx2* gene. Amplification was performed using the following thermal cycler (Perking Elmer 9600) program; pre incubation at 95 °C for 5min, then 30 cycles of 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C and final extension at 72 °C for 10min. Amplified gene products were separated and revealed using 0.8% agarose gel (Eurobio, Les Ulis, France) electrophoresis, in 1X TBE (Gibco BRL).

Plating efficiency

The plating efficiencies of media were determined as follows. *E. coli* strain was grown in brain heart infusion (Difco, Detroit, USA) and the broth was incubated at 37 °C for 4 h. The culture was diluted using sterile peptone water (BioMérieux, Paris, France) and dilutions were spread by a sterile glass rod on predried SMAC, BCMtm and VR-MUG plates in duplicate. The plates were incubated at 37 °C for 24 h. Presumptive colonies, like non-sorbitol fermenting (colorless) on SMAC, fluorescent in presence of UV on VR-MUG and a dark blue or black dome to raised colonies on BCMtm were counted. The percentage recovery of colonies was determined

For results expression, serotyping and detection of virulence factor by PCR were used as standard methods to evaluate media efficiency in this study. Strain of *E. coli* O157:H7 or non-O157 which carried the *stx* gene were considered as true positive (Tp). For each medium, sensitivity (Se), specificity (Sp) and predictive values (Pv) were calculated using expressions, $Se = \frac{Tp}{Tp+Fn}$, $Sp = \frac{Tn}{Fp+Tn}$ and $Pv = \frac{Tp}{Tp+Fp}$ respectively, where Tp is true positives, Fn is

the false negatives, Fp the false positive and Tn the true negatives.

Effect of temperature on growth of *E. coli* O157:H7

E. coli O157:H7 suspension was purchased in peptone water, (BioMérieux, Paris, France) and incubated 24 h at 37 °C. An equivalent of 10⁵ cells were introduced into series of tubes (25x15 mm) containing 15 ml of trypticase soy broth (pH 7.02) (Difco, Detroit, USA) and the tubes were incubated at different temperatures, 37 °C, 42 °C and 44.5 °C. Population of *E. coli* O157:H7 were enumerated by plating 0.1 ml inoculum suspension every 1 h on VRBL (BioMérieux, Paris, France) in duplicate. Colonies were counted after 24 h incubation at 37 °C. *E. coli* enumeration was made within the first 8 h of culture to reduce growth variation effect above this time and measure evolution of the population at the optimum growing phase alone.

Survival of *E. coli* O157:H7 in acid media

Series of tubes (6) containing 15 ml of trypticase soy broth (pH 7.02) were prepared. The pH was adjusted to 3.5, 4.5 and 5.5 using acetic acid (Merck, Darmstadt, Germany). An equivalent of 10⁵ cells of 24 h culture of *E. coli* O157:H7 in peptone water was transferred to the tubes. Suspensions were incubated at 42 °C and the optimum temperature was determined by the preceding test. Every 1 h, 0.1 ml of culture was streaked on VRBL (BioMérieux, Paris, France) plates which were incubated for 24 h at 37°C. Colonies were counted and the results of bacterial populations were expressed in logarithm of colonies formant unity (CFU).

RESULTS

Performance of culture media for *E. coli* O157:H7 isolation

Results of SMAC medium cultures revealed that 97% of non-sorbitol fermenting

Table 1: Serotypes of non-sorbitol fermenting *E. coli*.

Strains belonging to human		Strains belonging to Foods	
References	Serotypes	References	Serotypes
EH 118	O28	EA14	O92
EH 181	O15	EA51	O29
EH144	O157:HNM	EA57	O25
EH211	O68	EA712	O20
EH667	O86	EA50	O69
EH409	O121	EA47	O157:H7
EH138	O159:H21	EA111	O91:H30
EH173	Ound:H30	EA98	O50
EH413	Ound:H26	EA10	O40
EH309	O32	EA81	O31:H14
EH261	O80	EA (6)	ND
EH439	O38		
EH503	O110:H28		
EH (7)	ND		

EH (7): 7 strains of *E. coli* from human, EA (6): 6 strains of *E. coli* from foods; ND: strains non-O157 of undetermined serotype; Ound: strain belonging to he unknown serogroup.

E. coli produced characteristic colonies of serotype O157:H7. Two strains among these, as confirmed by Murex kit test, belonged to O157 group. One of these strains presented typical O157:H7 serotype and the other one was O157:HNM (Table 1). The gene of shiga-toxine2 (*stx2*) was detected in both of strains (Figure 1).

