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MICROBIOLOGICAL AND KINETIC DETECTION OF GRAM NEGATIVE BACILLI PRODUCING EXTENDED-SPECTRUM- B-LACTAMASES (ESBL) IN EMERGENCIES AND REANIMATION UNITS OF UNIVERSITY HOSPITAL CENTER, YALGADOUEDRAOGO, BURKINA FASO

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ABSTRACT

Background: Epidemiology of extended-Spectrum- β -lactamases has become worldwide, and our aim was to establish the prevalence of isolates producer in university hospital center Yalgado OUEDRAOGO particularly in reanimation and emergencies units.

Material and methods: Prospective study was drive during July 2009 to march 2012 in order to collect strains resisting to third generation of cephalosporin during diagnosis analysis of biological specimens. Susceptibility of bacteria to antimicrobial agents was evaluated by disc diffusion method. Production of extended-spectrum β -lactamases has been investigated by double disc diffusion and kinetic methods.

Results: 259 isolates which resisted at least to one of third generation of cephalosporins were collected. Among them 188 (72, 58 %) were positive to synergy test by a double disc diffusion method. The MICs of ceftriaxone determined by E-test were under than 50 μ g/ml, 100 μ g/ml et 256 μ g/ml for respect 81,57% ; 55,26% et 39,74% of isolates. Hydrolyze of β -lactam ring by bacterial extract followed at spectrophotometer showed speeds running at 0 to 0,090UAb.mn⁻¹ for both isolates. Extract of 171 bacterial strains positives to synergy test had hydrolyzed at least one of oxy-iminocephalosporins and were identified as producing extended- spectrum β -lactamases. Spices reported by this study were 99 *Escherichia coli* (57,89%) ; 28 *Klebsiella pneumonia* (16,37%) ; 15 *Enterobactersp* (8,77%) ; 19 *Pseudomonas aeruginosa* (11,11%) ; 4 *Citrobactersp* (2,33%) 2 *Acinetobactersp* (1,16%) , 3 *Proteus mirabilis* (1,75%) and 1 *Salmonella typhi* (0,05%).

Conclusion: This study showed that bacterial resistances by extended- spectrum β -lactamases are a reality in University Hospital center YalgadoOuedraogo. It calls about antibiotics prescription and hospital hygiene in order to reduce emergence and propagation of new resisting bacterial.

Keys words: microbial and kinetic analysis, Gram negative bacilli, extended-Spectrum- β -lactamase, emergencies, reanimation.

DETECTION MICROBIOLOGIQUE ET CINETIQUE DE BACILLES A GRAM NEGATIF PRODUCTEURS DE B-LACTAMASES A SPECTRE ELARGI DANS LES UNITES D'URGENCE ET DE REANIMATION AU CENTRE HOSPITALIER UNIVERSITAIRE, YALGADO OUEDRAOGO, BURKINA FASO

Résumé

Objectif : En raison du caractère mondial de l'épidémiologie actuelle des β -lactamases à spectre élargi (BLSE), notre objectif a été d'évaluer la prévalence des souches productrices au Centre Hospitalier Universitaire YalgadoOuedraogo en particulier dans les unités d'urgence et de réanimation.

Matériel et méthodes : Un criblage des isolats résistants aux céphalosporines de troisième génération a été conduit lors d'une étude prospective entre Juillet 2009 et Mars 2012. Les souches d'intérêt étaient les bacilles à Gram négatif isolés au décours de l'analyse des échantillons biologiques à visée diagnostic au profit des malades hospitalisés. La production de BLSE a été recherchée par des approches microbiologiques et cinétiques.

