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Evaluation of Drigalski agar supplemented with ceftazidime (2 mg/L) for selective isolation of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteria*

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The purpose of this study was to evaluate the performance of the Drigalski agar supplemented with 2 mg/L of ceftazidime (DC) for selective isolation of expanded-spectrum beta-lactamase (ESBL) producing *Enterobacteria* in clinical samples. The Drigalski agar supplemented with 2 mg/L of ceftazidime was compared with the chromID™ ESBL medium (bioMérieux, Marcy l'Etoile, France). Six strains from the collection of reference center for antibiotics of the Pasteur Institute of Paris and 247 clinical samples were used to calculate the performance and the intrinsic characteristics of DC medium, respectively. The main ESBL producing *Enterobacteria* consist of *Escherichia coli* (n = 31; 44.3%), *Klebsiella pneumoniae* (n = 19; 27.1%) and *Enterobacter cloacae* (n = 12; 17.1%). ESBL producers were confirmed by synergy testing. The sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (PPN) after 24 h of incubation at 37°C were respectively 98.6, 98.7, 98.6 and 98.7%. The study shows that DC agar is a sensitive and specific medium for selective isolation of ESBL producing *Enterobacteria*, but the character chromogenic agar chromID™ ESBL remains an additional advantage in the identification of strains.

Key words: Expanded-spectrum beta-lactamase (ESBL), Enterobacteriaceae, sensitive culture medium, specific culture medium, predictive culture medium.

INTRODUCTION

Beta-lactams are the main family of antibiotics that include the largest number of molecules and the most used in the world. This widespread use is due to their broad spectrum of activity, low toxicity, effectiveness and affordability for certain molecules (Livermore, 1995; Dosso, 2000). In recent years, many studies have shown

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Table 1. Reference strains.

Number	Strain	ESBL genes or Cephalosporinase genes
U2A 1446*	*Salmonella*sp	Tem1, SHV_{12}
U2A 2252*	*Klebsiellapneumoniae*	Ges 1
U2A 2239*	*Klebsiellapneumoniae*	Tem1
U2A 2240*	*Klebsiellapneumoniae*	DHA1
U2A 1528*	*Escherichia coli*	AAC6'
U2A 1878*	*Proteus mirabilis*	Cit/Fox
ATCC 25922	*Escherichia coli*	-

- : No resistance genes.

the increase around the world, of ESBL infections. The increase in the relative frequency of ESBL producing *Enterobacteria*, very epidemiogenic was observed in both treatment centers (Kim et al., 2002; Pitout et al., 2004) and community (Arpin et al., 2003; Woodford et al., 2004). In addition, the involvement of ESBL strains in both community and nosocomial infections are increasing (Colodner et al., 2004; Brigante et al., 2005; Paterson and Bonomo 2005; Guessennd et al., 2008). Multidrug resistance of ESBL is a step towards the therapeutic impasse due to the accumulation of acquired resistance to several classes of antibiotics. They are sensitive to only a small number of antibiotics (Dosso et al., 2000; Bronzwaer et al., 2002). These resistors are therefore a major public health problem with consequences for both the individual and the economy (Akoua et al., 2004; Guessennd et al., 2008). They are a significant cause of morbidity and mortality worldwide (Beaucaire, 1997). Currently, in Ivory Coast, there are very little data on the overall cost of treatment of infections caused by multi-resistant bacteria. The ESBL plasmid associated with fluoroquinolone resistance were studied in Ivory Coast in 2008 and this study showed a prevalence of 27.2% of *qnr* genes associated with producing extended-spectrum beta-lactamase (Guessennd et al., 2008). The recent emergence and spread of new types of ESBL pose an additional challenge for clinical microbiology laboratories for their detection (Livermore et al., 2005; Ben-Ami et al., 2006). Several phenotypic tests were recommended to search for and confirm ESBL, but these tests are only effective on bacteria isolated after culture (Pfaller and Segreti, 2006). The identification of an infection caused by ESBL promptly in a patient is a critical step in the management of patients to avoid treatment failure and spread of these bacteria, and for the detection of ESBL infections in several culture media existing in the market, such as chromID^{TM} ESBL medium (bioMérieux, Marcy l' Etoile, France). Although, the often too long delivery associated to with high cost, the expiration date often too short and often random conservation conditions are obstacles and difficulties that make these media inaccessible to most southern laboratories for routine research on ESBL. The objective of this study was to validate Drigalski agar supplemented with ceftazidime (2 mg/L) named (DC) to detect within a reasonable time (24 h), the presence of ESBL in clinical samples.

