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The Clinical Microbiolog Epidemiology and Infection Control Implications: Acinetobacter

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Abstract

In a previous study, we showed that Acinetobacter genomic DNA group 3 was the most common species among blood culture isolates and was commonly found on superficial carriage sites of the healthy and the sick, which are different findings from those reported in Europe and North America. We used amplified ribosomal DNA restriction analysis and pulsed-field gel electrophoresis to study further the molecular epidemiology of acinetobacters in our region. Over a study period of 6 weeks with 136 consecutive routine clinical isolates (1.33% of all specimens), genomic DNA groups 2 (Acinetobacter baumannii), 3, and 13TU were obtained from 59 of 69 positive patients. There is a significant difference in the specimen sources of the three genomic DNA groups, with group 13TU being significantly associated with the respiratory tract (chi-square exact test, $P = 0.0064$). Settle plates showed a significantly heavier environmental load from the intensive care unit (ICU) than from the four surgical wards examined (22 of 70 versus 76 of 120 plates with <5 colonies; chi-square test, $P < 0.0001$). Genomic group 3 accounted for 6 of 12 clusters of possibly related strains among patients, between patients and the ICU environment, and in the ICU environment. Genomic groups 2 and 3 accounted for 21% of the 132 genomically identified isolates recovered from 21 of 41 local vegetables, 53 of 74 fish and meat samples, and 22 of 60 soil samples. Group 13TU was present only in patients' immediate surroundings. The role played by the environment and by human carriage should be evaluated in order to devise a cost-effective infection control program pertinent to our situation of acinetobacter endemicity.

INTRODUCTION

Acinetobacter spp. are important nosocomial pathogens associated with a growing number of hospital-acquired infections worldwide (2, 14). In hot, humid areas, such as Hong Kong, Acinetobacter infection is endemic, with higher incidences of nosocomial infection, including bacteremia and pneumonia, than those reported elsewhere (2, 14, 28, 34, 35). The clinically important species, such as Acinetobacter baumannii (genomic DNA group 2), are intrinsically resistant to the first-line antimicrobial agents, e.g., ampicillin and cefuroxime. Acinetobacter spp. have a propensity to readily develop resistance to second- and third-line agents such as cefotaxime, ciprofloxacin, and imipenem, giving rise to therapeutic problems (2, 30, 32). Outbreaks of Acinetobacter infections, often caused by multiresistant strains, have been widely reported, commonly in intensive care units (ICUs) in North America and Europe. Epidemiological features and risk factors of these outbreaks have also been well described (2, 5, 8, 10, 14, 16, 17, 18, 20, 24, 36, 43). In contrast, there is a paucity of information in regions of Acinetobacter endemicity, such as Hong Kong (28, 34, 35). It has recently been shown that there is a significant difference between Hong Kong and Europe in the genomic DNA groups of isolates obtained from blood cultures and various superficial carriage sites (4, 7, 33). Species other than A. baumannii appear to be of greater epidemiological significance than was previously appreciated (7). This raises the question of whether identified risk factors of infection and control measures that are promulgated and practiced in areas where the infection is not endem-

(H5702-50; Hamamatsu). Removable light filters are positioned between the fiber end face and the PMT detector. The PMT provides a voltage signal proportional to the measured light intensity.

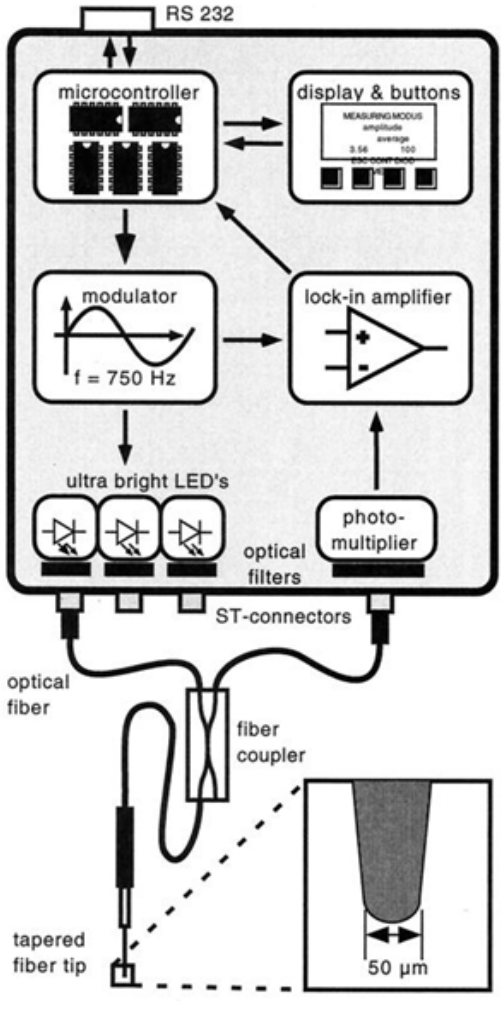

FIG. 1: Technical concept of the fiber-optic fluorometer.