Résultats : 259 isolats résistant à au moins une C3G ont été collectés dont 188 (72, 58 %) présumées productrices de BLSE objectivées par une image de synergie. La CMI de la ceftriaxone recherchée était supérieure à 50 μ g/ml, 100 μ g/ml et 256 μ g/ml pour respectivement 81,57% ; 55,26% et 39,74% des isolats. La vitesse moyenne d'hydrolyse des noyaux β -lactames par les extraits bactériens était supérieure à 0,090UAb.mn⁻¹ pour l'ensemble des souches. A l'issue des analyses cinétiques les extraits de 171 souches bactériennes précédemment positives au test de synergie ont hydrolysé au moins une oxy-imino céphalosporine. Ces souches ont été retenues comme productrices de BLSE. Les espèces rapportées sont: 99 *Escherichia coli*

(57,89%); 28 *Klebsiellapneumoniae*(16,37%); 15 *Enterobactersp* (8,77%); 19 *Pseudomonas aeruginosa* (11,11%); 4 *Citrobactersp* (2,33%) 2 *Acinetobactersp* (1,16%), 3 *Proteus mirabilis* (1,75%) and 1 *Salmonella typhi* (0,05%).

Conclusion : Cette étude a montré que le péril BLSE est une réalité au CHU Yalgado OUEDRAOGO. Elle interpelle sur les mesures d'hygiène hospitalière et sur les mesures de prescription des antibiotiques.

Mots clés : Détection microbiologique et cinétique, bacilles à Gram négatif, β -lactamases, spectre élargi, Urgences, Réanimation

INTRODUCTION

Infectious diseases are a major cause of loss of productive years of life in the world, and more than 45% of deaths in low-income countries [1]. Bacterial infections are responsible for 70 % of cases of mortality caused by microorganisms. [2] These data are certainly linked to the occurrence of bacterial resistance and could be unfortunately constantly increased with the emergence of new resistances. Control of bacterial resistance is justified nobly motivated and constitute a public health priority for WHO. The mechanisms of resistance are widely documented. The enzymatically resistance consist in the production of enzymic proteins for whose β -lactamases constitute a large family that inactive β -lactams antibiotics. The involved form of those enzymes called extended spectrum β -lactamases (ESBL) allow bacteria to resist to third or fourth generation cephalosporins [3]. ESBLs, worldwide documented are carried by several species of Gram negative bacilli for which a large proportion adapted to humans are responsible to clinical therapeutic failure. Molecular characterization of bacterial strains allows us to know the types of β -lactamases encoded. It is established that among main ESBL, *bla*CTX-M is more described than *bla* TEM and *bla* SHV. Faced with the global expansion of extended spectrum β -lactamase, clinical laboratories are most than ever called to screen isolates producing. Molecular methods conduce to determine *bla*-type but don't access to kinetic parameters which indicate the affinity between these enzymes and β -lactam antibiotics. Through this study we aimed to contribute to clear bacterial resistance by establishing extended-spectrum β -lactamase profile of Gram negative bacilli that resist oxyiminocephalosporins at University Hospital Center Yalgado, Ouedraougousing microbiological and kinetic methods.

I. MATERIALS AND METHODS

I.1. Clinical specimens and bacterial strains

Bacterial strains collected during a prospective study from July 2009 to March 2012 were Gram negative bacilli that resist third generation of cephalosporin. They were isolated during diagnosis analysis of biological specimens. Different clinical specimens such as blood, urine, pus, vaginal swam, ascitic fluid, peritoneal fluid, and stool and rachis fluid samples were collected from hospitalized patients of emergencies and reanimation units.

Samples were taken from infected patients who presented infectious evident symptoms like fever and purulent urine. Isolates were identified using conventional method [4] Identification of isolates was achieved using API 20E test trips (BioMerieux S.A., Marcy l'Etoile, France).

I.2. Antibiotic susceptibility testing and ESBL detection

Antibiotic susceptibility was tested by disk diffusion method [5], with antibiotic disks used to test Gram negative bacilli particularly monobactam: Aztreonam (30 μ g), third generation cephalosporin like cefotaxim (30 μ g), ceftriaxon (30 μ g), ceftazidim (30 μ g), and fourth generation cephalosporins: ceftapim (30 μ g). Antibiotics were tested on Petri plates containing Muller Hinton agar. Measurements of inhibition area determine the clinical categories (CA-SFM, 2012). Isolates that were resistant at least to one of the antibiotics in clinical test, using NCCLS methods [6] were collected, purified and conserved at -80°C for further analysis. In order to screen ESBL phenotypical profile, isolates were submitted for synergy test [7] between third generation of cephalosporins disks (cefotaxime or ceftazidime) and amoxicillin plus clavulanic acid.