MATERIALS AND METHODS

Collection of clinical samples

Clinical samples (78 urines samples, 24 blood cultures, 22 pus and 26 others) were provided by the Clinical Bacteriology Unit (CBU) of the Institute Pasteur in Ivory Coast. A total of 247 clinical samples from hospitalized and not hospitalized patients (community patients) were used from January 2013 to May 2013 for the study.

Medium to validate

The Drigalski agar (ref 64664 from Bio-Rad ® France) supplemented with 2 mg/L of ceftazidime was assessed as compared to chromID ® ESBL medium (43481 reference BioMérieux, Marcy l'Etoile, France) as a reference. Ceftazidime powder used in the experiment was provided by the manufacturer SIGMA reference C0690500.

Reference strains

Reference strains of ESBL-producing *Enterobacteria* (U2A) with different resistances genes and a strain of none ESBL -producing *Enterobacteria* (ATCC) used for the validation of culture media are from the Collection of the Centre of Reference for antibiotics at the Pasteur Institute in Paris (Table 1).

Reference strains culture

All reference strains (Table 1), lyophilized was revived in Brain Heart Infusion (BHI) for three hours. They were then transferred onto agar Eosin Methylene Blue (BIO-RAD, France) and then on ordinary agar. Susceptibility testing was performed by disk diffusion method on strains in which the 3rd generation cephalosporins, ceftriaxone (CRO), cefepime (FEP), cefotaxime (CTX), ceftazidime (CAZ) and aztreonam (ATM) were placed at a distance of 1.5 cm from center to center around the disk of amoxicillin + clavulanic acid (AMC) (Jarlier et al., 1988). This allowed the identification of the Expanded- Spectrum Beta-Lactamase (ESBL) production by the strain, resulting in a synergy image "champagne cork" (synergy testing).

Preparation of stock solution of ceftazidime

A quantity of 2 mg of ceftazidime was weighed then added to 5 ml sterile distilled water to give an initial concentration (Ci) of 0.4 mg/mL (ceftazidime stock solution). The antibiotic solution was stored in a freezer at -20°C.

Preparation of medium Drigalski + 2 mg/L of ceftazidime

After preparation of Drigalski agar according to the manufacturer's instructions, 20 mL were distributed in flasks. The flasks were autoclaved at 121°C for 15 min and then kept at 45°C in a hot-water bath.

Volume (Vi) of the stock solution of ceftazidime to be taken for each flask containing 20 mL of Drigalski agar was determined according to the following formula 1:

$$CiVi = CfVf \Longrightarrow Cf = \frac{CiVi}{Vf} \quad (1)$$

Ci = initial concentration of the antibiotic solution; Vi: volume of the initial solution to be taken for antibiotic; Cf: final concentration of Drigalski agar; Vf: volume of the final Drigalski agar.

For a Drigalski agar at a concentration of 2 mg/L of ceftazidime, 100 uL of the antibiotic stock solution at concentration of 0.4 mg / mL was incorporated in each flask containing 20 mL of Drigalski agar. After homogenization, the content of each flask was poured into a Petri dish of 90 mm.

Test sterility testing

This test was performed according to standard NF EN 1040. Sterility of media was assessed after incubation at 37°C and after storage in a refrigerator at 4°C. After preparation of each batch of Drigalski agar supplemented with Ceftazidime, two Petri dishes containing the first Petri dish casting, the last Petri dish casting were incubated at 37°C as sterility control batch. The absence of colonies on the agar showed the sterility of the culture media. The results were taken after 24, 48 and 72 h of incubation.

Sterility of Petri dishes stored in a refrigerator at 4°C and the reference medium Petri dishes (CHROMID™ ESBL) was monitored for a month. Every weekend, indicators such as the growth of microorganisms on agar plates (Petri dishes soiled) and the appearance of agar were noted (color, consistency agar, surface dried or not). Rate (T) Petri dishes rejected according to the different indicators was calculated using formula 2. Some characters such as the capacity of Drigalski agar supplemented of Ceftazidime to select ESBL, lactose fermentation and the color of the agar were also recorded.

$$T = \frac{n}{N} \times 100 \quad (2)$$

n: Number of Petri dishes corresponding to the indicator considered after a week; N: total number of Petri dishes stored.