The light intensity of the LEDs is modulated at a frequency of 750 Hz. Subsequently, the fluorescence signal detected by the PMT is also modulated at 750 Hz. A lock-in amplifier selectively amplifies only signals at this frequency in order to minimize the influence of ambient light or electronic noise. The electronics is controlled by a built-in microcontroller (V25; GME GmbH) that reads the fluorescence signal measured by the lock-in amplifier via an analogue-to-digital converter. A reference zero value obtained with the microprobe immersed in pure water is subtracted from each measurement. The results of the measurements are shown in digits on a small text display. Alternatively, a personal computer can be connected via a serial (RS 232) interface in order to control the measuring procedure by custom-made programs.

and probably is capable of reducing weight in human (9). Olax subscorpioidea is a plants used in Ivorian traditional medicine in the treatment of many diseases including jaundice and hepatitis. The plant is used in mixture with palm wine locally called "bandji" for their traditional therapeutic utilization (10). Researchers have proved that Olax subscorpioidea Oliv. has antidepressant effect on effect and this justified its use in the treatment of mental disorders (11).

MATERIALS AND METHODS

Collection of Plant Material

Olax subscorpioidea Oliv. roots were collected across farms in Akungba community in Ondo State. The plant part was authenticated at the herbarium of the Department of plant science and biotechnology Adekunle Ajasin University, Akungba Akoko, Ondo State. Nigeria.

Preparation of Extract

Olax subscorpioidea Oliv. Root was washed with sterile water and air dried for 10days, and then chopped into small pieces to increase surface area. 100 g of the root was soaked in 400 mL of ethanol for 7 days. After which it was filtered with whatman No. 1 filter paper. The solvent was recovered and the crude extract obtained using rotary evaporator. The crude extract was thus kept in refrigerator at 4oC for further screening (12).

Specimen Collection

Specimens were collected hospitals across Akoko region of Ondo state, Nigeria using standard collection techniques (Cheesebrough, 2006). Sterile cotton-wool swabs were used to collect samples from the wound of surgical patients with evident infection. The swabs were introduced gently into the wound sites and rotating the swab tips in the wound, taking care to avoid contamination of specimen with commensals from the skin, and then immersed immediately in a MacCartney bottle containing peptone water. The sample bottles were then transported to the Microbiology laboratory of Adekunle Ajasin University, Akungba-Akoko, Ondo state(12).

Isolation of Bacteria

At the laboratory, the inoculated peptone water was serially diluted to 107 and the bacteria isolated using pour plate method.Aliquot of 1 ml from 104 and 107 were dispensed into sterile petri dishes, molten agar was then added, swirled and allowed to solidify. The media used were MacConkey agar, Nutrient agar, Eosin Methylene Blue agar and Mannitol salt agar and were each prepared according to the instruction of the manufacturer. The agar plates were then incubated at 37oC for 24 hours.

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Isolation of Pure Isolates

After 24 hours of incubation, the colonies were subcultured by streaking each colony on the surface of freshly prepared agar plate. The plates were then incubated at 37oC for 24 hours. Distinct colonies were then picked into nutrient agar slant and stored in the refrigerator for further use.

Biochemical Test

Microbact (24E) kit identification

This kit was used for biochemical tests with the range of simple, standardized system, for the rapid identification of Gram- negative bacteria. Preparation of 18-24 hours old pure culture of the organism to be identified, oxidase test was performed which must be negative or positive for 24E kit, selected isolate colony was emulsified in saline, test strip was placed in holding tray and the back seal was peeled and addition of 4 drops of bacterial suspension to each well, addition of 2 drops of mineral oil (MB1093A) to the black wells, the seal was replaced and incubated at 350C + 20C for 18-24 hours, the tray was removed from incubator and appropriate reagents was added. The results was then recorded and interpreted using the MicrobactTM identification software package (Balows et al., 1991; Oxoid limited). A purity check was performed by inoculating a purity plate with 1 drop of bacterial suspension and incubated at 350C + 20C for 24 hours,Well 13 was read at 24-48hours for Enterobacteriaceae and at 48 hours for MGNB, Well 24 was interpreted differently at 24 hours and 48 hours, A nitrate reduction test was done in well 7 after reading the ONPG reaction, Performance was monitored by testing appropriate control strains (Oxoid Limited)(13).

