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Inhibitory action of clinical MDR bacteria by plant extracts to induce membrane permeability and nucleotides leakage

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***ABSTRACT*: Isolation and identification of multidrug-resistant bacteria (MDR) from a various medical specimens at Zagazig University Hospitals, Egypt has been reported. The identification of such bacteria morphologically, physiologically and biochemical test were identified as *Staphylococcus aureus, P. aeruginosa, K. pneumonia, E. coli, P. vulgaris* ,and *E. fecalis.* The most effective plant extracts were *Tamarindus indica*., *Rosmarinus officinalis*.,and *Hibiscus sabdariffa*., on the growth of selected bacteria. Potassium leakage after 100 min of exposure to plant extracts induced intracellular potassium ions by 11.3-13.5 ppm and 4.5-8.3 ppm for *Staphylococcus aureus* and *Pesudomonas aeruginosa* respectively. selective permeability of the cytoplasmic membrane, and is thus a primary indication of membrane damage The major damage to the bacterial cytoplasmic membrane was measured by leakage of nucleotides after 8 hours of plant extract exposure were recorded 0.3- 0.48 and 0.2-0.38 nm for *Staphylococcus aureus* and *Pesudomonas aeruginosa* respectively.**

***KEYWORDS: Tamarindus indica, Staphylococcus aurus*, MDR**

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I. INTRODUCTION

Transmission of pathogenic organisms that cause nosocomial infection to the community can occur in the number of different ways and particularly through health staff, visitors, and discharged patients. However, the spread and development of nosocomial infections are influenced by many factors which include: 1- The characteristics of the

microorganisms, including their level of antibiotic resistance, innate virulence, and amount (inoculum) of the microbial agent. 2- Patient susceptibility includes the elderly and neonates are more susceptible and have lower immune status as are those with trauma or who have surgery, these are associated with a decreased resistance to infection. 3- Environmental factors **(WHO, 2002 and Khan *et al.*, 2017)**.

Bassetti *et al*., (2013) reported that multi-drug resistance (MDR) is used to describe bacteria that resist one or more antibiotics in three or more antibiotic classes, while extreme drug-resistant strains are termed as resistant to all antibiotics.

Natural products have been found to have great effects in disrupting the bacterial membrane. It is likely due to the presence of lipophilic compounds such as cyclic hydrocarbons, terpenes and aromatics which are abundantly found in the plants. Damage to the membrane can take several forms: (i) physical disruption of the membrane; (ii) dissipation of the proton motive force (PMF) and (iii) inhibition of membrane-associated enzyme activities **(Dholvitayakhun *et al.*, 2017).**

The mode of action of the highly aromatic quaternary alkaloids, such as berberine and harmane, is also intercalation with DNA. Coumarins cause a reduction in cell respiration and tannins act on microorganism membranes as well as bind to polysaccharides or enzymes promoting inactivation **(Radulovic *et al.*, 2013).**

Gram-negative bacteria have innate multidrug resistance to many antimicrobial compounds owing to the presence of efflux pumps (**Blanco *et al.*, 2016**). **Garvey *et al.*, (2011)** indicated that extracts of different plants, used as herbal medicinal products, contain inhibitors of efflux pumps in Gram-negative bacteria. This is a very important finding, as it gives hope that plant secondary metabolites (PSMs) could be truly useful in fighting multidrug-resistant strains.

Although the exact pathways by which plant compounds exert their antimicrobial effects are not clearly defined. Several mechanisms include disruption of the bacterial cell membrane leading to loss of membrane potential, impaired ATP production, and leakage of intracellular contents. Furthermore, interruption of DNA/RNA synthesis and function, interference with intermediary metabolism, induction of coagulation of cytoplasmic constituents and interruption of normal cell communication lead to cell death **(Chen *et al.*, 2018)**. The aim of this study was to evaluate the inhibitory effect of plant extracts on the permeability of the bacterial membrane and nucleotides leakage of clinical MDR isolated from Zagazig University Hospitals, Egypt .

II. MATERIALS AND METHODS

Apparatus

2.1. Collection of samples: Samples were collected from hospital environments and medical specimens were collected from patients admitted to Zagazig University hospitals Faculty of medicine, Zagazig, Egypt, in the period from January to August 2016. The collected specimens were quickly transported under aseptic conditions to the Microbiology Laboratory at the Faculty of Science at Zagazig University according to **Murray *et al.* (2007)**.