Fertility testing

This test was also done according to Standard NF EN 1040. It was made with extended-spectrum beta-lactamase (ESBL) producing *Enterobacteria* (reference strains) and one reference strain non-producing beta lactamase (ATCC 25922). The comparison was made with a reference medium used for the selective isolation and presumptive identification extended spectrum beta-lactamase *Enterobacteria* producing (chromID™ ESBL ref 43481) Bio-Mérieux and synergy testing.

The enumeration of bacterial colonies on Drigalski supplemented with ceftazidime and the reference medium was made by plating after seeding 100 µL of 10^{-3} and 10^{-4} dilutions bacterial suspension calibrated to 0.5 McFarland using densimat ® of bioMérieux. The Petri dishes were incubated at 37°C for 24 h. The calculation of the number of colonies, expressing in Forming-Colony Unit (FCU/mL) per milliliter was adapted (Anonymous, 2006) and results were expressed as log10.

Calculation of performance Drigalski medium containing ceftazidime

The performance p (%) of Drigalski agar supplemented with ceftazidime on the strains tested, estimated relative to the reference medium chromID ESBL ®, is obtained by the following equation (Denton et al., 1998):

$$P(\%) = \frac{Log\,(FCU\ on\ CHROMID\ ESBL\ medium) - Log\,(FCU\ on\ Drigalski\ with\ antibiotic)}{Log\,(FCU\ on\ CHROMID\ ESBL\ medium)} \times 100$$

Any value of p < 0 means that the Drigalski agar supplemented with ceftazidime is more efficient than the reference medium (chromID ® ESBL). Otherwise, the comparison medium is most efficient on the test strains concerned. A standard deviation of 10% is allowed between the two compared media.

Determination of the intrinsic characteristics of Drigalski agar supplemented with ceftazidime

After doing mobility and morphology tests by microscopy of clinical samples, DC agar and chromID™ were seeded simultaneously. The incubation was performed at 37°C for 24 h. The strains were identified using the API 20E of bioMérieux. The production of extended-spectrum beta-lactamase by strains was confirmed by synergy testing.

Knowing the prevalence (p0) of the extended-spectrum betalactamase producing *Enterobacteriae* in clinical samples, the intrinsic characteristics such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), Youden index J, the accuracy E (the proportion of correct results) and likelihood ratio (LR) expressing the likelihood of a diagnosis in case of positive test were determined (Adjidé et al., 2009).

Sensitivity (Se): proportion of Drigalski supplemented containing ceftazidime with culture positive (true positives, TP) when the reference strains have growth "chromID ESBL positive".

Se = TP / (TP + FN) and 95% of Se = 1.96 ± √ In (Se * (1- Se) / n)

Specificity (Sp): proportion of Drigalski supplemented containing ceftazidime with negative culture (true negatives, TN) when the reference strains have no growth "chromID ESBL negative".

Sp = TN / (TN + FP) and 95% Sp = Sp ± 1.96 √ (Sp * (1- Sp) / n)

Positive predictive value (PPV): conditional probability that there are ESBL in the sample when the culture is positive:

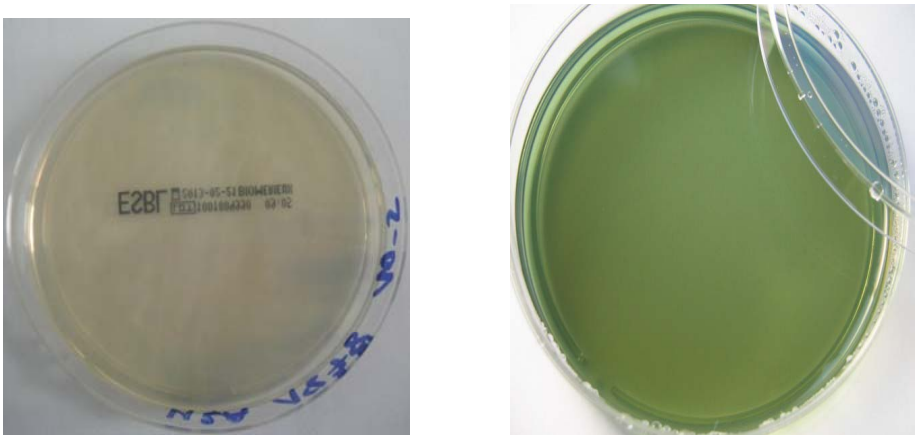
VPP = [TP / (TP + FP)] × 100 = P0 × Se / [p0 × Se + (1- p0) × (1 - Sp)]

Negative predictive value (NPV): conditional probability that there is no ESBL in the sample when the culture is negative.