Gram Stain

A loopful of sterile distilled water was dropped on a clean grease free slide by using a sterile inoculating loop after which an inoculum from the culture was mixed with the water on the slide. The smear was air dried and heat fixed by passing it quickly over a Bunsen flame. The smear was flooded with crystal violet solution for 60 seconds (one minute) and rinsed with water. The smear was again flooded with Lugol's iodine for 30 seconds and rinsed with water, 70% alcohol was poured on the slides for 15 seconds until the crystal violet had been completely washed off. It was then counterstained with Safranin for 60 seconds and allowed to dry. The slides were then observed under oil immersion objective lens (x100). Gram positive cells remained purple while Gram negative cells appeared red or pink (14).

Catalase Test

A drop of hydrogen peroxide solution was placed on a clean grease free slide. A flamed inoculating loop was used to place a loopful of an inoculum on the slide and gently mixed after which it was observed for bubbles or effervescence which is an indication of catalase positive organism (14).

Oxidase Test

A piece of filter paper was placed in petri dish and three (3) drops of freshly prepared oxidase reagent was added. Using a sterile glass rod, a colony of test organisms was removed from a culture plate and it was smeared on the filter paper. Oxidase positive organisms gave blue colour within 5 to 10 seconds, and in oxidase negative organisms, colour did not change (15).

Coagulase Test

A loopful of normal saline solution was placed on each glass slide and was emulsified. Human plasma was added to one of the suspension and was stored properly for 15 minutes while the other was left as control. Coagulase positive was indicated by clumping which did not re- emulsify (15).

Antibiotic Susceptibility Test and Antimicrobial Assay of the Extract

Antibiotic susceptibility tests were performed using Kirby-Bauer's disc diffusion method on Mueller-Hinton agar. The inocula were prepared from the stock cultures which were maintained in nutrient agar slant at 4^0C and subculture in nutrient broth using a sterilized wire loop. 1 ml of 10^{-4} normal saline dilution of a 24 hours broth culture was mixed with 19 ml of the agar in a sterile universal bottle and poured into sterile petri dish. The agar plate was left to solidify and the antibiotic disc were thus placed on the agar surface at minimum of 22 mm apart and were incubated overnight at 37°C for 24 hours.The diameter of zones of inhibition were measured and recorded in millimeter and the results interpreted according to the Clinical Laboratory Standard Institute (16) guidelines.

The antimicrobial screening ofthe Olaxsubscorpioidea Oliv. Root extract against the bacterial isolates was carried out using the agar well diffusion method. A stock concentration of 100 mg/ml was constituted by dissolving 1 g each, of the extracts in 10 ml of Dimethyl sulfoxide (DMSO) diluted with sterile distilled water in ratio 1:3. 50 mg/ml, 25 mg/ml and 12.5 mg/ml concentrations of the extracts were the prepared using dilution formula (C1V1=C2V2). 1 ml of10^{-4} normal saline dilution of a 24 hours broth culture was mixed with 19 ml of the agar in a sterile universal bottle and poured into sterile petri dish. The agar plate was left to solidify and wells were bored on them using 6 mm cork borer. 50 µl of each concentration of the extracts was poured into each well and incubated at 37°C for

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24 hours.The diameter zones of inhibition were measured and recorded in millimeter and the results interpreted according to the Clinical Laboratory Standard Institute (16) guidelines. Piperacillin/tazobactam (0.125 mg/ml) and Demethyl sulfoxide (DMSO) were used as positive and negative controls respectively.

Minimal Inhibitory Concentration and Minimal Bacteriocidal Concentration

The minimal inhibitory concentration (MIC) was determined using the tube dilution method. Graded concentrations of the extract was prepared using Mueller Hinton broth medium into differenttest tubes. The concentrations were 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml and 3.125mg/ml. standardized inoculum of 24 hours broth culture was inoculated into the test tubes was incubated at 37oC for 24 hours. After incubation, the test tubes were examined for sign of growth (turbidity) and the minimal concentration with no growth was recorded as the MIC (17).

The minimal bacteriocidal concentration (MBC) was determined by streaking out samples from the test tubes with no growth on the surface of freshly prepared nutrient agar. The plates were then incubated at 37oC for 24 hours, after which plates were observed for any bacterial growth. Again, the minimal concentration with no growth was taken as the MBC(17).