On BCMtm and VR-MUG media, 5 and 3 strains gave respectively presumptive colonies of *E. coli* O157:H7. Among these strains, two strains out of 5 presumptive observed on BCMtm medium are the true positive, as they belonged to O157 serogroup and carried shiga-toxin2 gene. Two strains out of 3 presumptive observed on VR-MUG medium also belonged to O157 serogroup and carried shiga-toxin2 gene. The sensitivity of the three tested media was 100%. On the other hand, specificities and predictive positive values were significantly different. Highest specificity value (97%) and predictive positive value (66%) were recorded on VR-MUG medium (Table 2). SMAC gave slightest value

of specificity (3%) and predictive positive value (5.4%).

Effect of temperature on growth of *E. coli* O157H7

Figure 2 shows the evolution of growth of *E. coli* O157 and *E. coli* non-O157 at 37 °C, 42 °C and 44.5 °C. At all the studied temperatures, microbial population increased during incubation time. However the rate of growth obtained at 42 °C for *E. coli* non-O157 was above that sourced at 37 °C and 44.5 °C. After 8 hours of growth, microbial population obtained at 42 °C was one log₁₀ (CFU/ml) higher than that obtained at 44.5 °C. The incubation of this strain at 42 °C is consequently favorable to the optimal bacterial growth. Microbial growth curves of serotype O157 have the same evolution with those obtained for *E. coli* non-O157. After 8 hours of growth time, log₁₀ (CFU ml⁻¹) value was 11 and 9.9 respectively at 42 °C and 44.5 °C. The growth was better at 42 °C than at 44.5 °C. The values obtained for *E. coli* non-



Figure 1: Agarose gel electrophoresis of DNA amplification products for shiga-toxin2 (*stx2*) gene detection.

M: Molecular weight marker; Pb: base peer; (1): O157:H7 positive reference; (2): O20; (3): O91:H31; (4): O86; (5): O27; (6): O159:H21; (7): Ound:H30; (8): Ound:H26; (9): O157:HNM; (10): O157:H7; (11): negative reference.

Table 2: Performance of SMAC, ChromoAgar and BCMtm for isolation *E. coli* O157, testing 38 non-sorbitol fermenting strains.

Culture media	Characteristics of Shiga-toxin producing <i>E. coli</i>			Performance of culture media		
	presumptive colonies of O157:H7	Serogroup O157	<i>stx</i> gene detected	Se (%)	Sp (%)	VPP (%)
SMAC	37	2	2	100	50,7	5,4
ChromoAgar						
BCM tm	5	2	2	100	94,7	40
VR-MUG	3	2	2	100	97,3	66

Se: sensitivity ; Sp: spécificity; Vpp: predictive positive value; VR-MUG: red violet 4-méthyl-umbelliferyl-β-D-glucuronide ; SMAC : MacConkey Sorbitol agar; *stx* : strain of *E. coli* producing at least one *stx* gene (*stx1* ou *stx2*).

O157 were above those obtained for *E. coli* O157.

Effect of acidic pH on the growth

The shape of the curves shows, for cultures at pH 5.5, an increase in microbial population with time for both *E. coli* O157

and *E. coli* non-O157 (Figure 3). The number of microbial cells after 8 h is $10 \cdot \log_{10}$ CFU ml⁻¹ for *E. coli* non-O157 and $9.2 \cdot \log_{10}$ CFU ml⁻¹ for *E. coli* O157.

At pH 3.5 and 4.5 the growth of *E. coli* was inhibited. The rate of growth of microbial population decreased from the second hour.