Table 2. Growth of reference strains after storage for 1 month at +4°C.

Reference strains	Growth	Lactose fermentation	Colonies size
*Salmonella*sp U2A 1446	Good	Lactose $^{-}$	Unchanging
K. pneumoniae U2A 2252	Good	Lactose $^{+}$	Unchanging
K. pneumoniae U2A 2239	Good	Lactose $^{+}$	Unchanging
K pneumoniae U2A 2240	Good	Lactose $^{+}$	Unchanging
E. coli U2A 1528	Good	Lactose $^{+}$	Unchanging
P. mirabilis U2A 1878	Good	Lactose $^{-}$	Unchanging
E. coli ATCC 29922	Complete inhibition	ND	ND

ND: Not determined.



(a) (b)

Figure 1. Appearance of chromID^{TM} ESBL medium (a) and Drigalski agar (b) before seeding.

VPN = [TN / (TN + FN)] × 100 = 1 - P0 × Sp / [p0×Sp + p0 × (1-Se)]

Youden index J = Se + Sp - 100

The correct E = (TP + TN) / (TP + FP + TN + FN)

Likelihood ratio LR = Se / (1- Sp)

RESULTS

Sterility test

Incubated Petri dishes comprising of the first and last casting Petri dish in each batch of prepared medium (50 Petri dishes) showed no sign of contamination after 24 h, 48 and 72 of incubation at 37°C in an oven.

After four weeks of storage in a refrigerator at 4°C, the appearance of agar remained the same and no growth of microorganisms on the media was observed.

The results of the culture of reference strains on the Petri dishes stored in the refrigerator are shown in Table 2.

The Drigalski agar have a green color when the pH is neutral and a blue color when the pH is in the range of basic pH (Figure 1).

Growth and colony size reference strains were respectively right and unchanging on different culture media after four (4) weeks of storage in a refrigerator at 4°C (Figure 2).

Fertility tests

The main strains ESBL producing *Enterobacteria* (ESBLE) isolated from clinical samples were *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* (Table 3).

Performance reflects the ability of the medium to selectively and quantitatively promote ESBL culture as compared to chromID^{TM} ESBL medium (Table 4).

Evaluation of Drigalski agar with clinical samples (ability to select ESBLE) was used to estimate the Se, Sp, VPP and VPN as well as the positive likelihood ratios

