Research Article

Identification and Characterization of OXA-48 Carbapenemase-Producing Enterobacteriaceae Clinical Isolates in Baghdad

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Abstract

Background: *Enterobacteriaceae* is the most frequent Gram-negative pathogens that are accountable for many serious infectious diseases. The emergence and widespread carbapenem-resistant *Enterobacteriaceae* have increased dramatically and have become a burden on public health with increasing challenge in treatment with classical antibiotics.

Objectives: The goal of this research was to detect of OXA-48 carbapenemase-producing *Enterobacteriaceae* clinical isolates using a new *in vitro* phenotypes technique "OXA-48 K-SeT assay".

Methods: A total of 40 *Enterobacteriaceae* strains was involved in this investigation. Antibiotics susceptibility rates were carried out by modifying Kirby-Bauer's disk diffusion technique. Biochemical tests were performed using the API 20 E kit. Identification of OXA-48 carbapenemase was tested by OXA-48 K-SeT assay and modified Calgary biofilm method were used for detection of biofilm formation.

Results: The majority of *Enterobacteriaceae* isolates were obtained from urine samples 23 (57.5%) followed by sputum and wound exudate samples 6 (15%), 5 (12.5%) respectively. The prevalence of infection with *E. coli* and *K. pneumoniae* was significant among age groups of 40 -59 years old (P <0.001), and patients with age below twenty or over sixty years old showed lower susceptibility to infection with *Enterobacteriaceae*.

The strains of *k. pneumoniae* obtained from urine samples exhibited a strong propensity to develop biofilms and it showed an excellent biofilm propensity score, whereas *E. coli* strains showed a lower propensity to form biofilm. In addition, *K. Pneumonia* was displaying a predisposition to resistant to OXA-48 carbapenemase with 7 (17.5%) positive for OXA-48 K-SeT, while only 4 (10%) *E. coli* exhibit positive results for OXA-48 K-SeT.

The most OXA-48-positive and OXA-48-negative of *Enterobacteriaceae* isolates showed markedly resistance to beta-lactamase inhibitor combination (amoxicillin-clavulanate and piperacillin-tazobactam) with different resistance rates were noted against cephalosporin groups (ceftazidime, cefotaxime, cefepime, and cefoxitin) and less resistance to monobactam groups (aztreonam) were observed, while no resistance was observed against colistin. The higher levels of antibiotic resistance correlated dramatically with increase biofilm-producing of *Enterobacteriaceae*, regardless of types of antibiotics (β-lactam and non- β-lactam antibiotics).

Conclusion: The findings of this study show that the OXA-48 K-SeT assay were exact phenotypic method and significant test for direct detection of OXA-48-producing *Enterobacteriaceae*. This study found clearly correlating of biofilm formation and increased level of antibiotic resistance in *Enterobacteriaceae* isolates. The quick recognition and confirmation of carbapenemases resistance in these bacteria is important for proper choice of antibiotics and avoidance multidrug-resistant pathogens.

Key words: Enterobacteriaceae, OXA-48 carbapenemase, Biofilm formation, Multidrug resistance

INTRODUCTION

carbapenemase-producing *Enterobacteriaceae* (CPE) have been spread and revealed broadly around the world, especially in the Middle East, Asia, and Europe.[1] The fast and exact discovery of anti-infective agents against these microscopic organisms is vital for contamination control.[2]

Carbapenem-resistant *Enterobacteriaceae* (CRE) are for the most part distinguished among *Klebsiella pneumoniae, Escherichia coli, Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Carbapenemases are compounds ready to hydrolyze almost all β-lactam antibiotics, including carbapenems and most of carbapenemases are plasmid-interceded and have been for the most part revealed in *Enterobacteriaceae*.[3] Carbapenemases are grouped into various molecular classes, class A enzymes "i.e., *Klebsiella pneumoniae* carbapenemase (KPC)" Class B enzymes, which incorporate metallo-β-lactamases "i.e., Verona integron encoded metallo-β-lactamase (VIM)", and class D enzymes "i.e., oxacillin hydrolyzing (OXA)" which are penicillinases equipped for hydrolyzing oxacillin and cloxacillin.[4]