Secondary Metabolite (Phytochemical) Screening of OlaxsubscorpioideaOliv. root

Qualitative Method of Analyses

Preliminary test / Preparation test

Plant filtrates were prepared by boiling 20 g of the fresh plant in distilled water. The solution was filtered through a vacuum pump. The filtrates were used for the phytochemical screening for flavonoids, tannins, saponins, alkaloids, reducing sugars, anthraquinones and anthocyanosides.

Test for Alkaloids

About 0.2gram was warmed with 2% of H2SO4 for two minutes, it was filtered and few drops of Dragendoff's reagent were added. Orange red precipitate indicates the present of Alkaloids(18).

Test for Tannins

One milliliter of the filtrate were mixed with 2m1 of FeC1, A dark green colour indicated a positive test for the tannins (18,19).

Test for Saponins

One milliliter of the plant filtrate were diluted with 2 ml of distilled water; the mixture were vigorously shaken and left to stand for 10min during which time, the development of foam on the surface of the mixture lasting for more than 10mm, indicates the presence of saponins (20).

Test for Anthraquinones

One milliliter of the plant filtrate was shaken with 10ml of benzene; the mixture was filtered and 5 ml of 10 % (v/v) ammonia were added, then shaken and observed. A pinkish solution indicates a positive test (20).

Test for Anthocyanosides

One milliliter of the plant filtrate was mixed with 5 m1 of dilute HCI; a pale pink colour indicates the positive test (21).

Test for Flavonoids

One milliliter of plant filtrate was mixed with 2 m1 of 10% lead acetate; a brownish precipitate indicated a positive test for the phenolic flavonoids. While for flavonoids, I m1 of the plant filtrate were mixed with 2m1 of dilute NaOH; a golden yellow colour indicated the presence of flavonoids(21,22).

Test for Reducing Sugars

One milliliter of the plant filtrate was mixed with Fehling A and Fehling B separately; a brown colour with Fehling B and a green colour with Fehling A indicate the presence of reducing sugars (23).

Test for Cyanogenic glucosides

This was carried out subjecting 0.5g of the extract 10ml sterile water filtering and adding sodium picrate to the filtrate and heated to boil (24).

Test for Cardiac glucosides

Legal test and the killer-kiliani was adopted, 0.5g of the extract were added to 2ml of acetic anhydrate plus H2S04(25)

Quantitative Method of Analyses

Saponins

About 20grams each of dried plant samples were ground and, put into a conical flask after which 100 ml of 20 % aqueous ethanol were added. The mixture were heated using a hot water bath. At about 55OC, for 4 hour with continuous stirring, after which the mixture were filtered and the residue re-extracted with a further 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 rnl of diethyl ether were added and then shaken vigorously. The aqueous layer were recovered while the ether layer was discarded. The purification process was repeated three times. 60 rnl of n-butanol were added. The combined n-butanol extracts were washed twice with 10 m1 of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage of the starting material (25,26).

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Fig 3.3: Antimicrobial screening of ethanol extract of Olax subscopioidea Oliv. rootat 25mg/ml.

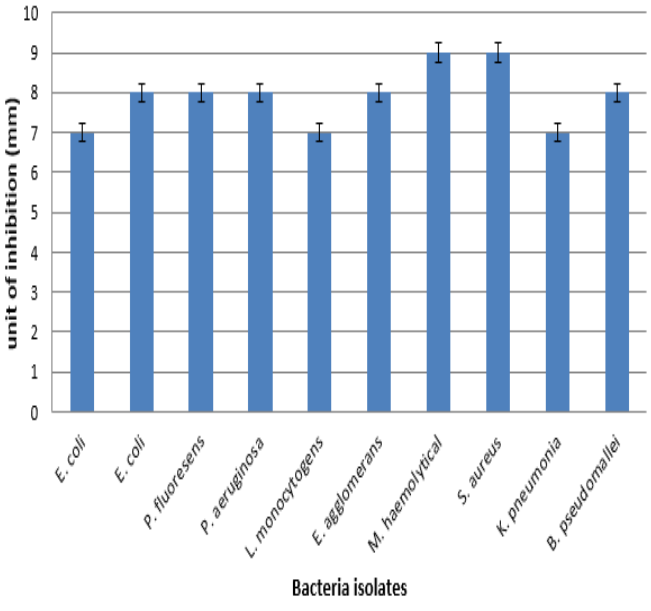

Fig 3.4:Antimicrobial screening of ethanol extract of Olax subscopioidea Oliv. rootat 12.5mg/ml.