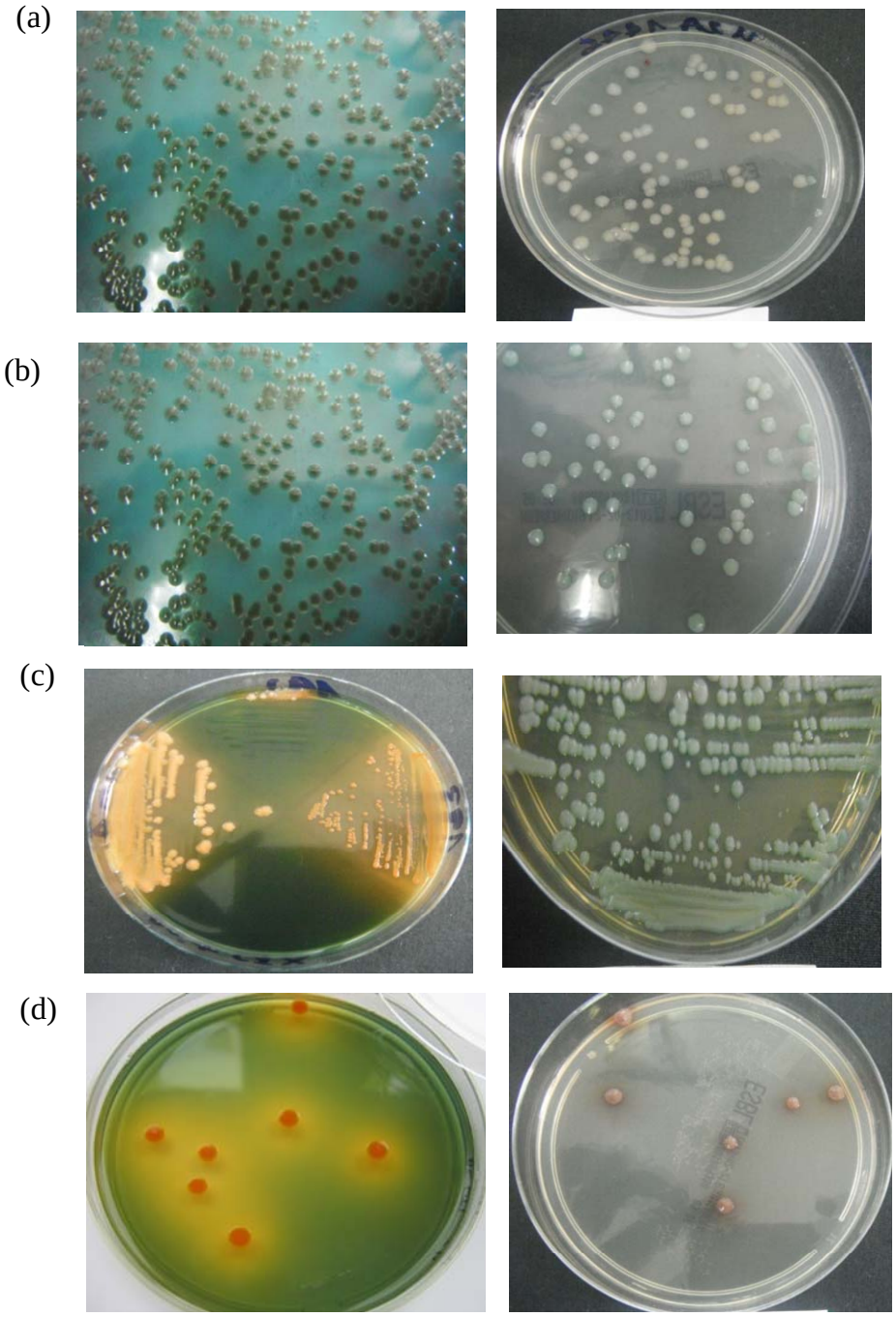


Figure 2. Appearance of reference strains on Drigalski agar supplemented with ceftazidime and chromID^{TM} ESBL before and after four weeks storage in the refrigerator. (a) *Salmonella* spp. U2A 1446; (b) *Proteus mirabilis* U2A 1878; (c) *Klebsiella pneumoniae* U2A 2239; (d) *Escherichiacoli* U2A 1528.

Table 3. Frequency of main strains ESBL producing *Enterobacteria* in clinical samples.

Enterobacteria	**Strain ESBLE/total number of ESBLE**	**Frequency (%)**
Escherichiacoli	31/70	44,3
Klebsiellapneumoniae	19/70	27,1
Enterobactercloacae	12/70	17,1

Table 4. Comparative Drigalski agar supplemented with ceftazidime and chromID^{TM} ESBL medium performance.

Reference strains	Number of viable bacteria obtained on each media Log10 (CFU / mL)		Performance agar Drigalski and chromID^{TM} ESBL
	Drigalski supplemented with Ceftazidime	chromID^{TM} ESBL	
*Salmonella*sp U2A 1446	7.78	7.76	- 0.26
K. pneumoniae U2A 2252	7.71	7.77	0.77
K. pneumoniae U2A 2239	7.45	7. 5	0.67
K pneumoniae U2A 2240	7.35	7.24	-1.52
E. coli U2A 1528	7.76	7.76	0.00
P. mirabilis U2A 1878	7.74	7.70	- 0.51
E. coli ATCC 29922	(-)	(-)	(-)

(-): No growth.

Table 5. Intrinsic characteristics of the medium Drigalski agar supplemented with ceftazidime on clinical samples.

Characteristics of Drigalski + ceftazidime as compared to CHROMID ESBL	Value
Sensibility (Se%) (IC 95%)	98.6
Specificity (Sp%) (IC 95%)	98.7
Predictive positive value (PPV%) (IC 95%)	98.6
Predictive negative value (PNV%) (IC 95%)	98.7
Accuracy E (%)	98.7
Positive likelihood ratio (L)	∞
Negative likelihood ratio (λ)	0
The Youden index (J%)	0.97

(L) and negative (λ) and Youden index (Table 5).

DISCUSSION

Lactose Drigalski Agar (Figure 1a) without antibiotic selective medium is similar to the differential Agar and MacConkey agar based media deoxycholate. It is used as a differential selective medium for Gram-negative bacilli in general (*Enterobacteriaceae* and certain non-fermenters such as *Pseudomonas*) and inhibits Gram-positive bacteria. It is recommended to use it with clinical samples such as urine and other samples that may contain a mixed microbial flora (Dupeyron et al., 1986).