Oxacillinase (OXA- 48) is a carbapenem-hydrolyzing class D β-lactamase which gives resistance to penicillins and frail imperviousness to carbapenems, initially depicted in *Klebsiella pneumoniae* disengages from the neighboring country (Turkey), while a limited number of distributed information about OXA-48-producing Gram-negative bacilli (GNB) from Iraq are available.[5]

Various phenotypic methods developed for the characterization of OXA-48-like carbapenemases in *Enterobacteriaceae* such as "modified Hodge test, MIC determination, acidometric, and combination disk tests". In addition to genotype methods "Polymerase chain reaction (PCR), loop-mediated isothermal amplification, microarrays, and DNA sequencing" as the gold standard methods.[6]

The objectives of this study were to investigate of OXA-48 carbapenemase-producing *Enterobacteriaceae* uses a new method depending on immunochromatographic lateral flow assay (the OXA-48 K-SeT assay) and study the characterization of virulence factor (Biofilm formation) and antibiotic susceptibility of these microorganisms' which collected from clinical samples of patients who were hospitalized in the Baghdad medical city.

PATIENTS AND METHODS

Sample collection and bacterial identification: The investigation of OXA-48 carbapenemases was performed with 40 clinical isolates of Gram-negative bacilli including twenty strains of *K. pneumonia* and twenty strains of *Escherichia coli.* The identification of all *Enterobacteriaceae* family group isolates were confirmed by Gram stain, cultural characteristics on different types of culture media including blood agar, MacConkey agar, and brain-heart infusion broth was used to isolate bacteria from blood samples. Biochemical tests using the API 20 E kit "BioMérieux, Marcy L'Etoile, France" were employed to complete the diagnosis. The bacteria isolate was collected from different clinical samples including urine (23), blood (3), sputum (6), wound swabs (5) and burns (3). The isolates were collected during the period between September 2016 and February 2017 from various patients who were hospitalized in the Baghdad medical city.

Antibiotic susceptibility testing: Antibiotic susceptibility of *Enterobacteriaceae* isolates were carried out by modifying Kirby-Bauer's disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines (7). One to three colonies from *Enterobacteriaceae* isolates were grown overnight on Müller-Hinton agar (Oxoid, UK) as optimal incubation temperature 37°C for 24 h. Cultures of *Enterobacteriaceae* were adjusted to 0.5 McFarland standards and streaking method was used to plated the bacterial suspension on Müller-Hinton agar by sterile swab. The outcomes were communicated as sensitive (S) or resistant (R) as indicated by the criteria prescribed by the (CLSI) 2014. The following antibiotics were tested: Gentamicin (GEN), Amoxicillin-clavulanate (AMC), Cefepime (FEP), Cefotaxime, Cefoxitin (FOX), Ceftazidime (CAZ), Aztreonam (ATM), Piperacillin-tazobactam (TZP), and Colistin (CST). All Antibiotics were used in this study were purchased from Oxoid (UK) and Bioanalyse (Ankara, Turkey) as presented in the table 1.

OXA-48 K-SeT® assay: The OXA-48 K-SeT assay developed by "Coris Bio-Concept, Belgium" to the identification of OXA-48 like carbapenemases of *Enterobacteriaceae* from a single colony on solid medium. The principle of this test depends on immunochromatography assay on nitrocellulose membrane with colloidal gold nanoparticles. A nitrocellulose layer is sharpened with a monoclonal antibody (Ab) coordinated against one epitope of the OXA-48 like carbapenemase.[5] The second monoclonal Ab coordinated against another epitope of the OXA-48 carbapenemase and then conjugated to colloidal gold nanoparticles, this conjugate complex is dried on a layer

(Fig 1).[8] The OXA-48 K-SeT was carried out according to recommendations by manufacturer's directions. Briefly, a single bacterial colony of Enterobacteriaceae was homogenized in ten drops of lysis buffer "Tris-HCl, NaN 3 (pH 7.5)" and three driblets from suspensions were connected to the specimen well. The outcome observable within 15 minutes as a reddish-purple band that creates on the strip test in the (T) position (Fig. 2).