In addition, MICs of ceftriaxone, antibiotic frequently used in clinical routine in our sanitary centers was performed as recommended by guide E-test AB BIODISK.

I.3. Extraction and kinetics activity of β -lactamase

From bacterial aliquot conserved 2 μ l of an inoculum were collected and suspended in 4ml of Luria Bertini solution. This bacterial suspension stirring, was cultured 24 hours overnight at 37°C. Bacterial culture resulting was centrifuged at 3000 rpm during 30 minutes. The bacterial pellet which resulted was suspended in 500 μ l of 100 mM phosphate buffer Ph7, and submitted to physical method of freeze / thaw cycles [8,9]. This treatment allowed to obtain periplasmic contents and enzyme solution was obtained after a final centrifugation at 8000 rpm for 20 minutes. For certain bacterial strains, the enzyme production was induced with a solution of cefoxitin 20 μ g /ml during culture. Different bacterial extracts obtained were tested for β -lactamase activity with nitrocefin. A reaction medium consisting of 50 mM phosphate buffer pH7, enzyme (5 to 10 μ l) and nitrocefin final concentration of 100 μ M/ml was carried out in a spectrophotometer's tank. The extracts with a β -lactamase activity were tested with third-generation

β -lactams in order to determine their hydrolytic profile (ESBL profile). For this purpose, solutions of antibiotics were used as substrate in a reaction medium where the final concentration of antibiotic varied increasingly (25 .mu.m, 50 .mu.m, 100 .mu.m and 75 μ M). The enzymatic activities were monitored at UV / VIS double beam Uvikon 923, XL appropriate wavelengths: 482 nm (nitrocefin) to 235nm (ampicillin, benzylpenicillin), at 260 nm (cefotaxime, ceftazidime, ceftriaxone, cefuroxime, cefepime). All these experiments were performed at 30 °C in 50mM phosphate buffer pH7. The initial velocity (V_0) of hydrolysis of each compound (expressed as absorbance units per minute) was evaluated according to the relationship $V_0 = \Delta A_\lambda / \Delta t$ wherein $\Delta A_\lambda / \Delta t$ represents the slope at the origin of the curve of variation Absorbance at the wave length λ relating to time.

I.4. Determining the magnitude of kinetic parameters related to bacterial extracts

Kinetic parameters K_m and V_m which represent respectively Michaelis constant linked to substrates and maximal velocity of enzymatic reaction were determined by Hanse linearization based on Michaelis equation.

II. RESULTS

II.1. Antibiotic susceptibility testing and ESBL detection

After the isolation procedures, identification and antibacterial susceptibility testing, 259 bacilli Gram-negative resistant to at least one of third generation cephalosporin were collected. The bacterial strains identified using biochemical characteristics grouped in galleries (API 20E, minimum galleries) were distributed in the bacterial species as follows: *Escherichia coli* (n = 132), *Klebsiella pneumoniae* (n = 43), *Pseudomonas aeruginosa* (n = 34), *Enterobacter* sp (n = 25), *Citrobacter* spp (n = 11) *Acinetobacter baumannii* (n = 7), *Proteus mirabilis* (n = 6) and *Salmonella typhi* (n = 1). On a Petri dish, these strains were found to be resistant to antibiotics targeted as cefotaxime, ceftriaxone, ceftazidime, cefepime, aztreonam and to a lesser extent to imipenem (Table IV). For these antibiotics to which we particularly interested, the diameter of the inhibition of bacterial growth zones varied between 0 and 22 mm. The tests synergy revealed among the 259 strains, 188 (72.58%) ESBL-producing objectified by a synergy picture (Figure 1). Antibiotic discussed in carrying out tests were Amoxicillin + clavulanic acid (20/10 ug) Ceftazidime (30 μ g) and Cefotaxime (30 μ g) cefepime (30 μ g), aztreonam (30 μ g). The distance between the antibiotic disks for the materialization of the synergistic picture in ESBL-producing strains varied from 10 to 15 mm. The study of the MIC of ceftriaxone ABBIODISKE-test, showed that the MIC was greater than 50 μ g/ ml, 100 μ g/ ml and 256 μ g/ml for 81.57%, respectively, 55.26% and 39.74% of isolates.