Enteric Gram-negative bacteria are differentiated lactose fermenters (yellow colonies) and non- lactose fermenting (blue colonies) through the combination of lactose and pH indicator (bromothymol blue) (Zajc-Satler et al., 1993). Supplemented with ceftazidime or cefotaxime, two extended-spectrum cephalosporins, lactose agar agar Drigalski can be used to isolate Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *E. cloacae* and *Citrobacter freundii*) producing beta-lactamase extended spectrum in hospitalized patients (De Champs et al., 1993; Komatsu, 2000).

All ESBLE reference strains used had good growth, good fermentation of lactose for lactose-fermenting strains. Colony size remained unchanged after seeding the DC agar kept in the refrigerator at 4°C (temperature controlled) for four weeks (Figure 2). These results reflect the stability of the DC agar when stored at a temperature between +2 and 8°C.

During the study period, the main ESBLE strains isolated from clinical samples (urine, blood culture and pus) were *E. coli* (44.3%), *K. pneumoniae* (27.1%) and *E. cloacae* (17.1%). These results are similar to those of Cady et al. (2006). Hélène et al. (2008) have isolated these bacteria during their work on the isolation of ESBL from rectal swabs, urine and bronchial aspirations on chromID^{TM} ESBL and ESBL Agar Medium (AES) in France.

These same bacteria were also described by Glupczynski et al. (2007) in their work on the comparison of chromID - Bx and Mac Conkey on different clinical samples.

Microbiological performance on reference strains are almost equivalent or even superior for some reference strains to the chromID ESBL agar used for the isolation of ESBL. The Drigalski agar supplemented with ceftazidime showed, from the viewpoint of the rate of contamination of clinical samples with ESBL, 46.7% during the study period. This rate is significantly higher than those observed in the hospital and community infections in many African countries (South Africa 15.9%, Cameroon 12%), Europe (4.7% in Northern Europe and 13.5% southern Europe) and Asia (4.8% in Korea , 12% in Hong Kong) (Pai et al., 1999; Ho et al., 2000; Bouchillon et al., 2004; Gangoué-Piéboji et al., 2005).

The intrinsic characteristics define, for a given medium, is its ability to find the desired item when present and not to believe that the item is present when it is absent. VPP (98.6%), VPN (98.7%), combined with the accuracy (98.7%), the positive likelihood ratio (∞) and the Youden index (0.97) are DC agar sensitive and specific medium, predictive and accurate for the detection of ESBL in clinical samples. DC agar has intrinsic characteristics of Se (98.6%), Sp (98.7%) and predictive values giving its capacities to isolate ESBL identical to those of the reference medium, chromID^{TM} ESBL. The intrinsic characteristics of the DC agar are higher than those observed by Hélène et al. 2008 on chromID ESBL and ESBL agar medium with sensitivities respectively of 88 and 85% after 24 h of incubation. By opposition, some characteristics such as sensitivity are almost identical to those found by Glupczynski et al. (2007) chromID agar-BX (97.7%), but lower than that of MacConkey agar supplemented 2 mg/L of ceftazidime (84.1%). Sánchez-Carrillo et al. (2009) showed values of sensitivity, specificity, positive predictive values, negative predictive value respectively to be 100, 92.6, 85.8 and 100%.

Some *Pseudomonas* strains resistant to ceftazidime were also isolated from DC agar and chromID^{TM} ESBL agar. No strains of Enterobacteriaceae producing a high-level cephalosporinase were isolated during this study. The production of expanded-spectrum beta-lactamase strains isolated on DC agar was confirmed by synergy testing according to the recommendations of the susceptibility committee of the French Society for Microbiology (CASFM, 2012).

Conclusion

This study showed that the Drigalski agar containing 2 mg/L of ceftazidime is a sensitive and specific medium for research of Enterobacteriaceae producing extended-spectrum beta-lactamase. Based on this good sensitivity and specificity, this medium can be introduced in routine laboratories for research on ESBL in organic products in southern countries. The use of DC agar could even be considered in the search for ESBL in various ecosystems such as hospital environment, hospital effluents, sewage and animals in low-income countries. In addition to the sensitivity and specificity, the chromogenic properties of the medium chromID ESBL confer an advantage as compared to our study agar, which guides the user in identifying different strains. In addition, the DC agar can be kept in the refrigerator for at least a month.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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