2.2. Isolation, purification, and identification of isolated bacteria: Bacterial isolates were isolated by streaked on nutrient agar medium until pure single colonies were obtained. Preliminary identification of bacteria was based on colonial morphology of the organisms and hemolysis on nutrient blood agar **(Farrell and Qlobinson, 1972)**,

changes in physical appearance in differential media, and enzyme activities of the organisms. Biochemical tests were performed on colonies from primary cultures for identification and characterization of the isolates. The purified bacterial colony was identified according to Bergey's Manual of Systematic Bacteriology **(Vos *et al.*, 2009).**

2.3 Antibiotic resistant test

The sensitivity of bacterial strains was determined for the following 14 antibiotics (Oxoid Ltd., UK): amikacin, imipenem, ofloxacin, nitrofurantoin, vancomycin, ceftriaxone, ciprofloxacin, azithromycin, amoxicillin/clavulanic acid, amoxycillin, oxacillin, sulphamethoxazole/trimethoprim, cefaclor, and cephalothin by using disk diffusion assays **(Bauer *et al.*, 1966)** according to Clinical and Laboratory Standards Institute, 2019. MDR was defined as an resistance to at least one agent in three or more antimicrobial categories **(Magiorakos *et al.*, 2012)**.

2.4. Antibacterial activities of plant extracts against isolated bacteria.

The antibacterial activities of medicinal plant extracts were determined using the disc diffusion method according to **Abdallah (2016).** And according to **Kawther, (2007)** calculated susceptibility of bacteria to plant extract as values of inhibition zones as no inhibition zone-9mm (resistant), 10-15mm (intermediate), and ≥ 16mm (sensitive).

2.5. Phytochemical screening of plants extracts:

The most active extracts were analyzed qualitatively for the presence of different classes of phytochemicals: flavonoids, quinones, phenols, coumarins, tannins, terpenoids, alkaloids, and saponins according to standard procedures **(Trease & Evans, 2002)**

3.4. Inhibitory effect of clinical bacteria by membrane permeabilization.

The effects of plant extracts on bacterial membrane integrity were investigated by measuring the leakage of potassium ions (K+) and 260nm-absorbing cellular components (nucleic acids) originally present in the cytoplasm of bacterial cells by using ultraviolet spectrophotometer Beckman DU640 UV/Vis, USA.

a. Potassium leakage:

Potassium ions (K+) leakage was measured according to the method described by **Ultee *et al.*, (1999) and Rodriguez *et al.*, (2004).** The bacteria were grown overnight in nutrient broth in a shaking incubator at 37°C and 150 rpm. Cells were then washed three times in 0.9% NaCl by centrifugation at 4000 rpm for 10 min, then resuspended in glycyl-glycine (Sigma, USA) buffer solution (1 mmol/L, pH 6.8). Cell suspensions were adjusted to a density of 1.0 at an optical density 600nm. The bacteria were treated with the plant extracts after that incubated in a shaking incubator at 37°C and 150 rpm. Samples (2ml) of cell suspension were removed at 0, 10, 20, 40, 60, 80, and 100 min, and filtered through a 0.22-µm pore-size membrane (Sartorius, Gottingen, Germany) to remove bacteria. The potassium concentration in the supernatant was measured using an atomic absorption spectrophotometer (900T, Perkin-Elmer Ltd, UK) set to read absorbance at 766.5 nm compared with control zero time. Experiments were conducted in triplicate and mean values were calculated.

b. Nucleotides leakage:

Bacterial cell membrane integrity was also assessed by measuring the leakage of nucleotide upon treatment with plant extracts **(Oliveira *et al.*(2015)**. A culture of the test strains was grown overnight in nutrient broth in a shaking incubator at 37°C and 150 rpm, washed twice with 0.9% sterile saline and resuspended in PBS (pH 7.4) to keep it at a final density of 10^8 CFU/ml. The bacterial suspensions were incubated with the extracts at 37°C and 150 rpm for different periods (1, 2, 4, 6, and 8h). Negative controls, cells treated with distilled water, were tested under the same conditions. After incubation, the mixture was filtered through a 0.22µm pore-size membrane to remove bacteria and the absorbance of the filtrate was determined by UV-spectrophotometer at 260nm. Nucleotide leakage an indicator of damage in the cytoplasmic membrane.