Biofilm formation assay : The ability of *Enterobacteriaceae* isolates strains to form biofilms was investigated by modifying Calgary biofilm technique.[9] Briefly, bacterial strains of *Enterobacteriaceae* were grown for 24 h at 37°C and were diluted with Lysogeny broth (LB) to get a final concentration of 1 x 105 CFU/ml. Then, Two-hundred microliter were added to each well of a sterile 96-well Microtiter plate and incubate overnight at optimum temperature. The wells were washed twice with two-hundred microliter of 1X Phosphate Buffered Saline (PBS Buffer) and biofilms were fixed by incubation at 80 °C for 1 h. biofilm formation was stained with 1% crystal violet at room temperature for 20 minutes, followed by washing in water three to four times and destained of biofilm with 95% ethanol for 30 min at 37°C. The absorbance of 570 nm was applied to measure the biofilm formation of *Enterobacteriaceae* isolates using microtiter plate reader (Huma Reader-HS, Human GmbH, Wiesbaden, Germany). The main of three independent tests was scored in respect to the absorbance (A 570 ≥ 0.4 = +++; 0.3 = ++; 0.2 =+; ≤ 0.1 = +/-).[10]

Statistical analysis: Graphpad PRISM® 6 software "GraphPad Software, Inc., La Jolla, CA, USA" was applied to data analysis. Student's t-test was dependent for calculate of P-values. All information was accepted from no less than two independents experiments. $P < 0.05$ was regarded as statistical significant.[11]

Table 1: Antimicrobial disks used against Enterobacteriaceae isolates.

Class	Antimicrobial	Symbol	Concentration µg/ml
polymyxins	Colistin	CST	10
Aminoglycoside	Gentamicin	GEN	15
Cephalosporins	Cefepime	FEP	30
	Cefotaxime	CTX	30
	Ceftazidime	CAZ	30
	Cefoxitin	FOX	30
Monobactams	Aztreonam	ATM	10
β-lactamase Inhibitors	Piperacillin-tazobactam	TZP	20/10
	Amoxicillin-clavulanate	AMC	20/10

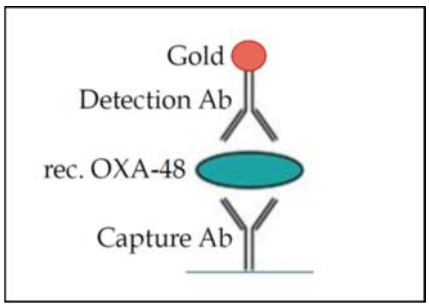

Figure 1: Principle of the OXA-48 K-SeT assay (8).

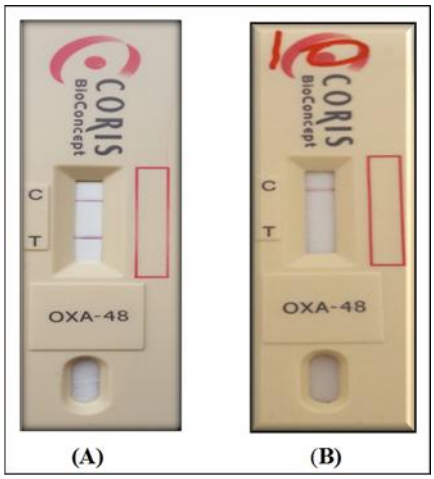

Figure 2: OXA-48 K-SeT assay used for recognition of OXA-48 like carbapenemases. A) Represents the positive result. B) Represents the negative result.

RESULTS

During the period of this study, a total of forty *Enterobacteriaceae* included twenty *E. coli* and twenty *K. pneumoniae* clinical isolates from patients who are in hospitals of medical city of Baghdad. The majority of *Enterobacteriaceae* isolates were obtained from urine samples 23 (57.5%) followed by sputum and wound exudate samples 6 (15%), 5 (12.5%) respectively, while the fewer isolates of *Enterobacteriaceae* were collected from blood 3 (7.5 %) and burn samples 3 (7.5 %) as presented in table 2.

Table 2: Distribution of Enterobacteriaceae isolates according to the type of samples.