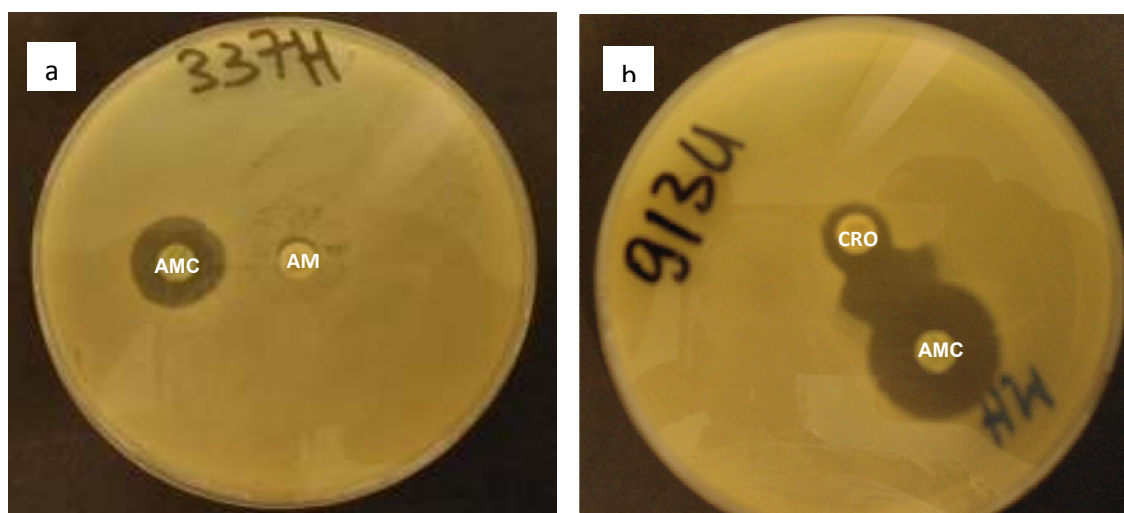


Figure: 1 a) Picture showing *E. coli* suspected as ESBL producing, b) Picture of synergy showing ESBL production by *E. coli* on Petri plate

II.2. Detection of ESBL by kinetic approach

II.2.1. Study

Hydrolytic Profile Of Bacterial Crude Extracts: At the end of kinetic analysis of bacterial crude extracts, the rates of hydrolysis of the β -lactam nuclei ranged from 0 to

0,69 U abs.min⁻¹ for all extracts tested with nitrocefin. Table I For total of 259 extracts analyzed, 227 showed a β -lactamase activity with Nitrocefin; while the activity was null for 32 extracts. Table II. In our experimental conditions, 171 bacterial extracts in addition to cefinase activity showed a β -lactamase

activity with at least a third generation cephalosporin indicating that the vast majority of host strains would be extended spectrum- β -lactamase- producing. The figures below are some examples that show the progression of the hydrolysis of β -lactam antibiotics by bacterial crude

extracts. In figures 2 and 3, the plots reflect changes in concentrations of the products formed (positive slope) or those of the disappearing substrate (negative slope).

N° Extract	$V_0(\text{U Abs. mn}^{-1})$					
	Nitrocefin (CPR)	Ceftriaxon(RO)	Cefotaxim(CTX)	Ceftazidin(CAZ)	Cefepim(FEF)	Imipenem(IPM)
1226Uro	0,360	0,028	0,072	0,018	0,042	0
450H	0,123	0,047	0,056	0,030	0,029	0
176Uro	0,073	0,037	0,063	0,014	0,051	0
224P	0,598	0,046	0,0687	0,022	0,041	0
565P	0,194	0,031	0,0615	0,011	0,021	0