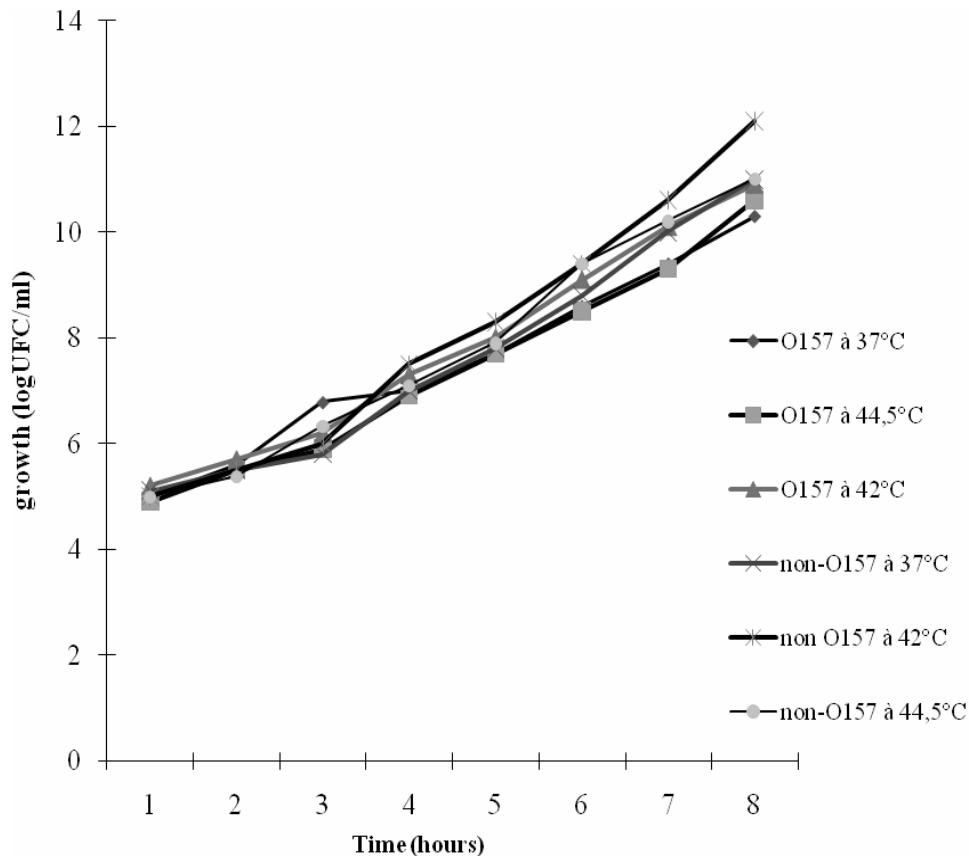


Figure 2: Growth of *E. coli* O157 and *E. coli* non-O157 according to the temperature.

Microbial population obtained after two hours decreased comparatively to the initial population and led to the death of microbial cells particularly at pH 3.5 and for *E. coli* non-O157 strain.

DISCUSSION

Performance of *E. coli* O157 isolation media

Sorbitol MacConkey agar (SMAC) is a selective culture medium most frequently used for isolation of *E. coli* O157:H7 (Griffin, 1995). However, previous studies have revealed the low sensitivity of this culture medium (Chapman et al., 1994; Sanderson et al., 1995). The results of the present work confirm this observation. Framatico et al. (1993) demonstrated that SMAC agar is not

suitable for sorbitol fermenting *E. coli* O157:H7. Novicki et al. (2000) have found 50% of sensitivity with the same culture medium. Moreover, we also demonstrated a low specificity of the SMAC agar because only two of 38 studied strains of *E. coli* have been confirmed *E. coli* O157. To improve the sensitivity of the SMAC agar, Onoue et al. (1999) proposed a selective enrichment with Vancomycin, Potassium Tellurite or Cefixim. However, another procedure consisting of the use of two selective isolation media could be examined in the future. Conventional methods for the detection of *Salmonella* recommend the use of selective enrichment and two selective culture media for isolation of strain (ISO 6579, 1990; CUMAIRA, 1994). A similar process should improved sensitivity,