III. RESULTS

3.1.Distribution of collected isolates:

One hundred bacterial isolates were selected as the most dominant from collected samples. Table 1 includes the isolate number, and source of isolation, bacterial isolates were distributed 61% of Gram negative and 39% of Gram positive bacteria. The Gram negative bacterial isolates were isolated from urine samples of patients with urinary tract infections, wound infections (pus), respiratory tract infections (sputum), and blood infections (blood). The highest isolates of Gram negative bacteria (29) were isolated from urine and the lowest number of Gram negative bacteria were isolated from blood (5). The highest isolates of Gram positive bacteria (17) were isolated from pus and the lowest isolates of gram positive bacteria were isolated from blood (3).

Table (1): Distribution of bacterial isolates according to their Gram's stain reaction and source of isolation :

Source of isolation	Gram positive isolates		Gram negative isolates		Total	
	No.	%	No.	%	No.	%
Urinary tract infections (urine)	5	14.7	29	85.3	34	34
Wound infections (pus)	17	65.4	9	34.6	26	26
Respiratory tract infections (Sputum)	10	62.5	6	37.5	16	16
Blood infections (blood)	3	37.5	5	62.5	8	8
Hospital environment	4	25	12	75	16	16
Total	39	39	61	61	100	100

(% of Gram +ve or –ve) = no. of isolates of source/total no. of source ×100.

3.2. Identification of the most dominant bacterial isolates:

The most dominant bacterial isolates bacterial isolates (n = 42) were selected as the most dominant from different sources and then identified using biochemical and morphological techniques.

3.2.1. Biochemical and morphological identification:

Morphological, physiological, and biochemical tests were conducted to identify the MDR bacterial isolates on genus and species levels (Table 2). The staining reactions and the culture were recorded. According to the keys of identification protocols, the tested isolates were divided into the following six groups: *Escherichia coli* (I), *Klebsiella pneumonia* (II), *Proteus vulgaris* (III), *Pseudomonas aeruginosa* (IV), *Staphylococcus aureus* (V), and *Enterococcus faecalis* (VI).

The frequency of these bacteria within multi-drug resistant bacterial isolates is shown in Table 3. *Escherichia coli* was found to be the most frequent pathogen (31%), followed by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* with percentages of 21.4, 16.7 and 14.3% respectively. On the other hand, *Proteus vulgaris* was the least frequent pathogen among MDR isolates (7.1%) followed by *Enterococcus faecalis* (9.5%).

Antibiotic susceptibility tests:

The collected bacterial isolates towards different 14 antibiotics by using a standardized disc diffusion method. the results are in tables (4) .The tested isolates were highly susceptible to imipenem with susceptibility percentage of (89%) so it represented the most effective antibiotic followed by amikacin, ofloxacin, ciprofloxacin, and nitrofurantoin with 76, 67, 60, and 48% susceptibility, respectively. on the other hand, the data showed that 86% of bacterial isolates were resistant to oxacillin while 75, 74 and 69% of bacterial isolates were resistant to amoxycillin, cephalothin, and sulphamethoxazole/trimethoprim, respectively.

Table (2): Biochemical tests and morphological characters for identification of 42 multi-drug resistant bacterial isolates:

Test	Group I	Group II	Group III	Group IV	Group V	Group VI
Gram's stain	- ve	- ve	- ve	- ve	+ ve	+ ve
Shape of cell	Bacilli	Bacilli	Bacilli	Bacilli	Cocci	Cocci
Arrangement	Short rods	Short rods	Short rods	Rods	Irregular clusters	Diplococci
Motility	+ ve	- ve	+ ve	+ ve	- ve	- ve
Catalase	+ ve	+ ve	+ ve	+ ve	+ ve	- ve
Oxidase	- ve	- ve	- ve	+ ve	- ve	- ve
Coagulase	- ve	- ve	- ve	- ve	+ ve	- ve
Indole	+ ve	- ve	+ ve	- ve	- ve	- ve
Citrate	- ve	+ ve	- ve	+ ve	- ve	+ ve
MR	+ ve	- ve	+ ve	+ ve	+ ve	+ ve