Sample type	*Enterobacteriaceae*		Total n (%)
	E. coli n (%)	*K. pneumoniae* n (%)	
Urine	10 (25)	13 (32.5)	23 (57.5)
Sputum	3(7.5)	3 (7.5)	6 (15)
Blood	2(5)	1(2.5)	3 (7.5)
Wound exudate	3(7.5)	2(5)	5 (12.5)
Burn	2(5)	1(2.5)	3 (7.5)
Total	20 (50)	20 (50)	40 (100)

The results showed that the prevalence of infection with *E. coli* and *K. pneumoniae* was significant among age groups of 40 -59 years old ($P < 0.001$). The patients with age below twenty or over sixty years old showed lower susceptibility to infection with *Enterobacteriaceae* strains. Moreover, female subjects were at high risk to be infected with E. coli rather than being infected with *K. pneumoniae* and significant difference between male and female was detected in infected patients with *Enterobacteriaceae* species in different age groups ($P < 0.001$) as indicated by the results in the figure 3.

All *Enterobacteriaceae* isolates were tested for capability to form biofilms by using a Calgary method with some modification. The results of presented study showed that most *Enterobacteriaceae* species obtained from urine samples exhibited a strong propensity to develop biofilms with significant differences with other samples ($P < 0.001$). On the contrary, *Enterobacteriaceae* species isolated from sputum, wound exudate, blood, and burns showed lower portability to produce biofilms with no significant difference between the samples was observed ($P > 0.05$) (Fig. 4).

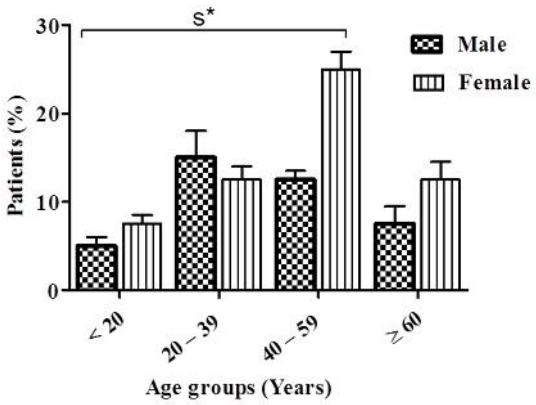

Figure 3: Distribution of the patients according to age groups (Years). S* = Significant ($P < 0.001$).

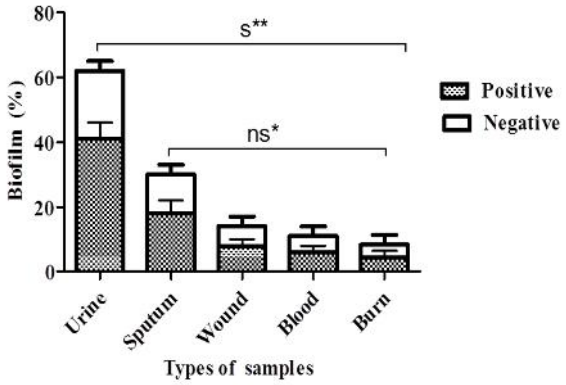

Figure 4: Correlation between types of samples and biofilm formation of *Enterobacteriaceae* isolates. ns* = non-significant ($P > 0.05$), S = Significant ($P < 0.001$).**

On the other hand, *K. pneumoniae* showed an excellent biofilm propensity score (+++), whereas *E. coli* strains showed a lower propensity to form biofilms (Fig. 5) with no significant differences between both strains in the propensity score for biofilm formation ($P > 0.05$).

The OXA-48 K-SeT test "Coris BioConcept, Belgium" depends on immunological catch the specific two epitopes of the OXA-48 variants "OXA-48, OXA-181, OXA-204, OXA-232, and OXA-244" by employing the colloidal gold nanoparticles adherence to a nitrocellulose membrane with a lateral-flow device . According to the results presented in this study, *K. pneumonia* was displaying a predisposition to resistant to OXA-48 carbapenemase with 7 (17.5 %) of these strains were positive for OXA-48 K-SeT (Table 3), while only 4 (10%) *E. coli* exhibit positive results for

OXA-48 K-SeT test and statistical analysis displayed no important differences between *Enterobacteriaceae* isolates in production of OXA-48 carbapenemase (P > 0.05).