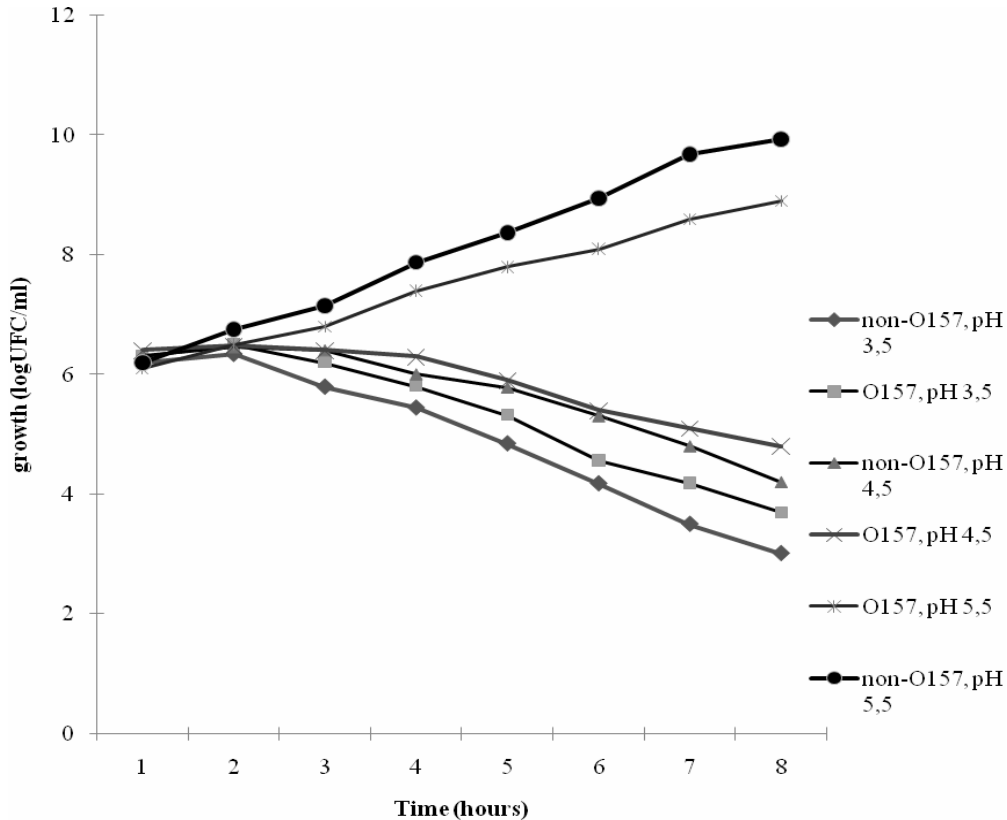


Figure 2: Growth of *E. coli* O157 and *E. coli* non-O157 according to the temperature.

using SMAC with BCMTMO157 or VR-MUG. Indeed, according to the results of our study, BCMTMO157 and VR-MUG have a better performance than SMAC. Using these two culture media (BCMTMO157 and VR-MUG), the presumptive colonies obtained are distinct from others and the number of false positives is reduced.

Effect of temperature and of pH on the growth of *E. coli* O157:H7

In the conventional method of investigating for *E. coli*, incubation of culture media was performed at 44-45 °C as recommended by Mackenzie et al. (1948). This temperature of incubation, according to some authors such as Doyle and Schoeni (1984); Raghubeer and Matches (1990), favors only a slight growth of *E. coli* O157:H7

strain. The *E. coli* O157:H7 strain studied in this report did not present a slight growth at 44.5 °C contrary to previous data. The results obtained are similar to that obtained by Palumbo et al. (1995) who also observed a good growth of strains O157:H7 at 45 °C on selective medium for *E. coli*. However, the estimation of microbial population allowed one to observe that 44.5 °C is not optimal growth temperature. After 8 hours of incubation, the rate of growth was more abundant at 37 °C or 42 °C than that obtained at 44.5 °C.

The decrease in the growth rate at 44.5 °C could be linked to the fragility of *E. coli* O157, compared to the classic coliform strains which have thermo tolerant characteristics. Indeed, according to Doyle et al. (1997) *E. coli* O157 does not resist to the traditional

food technologies processing. This microorganism could be destroyed in treated beef meat during 4 min at 57.2 °C or during 10 seconds at 64.3 °C. Linton et al., (1999) also showed that *E. coli* O157 could be inactive in orange juice (pH 3.4-4.5) during 5 min at 20 °C at a pressure of 550 Mpa. Our results are thus in agreement with previous data indicating that 41 or 42 °C is the optimal growth temperature for the investigation of *E. coli* species (Leclerc, 1997; EN-ISO, 1999).

The behavior was the same for *E. coli* O157:H7 which grows at 44.5 °C and whose optimum growth is observed at a temperature of 42 °C or 37 °C. Moreover, the advantage of using 42 °C as incubation temperature is that this condition of culture can facilitate the isolation of species that should not tolerate temperatures of 44.5-45 °C.

Since *E. coli* O157:H7 has been isolated from cider or from unpasteurized apple juice with high titrable acidity and pH near 4, the investigations showed that those micro organisms could be active in acidic products (Zhao et al., 1993). The survival of this pathovar in an acidic environment (pH 3.98) during 42 days at 5 or 25 °C has been previously demonstrated by Ryu and Beuchat (1998).

In this study, there was no active growth of *E. coli* O157 in environments where the pH was below 4.5. The viable microbial population is progressively inhibited and becomes completely inactive after the 2nd hour at 42 °C, in the medium of pH 3.5 and 4.5. Studied strains are consequently sensitive to the very acidic environment.

Based on the property of *E. coli* O157 to grow in acidic medium, Fukushima et al (2000) proposed a technique for selective detection of these pathovars. But this technique should not be suitable for the growth of *E. coli* O157 serotype studied that does not tolerate acidic environments.

The potential for *E. coli* O157:H7 to tolerate acidic conditions must be related to low temperatures. Several authors (Zhao et Doyle, 1994; Conner et Kotrola, 1995) have observed this property in case of processing or preserving foods at low temperature (temperature < 25 °C).

Conclusion

The culture media such as SMAC, VR-MUG and BCMtm are all sensitive for primary or basic identification of non-sorbitol fermenting *E. coli*. However, the specificity of these selective media is varied. The use of VR-MUG agar reduces the number of false positive cultures. Strain of *E. coli* O157:H7 isolated in Ivory Coast showed an optimal growth at 42 °C and did not tolerate acidic environments with a pH below 4.5. Cultural and physiological characteristics resulted from this study could contribute to the standardization of isolation and identification methods of shiga-toxin producing *E. coli* O157.

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