VP	- ve	+ ve	- ve	- ve	+ ve	+ ve
H2S production	- ve	- ve	+ ve	- ve	- ve	- ve
Urease	- ve	+ ve	+ ve	+ ve	+ ve	+ ve
Nitrate reduction	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Blood hemolysis	γ-hemolysis	γ-hemolysis	α–hemolysis	β-hemolysis	β-hemolysis	γ–hemolysis
Gelatin liquification	- ve	- ve	+ ve	+ ve	- ve	- ve
Identification	*E. coli*	*K. pneumoniae*	*P. vulgaris*	*P. aeruginosa*	*S. aureus*	*E. faecalis*

MR =Methyl red test, VP = Voges-Proskauer reaction - ve = Negative, + ve = Positive

Table (3): Frequency of different bacterial groups with in isolates:

Group no.	Bacterial species	Isolates no.	Total number	Percentage %
I	***Escherichia coli***	1, 4, 7, 15, 19, 25, 26, 30, 31, 3, 35, 83 and 86	13	31
II	***Klebsiella pneumoniae***	6, 43, 61, 65, 70 and 94	6	14.3
III	***Proteus vulgaris***	13, 33 and 98	3	7.1
IV	***Pseudomonas aeruginosa***	10, 17, 46, 50, 53, 56, 57, 79 and 93	9	21.4
V	***Staphylococcus aureus***	28, 34, 36, 38, 52, 64 and 89	7	16.7
VI	***Enterococcus faecalis***	22, 41, 71 and 75	4	9.5
Total			42	100

Table (4): Resistance of bacterial isolates against different antibiotics:

Antibiotic	**Symbol conc. (µg/disc)**	**Resistant (R)**	
		No.	**%**
Imipenem	IPM (10)	8	8
Amikacin	AK (30)	14	14
Ofloxacin	OFX (5)	32	32

Ciprofloxacin	CIP (5)	32	32
Nitrofurantoin	F (300)	44	44
Vancomycin	VA (30)	53	53
Ceftriaxone	CRO (30)	45	45
Azithromycin	AZM (15)	62	62
Amoxicillin/clavulanic acid	AMC (30- 20/10)	62	62
Cefaclor	CEC (30)	50	50
Amoxycillin	AX (25)	75	75
Oxacillin	OX (1)	86	86
Sulphamethoxazole/trimethoprim	SXT **(23.75/1.25)**	69	69
Cephalothin	CL (30)	74	74

Total bacterial count =100 isolates

% Resistant = No. of Resistant isolates/ Total count of isolates *100

4.1. Antibacterial activity of plant extracts against multidrug-resistant (MDR) bacteria:

Aqueous plant extracts which were derived from different parts of seven medicinal plant species traditionally used in Egyptian folk medicine were tested for their antibacterial activity against the same MDR. The inhibition zone diameters of aqueous extracts of tamarind were the most active one against Gram negative and Gram positive bacteria with inhibition zone diameters ranging from 18- 25mm for aqueous extracts. table (5) followed by roselle, rosemary and ginger which produced inhibition zone diameters in aqueous extracts ranging from 16- 21 mm, 0-17 mm, and 0-15 mm, respectively.

Moreover, the results indicated that Gram positive bacteria were more susceptible than Gram negatives to the tested extracts. The most susceptible bacteria were *E. faecalis*, *Staph. aureus* and *P. vulgaris* respectively. On contrary, the least susceptible bacterial strains were *P. aeruginosa*, *E. coli*, and *K. pneumoniae* respectively.

Table (5): Diameter of inhibition zone of aqueous plant extracts against MDR bacteria.