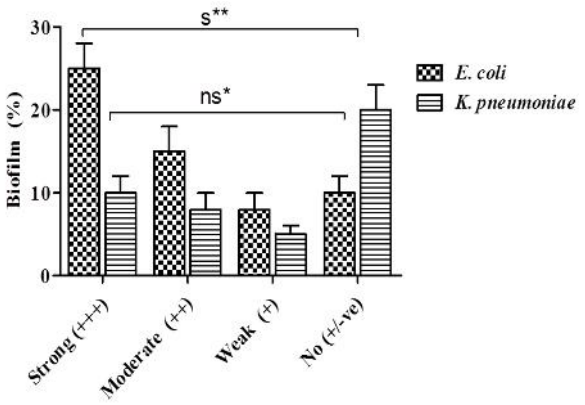

Figure 5: Biofilm propensity score of Enterobacteriaceae isolates. ns* = non-significant (P > 0.05), S = Significant (P < 0.001).**

On the other hand, the results obtained from table 4 indicated that most *Enterobacteriaceae* isolates from females' samples showed positive results for both OXA-48 K-SeT test and biofilm formation 6 (15%) and 16 (40%) respectively.

In this study, the most OXA-48-positive *Enterobacteriaceae* isolates showed markedly resistance to beta-lactamase inhibitor combination "amoxicillin-clavulanate and piperacillin-tazobactam" Moreover, the different resistance rate was noted against cephalosporin groups, including ceftazidime, cefotaxime, cefepime, and cefoxitin, while less resistance to monobactam group (aztreonam) and gentamicin were observed.

Table 3: The results of the OXA-48 set for *Enterobacteriaceae* isolates.

Enterobacteriaceae	Status OXA-48 K-SeT		**Total n (%)**
	Positive n (%)	**Negative n (%)**	
K. pneumoniae	7 (17.5)	13 (32.5)	20 (50)
E. coli	4 (10)	16 (40)	20 (50)
Total	11 (27.5)	29 (72.5)	40 (100)

Table 4: Distribution of patients, according to results of OXA-48 set and biofilm formation.

Sex	**Status (OXA-48 K-SeT)**		**Biofilm**	
	Positive n (%)	**Negative n (%)**	**Positive n (%)**	**Negative n (%)**
Males	5 (12.5)	11 (27.5)	9 (22.5)	7 (17.5)
Females	6 (15)	18 (45)	16 (40)	8 (20)
Total	11 (27.5)	29 (72.5)	25 (62.5)	15 (37.5)

Additionally, OXA-48-negative of *Enterobacteriaceae* isolates were also resistant to combination of beta-lactamase inhibitor "Amoxicillin-clavulanate and Piperacillin-tazobactam" and resistance against cephalosporin a group (ceftazidime, cefotaxime, Cefepime and cefoxitin) were also observed. On the contrary, no resistance was observed against colistin for both OXA-48- positive and OXA-48-negative of *Enterobacteriaceae* isolates strains.

The results regarding bacterial biofilm formation and antibiotic susceptibility test clearly indicated that higher levels of anti-microbial resistance related significantly with increase biofilm-producing of Enterobacteriaceae state, regardless of types of antibiotics (beta-lactamase and non- β-lactam antibiotics) are appeared in table 5.

Table 5. The percentage rate of antibiotics susceptibility test and biofilm formation propensity score of *Enterobacteriaceae* isolates.

Strain groups and antibiotics	Resistant (%)	Sensitive (%)	Biofilm Propensity score
OXA-48 type producing *Enterobacteriaceae* isolates			
Cefepime	49.2	50.8	+++
Cefotaxime	56.1	43.9	++
Ceftazidime	45.5	54.5	++
Aztreonam	50.8	49.2	++
Gentamicin	55.4	44.6	+
Amoxicillin-clavulanate	88.9	11.1	++
Cefoxitin	15.9	84.1	+/-
Piperacillin-tazobactam	97.2	2.8	+/-
Colistin	2.3	97.7	+/-
Non-OXA-48 type producing *Enterobacteriaceae* isolates			
Cefepime	90.5	9.5	++
Cefotaxime	96.9	3.1	++
Ceftazidime	94.4	5.6	+
Aztreonam	80.5	19.5	+
Gentamicin	62.1	37.9	+
Amoxicillin-clavulanate	90.1	9.9	+/-
Cefoxitin	88.2	11.8	+/-
Piperacillin-tazobactam	100	0	++
Colistin	12.4	87.6	+/-
Cefepime	49.2	50.8	+++
Cefotaxime	56.1	43.9	++