TABLE I: RATE OF HYDROLYSIS OF SUBSTRATES WITH SOME BACTERIAL EXTRACTS

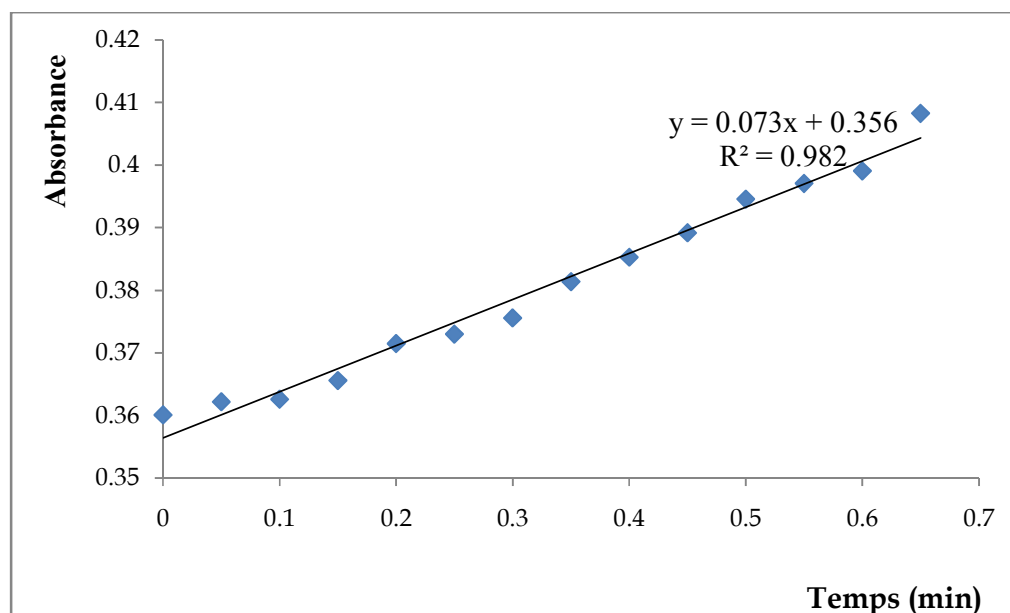


FIGURE 2: MONITORING THE KINETICS HYDROLYSIS OF NITROCEFEN ($100 \mu\text{M}$) IN FUNCTION OF TIME WITH AN EXTRACT OF *S. typhi* 176 URO. $V_0=0.073 \text{ U Abs. mn}^{-1}$

II.2.2. Determining the magnitude of the kinetic parameters bacterial crude extracts
 Linearization of Michaelis expression of the initial velocity $V_0: V_0 = \frac{V_m[S]}{K_m + [S]}$ by Hanes's method allowed to obtain new expression of the write speed as

$\frac{[S]}{v_0} = \frac{1}{V_m}[S] + \frac{K_m}{V_m}$. This linearization leads to approach magnitude order of kinetic parameters K_m and corresponding V_m . Table III gives an application example with the crude enzymatic extracts from the strain 224P.