Bacterial isolate	Inhibition zone (mm)						
	Ginger	Tamarind	Lavender	Rosemary	Roselle	Mustard	Chamomile
S. aureus	14(I)	23(S)	9(R)	14(I)	19(S)	13(I)	13(I)
E. faecalis	15(I)	25(S)	11(I)	17(S)	21(S)	14(I)	13(I)
E. coli	0(R)	18(S)	0(R)	0(R)	18(S)	0(R)	0(R)
K. pneumoniae	12(I)	21(S)	0(R)	15(I)	20(S)	10(I)	10(I)
P. aeruginosa	0(R)	19(S)	0(R)	0(R)	16(S)	0(R)	0(R)
P. vulgaris	0(R)	21(S)	0(R)	15(I)	18(S)	11(I)	0(R)

4.2. Phytochemical screening of plants extracts:

The results in a table (7) indicated that: phenols, flavonoids, alkaloids, and terpenoids are present in all tested extracts while coumarins are present only in chamomile and mustard extracts. Furthermore, quinones were absent in roselle, lavender, and mustard; tannins were absent in chamomile and lavender; saponins were absent in rosemary and lavender

Table (6): Phytochemical constituents present in the plants extracts:

Phytochemical analysis	Rosemary	Roselle	Tamarind	Chamomile	Lavender	Mustard	Ginger
Phenols	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Quinones	+	-	+	+	-	-	+
Coumarins	-	-	-	+	-	+	-
Tannins	+	+	+	-	-	+	+
Saponins	-	+	+	+	-	+	+
Alkaloids	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+

4.3. Mechanisms of antibacterial action of plant extracts:

4.3.1. Membrane permeabilization:

a. Potassium leakage:

The obtained results in tables (7 and 8) indicated that, after exposure to extracts, the leakage of potassium ions in both bacterial strains increased immediately and rapidly in the first 60 minutes, thereafter the values were almost unchanged. In response to the extracts, in the case of *S. aureus*, the extracts induced a marked elevated leakage of intracellular potassium (11.3-13.5 ppm) after 100 min of treatment. In the case of *P. aeruginosa*, some leakage of extracellular potassium reached 4.5-8.3 ppm after 100 min of contact with the extracts. Furthermore, *S. aureus* was observed to possess greater sensitivity to the extracts (higher potassium leakage) subsequently greater damage to the cell membrane was observed compared with control.

Table (7): Effect of plants extracts on the amount of K+ released from *S. aureus*:

Exposure time (min)	Extracellular K+ concentration (ppm)			
	Control	Tamarind	Rosemary	Roselle
0	0	0	0	0
10	0.34	4.8	3.2	5.1
20	0.35	7.5	5.1	6.8
40	0.52	10.9	7.7	9.1
60	0.56	12.8	10.5	13.2
80	0.61	13	11	13.3
100	0.65	13.2	11.3	13.5

Table (8): Effect of plants extracts on the amount of K+ released from *P. aeruginosa*:

Exposure time (min)	Extracellular K+ concentration (ppm)			
	Control	Tamarind	Rosemary	Roselle
0	0	0	0	0
10	0.18	1.8	0.82	1.5
20	0.21	2.6	1.4	2.8
40	0.30	5.4	2.9	5.2
60	0.33	7.5	3.7	7.1
80	0.37	8.1	4.0	7.5
100	0.41	8.3	4.5	7.9

b. Nucleotides leakage:

Major damage to the bacterial cytoplasmic membrane in response to exposure to the most effective plant extracts (tamarind, roselle, and rosemary) at the MIC concentrations was determined by measuring the leakage of nucleotides. The obtained results in tables (9 and 10) indicated that, after addition of the extracts, the nucleotide leakage increased rapidly after one hour and increased gradually from the 4th to the 8th hour with maximum effects observed at the 8th hour (0.48) at *S. aureus*. A stronger correlation between bacterial nucleic acid leakage and duration of exposure was observed. Furthermore, the damage to the cell membrane of *S. aureus* was greater than that of *P. aeruginosa.* Also, tamarind extract showed the best effects followed by roselle and rosemary, respectively compared with control.