DISCUSSION

Enterobacteriaceae are regular pathogens for human and can cause an expensive scope of sicknesses including urinary tract infection, respiratory tract infection, circulatory system contamination, skin and delicate tissue diseases in both community and healing facility settings.[12]

Multidrug-resistant in *Enterobacteriaceae* is an imperative general medical issue and carbapenem-resistant *Enterobacteriaceae* is progressively isolated from nosocomial contamination and community-acquired, in addition, these microorganisms spread clonally from individual to individual or genes that encode for carbapenemase can diffused evenly among disengages colonies.[13]

Iraq and its neighboring countries have a geographical significance to recognition of carbapenemase producing microorganism and a high level of multidrug-resistant (**MDR**) *Enterobacteriaceae were* recorded. [14]

Recently epidemiological information has emphasized that the commonest rate of OXA-48 variations are quickly expanding and that OXA-48 is at present turning into the widespread carbapenemase class in *Enterobacteriaceae* in numerous countries in the world such as Asia (Taiwan, India), Middle East (Lebanon), Mediterranean countries (Slovenia, and Greece), North African (Libya, Tunisia, Algeria, and Morocco), European areas (Switzerland, Belgium, and Germany), Netherland and United Kingdom.[12,13]

The most important *bla* OXA-48 gene was first recognized in *klebsiella pneumoniae* from clinical isolates in Istanbul. Yet, at present, numerous different types of *Enterobacteriaceae* are known to have this gene, for example, *Proteus mirabilis, K. oxytoca, Serratia marcescens, E. coli, C. freundii, Morganella morganii* and *Enterobacter* spp.[15]

In this analysis, greater part of OXA-48 production *Enterobacteriaceae* isolated were *k. pneumoniae,* this is in line with what was recently published by Abdulla, Anwar Ali, *et al.* in our country (Babil Governorate).[16] Moreover, the results of this study are consistent with the results were documented by Gauthier, Lauraine, *et al.*[17] and Aqel, Amin A., *et al.*[18] in neighboring countries (Jordan and Turkey). Furthermore, was predominant in *Enterobacteriaceae* isolated from clinical samples with 100% sensitivity and specificity for OXA-48 immunochromatographic test.

In this study, colistin was found as more active anti-microbial agent most *Enterobacteriaceae* isolated,

followed by cefoxitin. Similar to this result reported by that found Colistin was high sensitivity against the *Enterobacteriaceae* in their evaluation.

On the other hand, In the present study number of multidrug-resistant in OXA-48-producing *Enterobacteriaceae* isolates were barely greater than OXA-48 negative ones. This confirms the findings of previous studies by Tsakris, Athanassios, *et al*.,[19] that founds the most OXA-48 producing *Enterobacteriaceae* showed multidrug-resistant to fluoroquinolones and aminoglycosides, this can be due to mechanical carbapenem resistance in *Enterobacteriaceae* either generation of carbapenem-hydrolyze carbapenemases and /or upregulation efflux pump.

Biofilm development has regularly been related with destructiveness in numerous pathogenic microorganisms. In *E. coli* and *k. pneumoniae* there is an upregulation of destructiveness considers strains that can structure solid biofilms. In this study, multidrug-resistant of OXA-48 producing *Enterobacteriaceae* isolates were more likely to develop biofilms compared to OXA-48 non-producing *Enterobacteriaceae* strains. This phenomenon has been confirmed by recent study by Amuthamani , Subramaniyan *et al*.[20] where they found a close relationship between drug resistance in Gram-negative and the production of the biofilms. This might be as a result of the specific method of activity of antibiotics, i.e. the solubilization of the microbial layer, which is progressively inadequate against biofilm, in spite of the fact that sub- lethal levels of antibiotics really restrain biofilm production of most Gram-negative bacilli strains.[21,22]

Conclusions

The current study shows that the OXA-48 K-SeT assay was a good phenotypic strategy for direct and rapid affirmation of OXA-48 producing *Enterobacteriaceae* species specially in *E. coli* and *k. pneumoniae*. In addition, this study found clearly correlating of biofilms formation and increased multidrug resistance in *Enterobacteriaceae* isolated strains which leads to difficult in treatment and control of diseases. Therefore, rapidly recognition of OXA-48 producing *Enterobacteriaceae* is important for optimization of anti-microbial therapy and avoidance of medical institution outbreaks.

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