Table 3.5 shows the minimal inhibitory concentration (MIC) and minimal bacteriocidal concentration (MBC) of the extract. MIC ranges from 25mg/ml to 100mg/ml while MBC was between 50mg/ml and 200mg/ml. This table is well explained in Fig. 3.5 to 3.6 below in which the zones of inhibition of the organisms were represented by bar chart.

Table 3.6 shows the qualitative phytochemical screening of the ethanol extract of Olax subscorpioidea Oliv. root. Flavonoid, tannins, saponins, alkaloids, reducing sugars, steroid, phenol, terpenoid, pyrrolozidine alkaloid, glycoside and cardiac glycoside were present while anthraquinones and volatile oil were not detected.

Table 3.7 present the quantitative phytochemical screening of the ethanol extract of Olax subscorpioidea Oliv. root. Glycoside and terpenoid were most present with a concentration of 14.01 while saponins were least present with a concentration of 3.21.

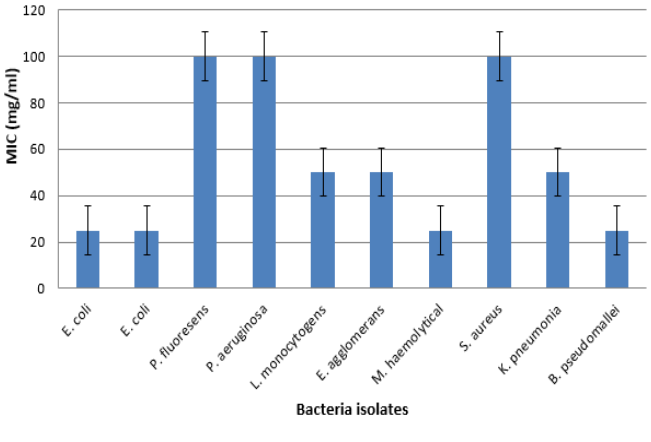

Fig 3. 5: Minimal inhibitory concentration MIC (mg/ml)

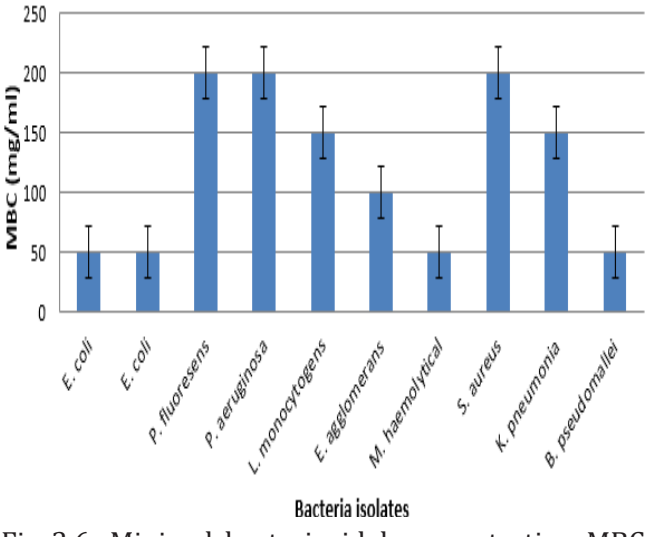

Fig 3.6: Minimal bacteriocidal concentration MBC (mg/ml)

Table 3.6: Qualitative phytochemical (secondary metabolite) screening of ethanol extracts of Olax subscorpioidea Oliv. root

Constituent	Presence
Alkaloids	+
Glycoside	+
Steroids	+
Anthraquinone	ND
Phenol	+
Tannins	+
Saponin	+
Flavonoids	+
Pyrrolizidine alkaloids	+
Reducing sugar	+
Terpenoid	+
Volatile oil	ND
Cardiac glycosides	+

KEY: + = Present, - = Absent and ND = Not Detected

Table 3. 7: Quantitative phytochemical (secondary metabolite) screening of ethanol extracts of Olax subscorpioidea Oliv. root

Constituent	Quantity
Alkaloids	13.89
Glycoside	14.01
Steroids	9.71
Anthraquinone	9.75
Phenol	7.51
Tannins	7.60
Saponin	3.21
Flavonoids	4.21
Pyrrolizidine alkaloids	4.00
Reducing sugar	13.89
Terpenoid	14.01
Volatile oil	9.71
Cardiac glycosides	9.75

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