Table (9): Effect of plant extracts on the number of nucleotides released from *S. aureus*:

Exposure time (h)	Absorbance at 260 nm			
	Control	Tamarind	Rosemary	Roselle
0	0.00	0.00	0.00	0.00
1	0.00	0.12	0.00	0.00
2	0.00	0.21	0.10	0.17
4	0.01	0.36	0.16	0.25
6	0.03	0.41	0.23	0.30
8	0.05	0.48	0.30	0.39

Table (10): Effect of plant extracts on the number of nucleotides released from *P. aeruginosa*:

Exposure time (h)	Absorbance at 260 nm			
	Control	Tamarind	Rosemary	Roselle
0	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00
2	0.00	0.11	0.03	0.09

4	0.00	0.21	0.08	0.20
6	0.02	0.27	0.14	0.29
8	0.03	0.35	0.20	0.38

V – DISCUSSION

The World Health Organization reported that 55 million people died worldwide in 2011, with one-third of the deaths owing to infectious diseases **(WHO, 2011).** Hospitals are a critical component of the antimicrobial resistance problem worldwide. The combination of highly susceptible patients, intensive and prolonged antimicrobial use and cross-infection have resulted in nosocomial infections with highly resistant bacterial pathogens **(Khan *et al.*, 2017).**

Bacterial isolates were distributed as 61% Gram negative bacterial isolates and 39% Gram positives. The highest Gram negatives isolated from urinary tract infections (urine) was 29. The highest Gram positive isolates collected from wound infections (pus) was 17. A similar predominance of Gram-negative organisms was found in result obtained by **Doyle *et al.*, (2011)**. Also, these results are in line with that of **Abou-Zied, (2011)** who demonstrated that Gram negative bacteria represented 53 % of total identified clinical bacteria while Gram positive represented 47%. Likewise, in developing countries, a systematic review of 220 studies from 1995 to 2008 across 6 regions (Africa, the Americas, Europe, Southeast Asia, eastern Mediterranean, and western Pacific) found that the most common bacteria causing health-care-associated infections (HAIs) are Gram-negative bacilli **(Allegranzi *et al.*, 2011).** However, a predominance of Gram-positive organisms was noted in **Vincent *et al.*, (2006)** study**.** Isolates antibiotic resistance patterns were assessed and traditional antimicrobial susceptibility test revealed that the most effective antibiotic was imipenem followed by amikacin, ofloxacin, ciprofloxacin, and nitrofurantoin, respectively. Imipenem was previously reported to be the most effective and similar effects of these antibiotics categories were also reported **(Reddy, 2016 and Abeer *et al.*, 2020)**

In a study by **Gupta *et al.* (2014),** tamarind fruit extract was active against all the test Gram-positive bacteria isolates with inhibition zones 16-19 mm. It also inhibited the growth of all Gram-negative bacteria isolates but produced an inhibition zone greater than 15 mm only in the case of *P. aeruginosa* and *Salmonella* sp. as similar results were obtained **(Abdulhamid *et al.*, 2016**).

Potassium leakage after 100 min of exposure to plant extracts induced intracellular potassium ions by 11.3-13.5 and 4.5-8.3 ppm for *Staphylococcus aureus* and *Pesudomonas aeruginosa* respectively. The major damage to bacterial cytoplasmic membrane was measured by leakage of nucleotide after 8 hours of plant extract exposure were recorded 0.3-0.48 and 0.2-0.3 nm for *Staphylococcus aureus* and *Pesudomonas aeruginosa* respectively. The cytoplasmic membrane is undoubtedly a major target of action of antibacterial agents. Damage to bacterial membrane, if it occurs to a certain extent may lead to the release of cytoplasmic constituents of the cell that can be monitored. Large molecules such as DNA, RNA, and other materials may leak after the leakage

of small molecules such as sodium, potassium, and phosphate **(Gyawali *et al.*, 2015).** Since the leakage of potassium ions occurs rapidly after treatment, it is proposed that the efflux of this ion is one of the first indications of the disruption of the selective permeability of the cytoplasmic membrane, and is thus a primary indication of membrane damage **(Oliveira *et al.*, 2015 and Ajiboye *et al.*, 2016).**

5. CONCLUSION

A high proportion of bacterial strains isolated from patient samples of infection showed antibiotic resistance. Among other things, this suggests that considerable precaution should be taken at all stages of the health care system to minimize the transmission of MDR bacteria among patients and medical staff in both in- and out-patient settings in the hospital and community. Moreover, regular monitoring of antimicrobial resistance patterns from each clinical sample could be very valuable. Antibiograms like the one developed here, if made available to clinicians and policymakers, could be quite useful.

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Conflict of interest

All authors declare that they have no conflict of interest.

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