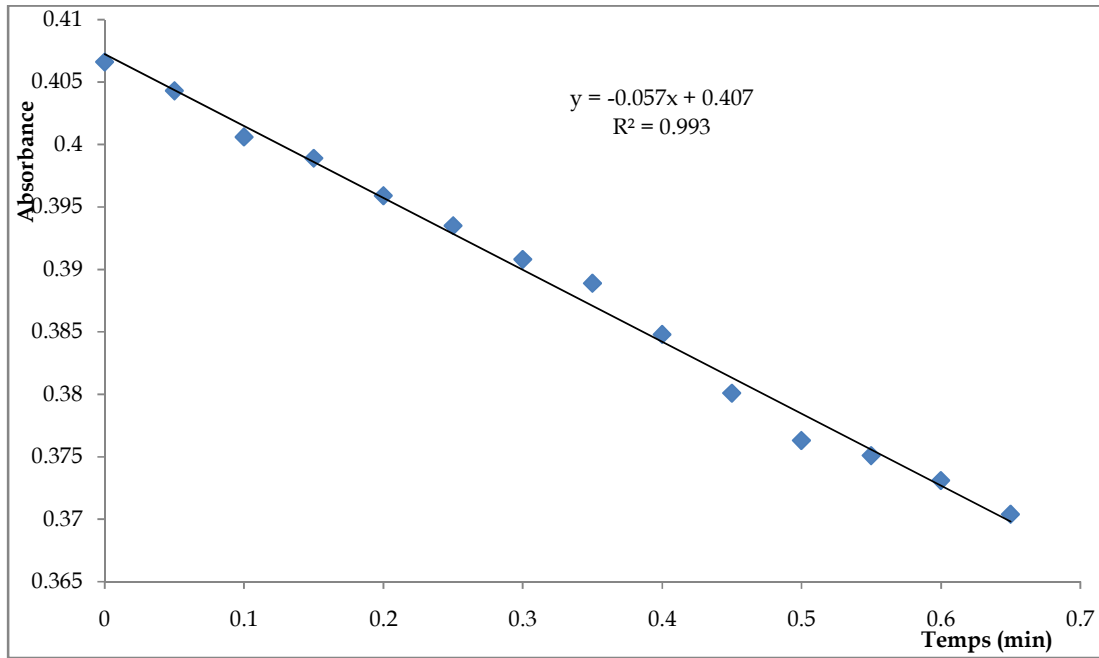


FIGURE3: MONITORING OF THE KINETICS HYDROLYSIS OF CEFOTAXIM (25 μM) IN FUNCTION OF TIME WITH AN EXTRACT OF *ENTEROBACTER SP 224P*. $V_0=0.057 \text{ UABS.MN}^{-1}$

TABLE II: HYDROLYSIS ANTIBIOTICS SUBSTRATES RATES BY BACTERIAL EXTRACTS

	Nitrocefin (CPR)	Cefotaxim (CTX)	Ceftriaxon (CRO)	Cefepim (FEP)	Ceftazidin (CAZ)	Imipenem (IPM)
Extracts analysed number	259	227	227	98	227	74
Hydrolysis rates of substrates (%)	$n=227$ 87,64	$n=142$ 62,55	$n=93$ 40,96	$n=16$ 16,32	$n=46$ 20,26	$n=0$ 0

TABLE III: VALUES LINKING CONCENTRATION TO INITIAL VELOCITIES OF HYDROLYSIS OF CEFOTAXIME

$V_0 \text{ UAbs.mn}^{-1}$	$1/V_0$	$[S] \mu\text{M}$	$[S]/V_0$
0.040	25	6,25	156,25
0.057	17,5438	25	438,595
0.084	11,9076	50	595,38
0.094	10,6382	75	797,865

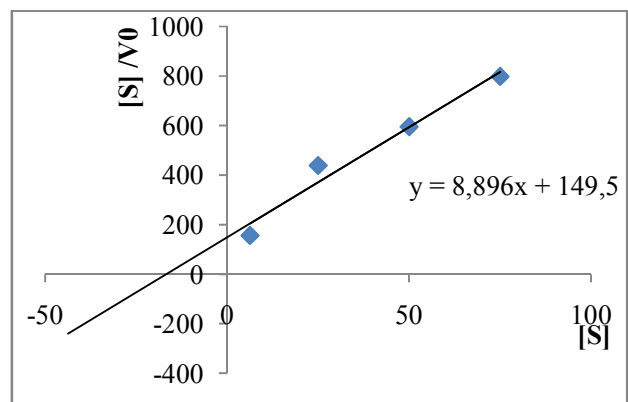


FIGURE 4: PLOT $[S]/V_0$ ACCORDING TO $[S]$ FOR CEFOTAXIME

According to Hanse's linearization, the slope of the plot (Figure 4) intersects the axis at point K_m

$/V_m$ and that of the point abscises k_m . Solving the equation $y = 8,896x + 149.5 = 0$ to determine the K_m value. From the foregoing, the kinetic parameters K_m and V_m linked to enzyme extracted from the strain 224P for cefotaxim were: $k_m = 16,80\mu M$ and $V_m = 0,112 \text{ UAb.mn}^{-1}$. Similar analysis allowed determining K_m and V_m relevant to ceftriaxon linked crude extract of strain 224P (*Enterobactersp*). For ceftriaxon parameters values were: $K_m 17,61\mu M$ and $V_m = 0.109 \text{ UAbs.mn}^{-1}$.

It was found that 171 bacterial strains have been implicated in both the synergy tests and kinetic analysis as producing ESBL. Note that of the 188

bacterial strains implicated by the synergy test 17 strains haven't any kinetic activity with third generation cephalosporin. Strains producing ESBL were retained in 66.02% of all collected resistant strains. The species were reported in order of quantitative importance: 99 *Escherichia coli* (57.89%); 28 *Klebsiellapneumoniae* (16.37%); *Enterobactersp* 15 (8.77%); 19 *Pseudomonas aeruginosa* (11.11%); 4 *Citrobactersp*(2.33%) 2 *Accinetobactersp* (1.16%) and *Proteus mirabilis* 3 (1.75%) and 1 *Salmonella typhi* (0.05%). Distribution of extended spectrum bacterial strains producing in biological samples are presented in table 4

TABLE IV: DISTRIBUTION OF ESBL-PRODUCING STRAINS BY BIOLOGICAL SAMPLE ANALYZED

Strains	Urines	Stools	Blood	PV	Pus	LCR	Total	Pourcentage
<i>Escherichia coli</i>	71	00	06	06	14	02	99	57,89
<i>Klebsiellapneumoniae</i>	11	00	07	01	09	00	28	16,37
<i>Enterobactersp</i>	08	00	02	01	04	00	15	8,77
<i>Proteus mirabilis</i>	01	00	00	00	02	00	03	1,75
<i>Pseudomonas aeruginosa</i>	08	00	01	00	10	00	19	11,11
<i>Salmonella typhi</i>	01	00	00	00	00	00	01	0,05
<i>Citrobactersp</i>	04	00	00	00	00	00	04	2,33
<i>Acinetobacterbaumannii</i>	02	00	00	00	00	00	02	1,16
Total	106	00	16	08	39	02	171	100
Pourcentage	61,98	00	9,35	4,67	22,80	1,16	100	

PV: vaginal swab. LCR: cerebrospinal fluid

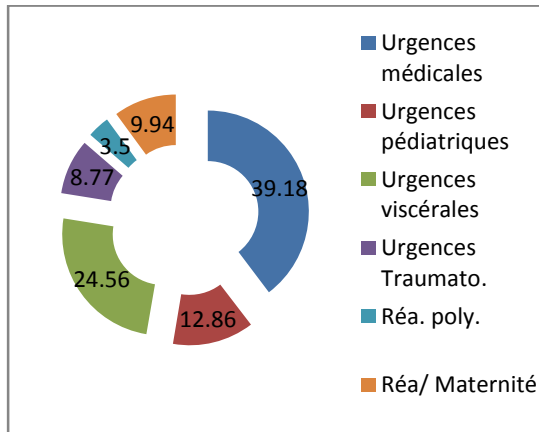


FIGURE 5: DISTRIBUTION OF PROPORTIONS (%) OF BACTERIAL STRAINS IN CLINICAL UNITS

III. DISCUSSION

III. 1. Antibiotic susceptibility testing and ESBL detection

Microbiological detection of ESBL production based on the synergy test is today a routine practice of laboratory diagnostics in low-income countries. Performed with the utmost care, it allows us to incriminate bacterial strains for which special attention should be given when prescribing antibiotics. ESBL phenotype of these strains is very often confirmed by an increase in MICs of antibiotics. Of 259 resistant strains we collected, 188 (56.7%) showed an ESBL phenotype on plates. The MIC values of ceftriaxone and cefotaxim we have determined for these strains were of the same order of magnitude as those determined by other authors for ESBL-producing strains [10, 11]. However, factors such as the distance between the discs of antibiotics for the materialization of the synergy image, the additional production of other β -lactamases (AmpC, MBL) can influence the result of the synergy test [12]. The study of the kinetic activity of the bacterial extracts has allowed incriminating 171 ESBL-producing strains among the 259. It was revealed that the extracts of 17 yet positive bacterial strains in synergy test hydrolyzed no third generation cephalosporins. The beta-lactamase strains were therefore not producing ESBL [13]. Detecting the ESBL by the enzymatic method could be considered a correction method for the synergistic test. In addition, this same method, if it does not determine the type of beta-lactamase in the presence of genes as molecular methods, allows through the values of the kinetic parameters to specify the nature of the affinity of the enzyme for antibiotics.

III.2. Frequency and biological distribution of ESBL producing strains

ESBL producing strains collected in our study are spread into bacterial species frequently reported by several authors. The species *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*,

Enterobacter sp., *Pseudomonas aeruginosa*, *Citrobacter* sp., *Accinetobacter baumannii* and *Salmonella typhi* we collected are very often cited by several authors as responsible for nosocomial infections [14]. ESBL-producing strains accounted for 66, 02% of all collected strains. This result was beyond those reported in Ghana (49, 3%)[15], South Africa (36, 1%) [16] and (5, 4 to 25%) in Europe [17]. Among ESBL-producing bacteria species we have identified *E. coli* represented the major species (57,89% of all our strains). This statement was similar to the results of other workers [18, 19, 20, and 21]. Note that *Escherichia coli* is the most representative of commensal enterobacteria. Feces contain 13×10^6 per gram in healthy subjects and this figure reached in sick subjects to 10^{11} [22]. The anatomical proximity of urinary and genital openings with the anal opening could facilitate the anogenital spread of this bacterium, which by ascending way affects various organs of the urogenital system. This observation is more pointed in the emergency and reanimation units, where the condition of patients is associated with a lower level of hygiene.

III.3. Ecological niche of ESBL-producing strains detected

From results of our study, medical emergencies unit followed by those of Visceral Reanimation and Pediatric Emergencies appear to be most sources of ESBL producing strains. However it should be noted that in context of our country, the position of the medical emergencies unit can be relative, given its place in the organizational system of hospitals. This unit represents for many others the entry and where complementary diagnosis is often required before the transfer of patients. From the above, Visceral Emergency unit prove to be the ecological niche of primary importance among the short-term hospitalization. In this unit as its name suggests, pathologies consist of visceral infections or visceral diseases complicated infections due to reputed ESBL producing *Enterobacteriaceae*. Intensive care unit of motherhood takes third place after Pediatric Emergencies. In both units, promiscuity and of individual and hospital hygiene conditions could explain the persistence of nosocomial strains and spread of ESBL. Reanimation and Intensive Care Services are indexed as excellent places for selective pressure of resistant strains by the indiscriminate use of antibiotics [23]. However, from our results polyvalent reanimation unit, recorded fewer cases of infections related to ESBL producing strains. This could be related to good hygiene and safety care practices, but it should be noted that the samples referred to diagnosis from this service were infrequent. In France, in 2009, the relative risk of acquiring a bacterial strain producing ESBL in clinical units of a university hospital was established as follows: Emergency (0, 46), Intensive care (1,78), Medicine (0,67), Surgery (0,97), Paediatrics (0,6), Haematology (3,07), Maternity (0,43)[24].

Conclusion

Detection of ESBL-producing bacterial strains is an important global concern. If nowadays molecular detection methods are the most recommended, it is indisputable that they remain inaccessible to low-income countries. Phenotypical procedures are common practices in diagnostic laboratories. The enzyme method of detection of the production of β -lactamases that we have proposed is similar to cefinase tests performed in the clinical laboratory. The improvement of our process is that it provides opportunities to obtain kinetic parameters of enzymes that assess their affinity with antibiotics substrates. Through both microbiological and kinetic methods, we were able to establish data on bacterial species frequently responsible for antibiotic resistance in emergency units at the University Hospital Center, Yalgado, OUEDRAOGO. The affinity of the bacterial extracts for cefotaxim suggests that the major part of the

strains harbors CTX-M type ESBLs. Urine samples from units of medical emergencies and visceral surgery reanimation appeared respectively as sources and ecological purveyors of ESBL-producing strains. The challenge lies in the development of reliable mechanisms to control the spread of these strains that contribute to high morbidity and mortality in our hospitals. These mechanisms should take into account a good hospital hygiene policy and also antibiotics management.

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