Isolation and Identification of Acinetobacter baumannii Clinical Isolates using Novel Methods

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Abstract:

Acinetobacter baumannii has emerged over the last decade as a significant opportunistic pathogen. Although it is generally associated with benign colonization of hospitalized patients. The purpose of this study was undertaken to isolate and identify of *A. baumannii* from clinical infections using different laboratory methods.

A total of 520 clinical samples (wound pus, urine, respiratory secretion, blood) were collected from February to June, 2011. *A. baumannii* isolates were preliminary identified according to the morphological characteristics and traditional biochemical test. Then confirmed by using API 20E and API 20 NE multi-test system. Isolates were further identified on molecular level using 16S rRNA specific gene by polymerase chain reaction (PCR). All PCR positive isolates were examined to grow on CHROMagar *Acinetobacter*/MDR medium and their antibiotics susceptibility.

A total of 335 Gram negative bacilli isolates were obtained according to the morphological characteristics and traditional biochemical test. Only 23(6.8%) were identified as suspected *A. baumannii*. Ten (43.4%) isolates revealed very good identification level by API 20E system. While, API 20 multi-test system for non enteric bacteria identified only 12(52.1%) isolates belonged to *A. baumannii*. The same isolates harbored positive bands in PCR experiments with specific primers for16S rRNA gene. *A. baumannii* isolates were distributed as 6(50%) recovered from urine, 4(33.3%) from lower respiratory secretion and only two (25%) were obtained from wound pus, no *A. baumannii* isolates were isolated from blood samples in this study. Hence, 6 (50%) of isolates were gave overnight pure growth on CHROMagar *Acinetobacter*/MDR medium with resistant to representatives of three or more classes of antibiotics. The present findings suggest that the PCR using16S rRNA gene CHROMagar were gold standard method for detection of multi-drug resistant *A. baumannii* isolates among patients in Najaf hospitals.

الخلاصة:

هدفت الدراسة إلى عزل وتشخيص بكتريا *Acinetobacter baumannii* من إصابات سريريه مختلفة باستخدام الطرق المختبرية التقليدية والجزيئية والعزل على الأوساط الخاصة. جمعت ٥٢٠ عينة سريريه (مسحات الجروح، الادرار، افرازات المجاري التنفسية، الدم) خلال الفترة من شباط إلى حزيران من العام ٢٠١١ من المرضى الراقدين والمراجعين لثلاث مستشفيات رئيسية في النجف . تم التشخيص الأولي لعزلات بكتيريا *A. baumannii* اعتمادا على الخصائص المظهرية للبكتريا وفعاليتها الكيموحيوية . كما تم إجراء الفحوصات التأكيدية للتشخيص باستخدام أنظمة التشخيص للاختبارات الكيموحيوية المتعددة API 20E و API 20 NEالخاصة بالبكتريا المعوية وغير المعوية . تم تشخيص بكتريا *A. baumannii* ولأول مرة على المستوى الجزيئي باستخدام تقنية تفاعل البلمرة المتسلسل PCR للتحري عن وجود ألجين الوراثي المشفر للجزء الرايبوسومي ١٦S من الحامض النووي rRNA الخاص بهذه البكتريا. زرعت العزلات الحاملة للجين على الوسط التشخيصي CHROMagar *Acinetobacter*/MDR كطريقة تأكيدية وتشخيصية للعزلات المتعددة المقاومة للمضادات الحيوية بالمقارنة مع طريقة الاقراص. تم عزل ٣٣٥ عزلة بكتيرية سالبة لصبغة غرام حسب نتائج الفحوصات المظهرية والكيموحيوية . كان من بينها ٢٣ (٦.٨%) عزلة مشتبه بها تابعة لبكتريا *A. baumannii* . أبدت نتائج التشخيص باستخدام نظام API 20E إن ١٠ (٤٣.٤ %) كانت تابعة لنفس البكتريا وبمستوى تشخيصي عالي ، في حين أظهرت ١٢(٥٢ %) عزلة نتائج موجبة باستخدام نظام API 20NE. كما كشفت تجربة PCR للتحري عن ألجين الوراثي 16S rRNA إن العزلات الموجبة لفحص API 20NE كانت حاملة لهذا الموروث. ستة عزلات (٥٠%) منها كانت مقاومة لاكثر من ثلاث اصناف من المضادات الحياتية ،كما و أعطت نموا واضح على الوسط التشخيصي CHROMagar *Acinetobacter*/MDR . خلصت الدراسة إلى وجود بكتريا *A. baumannii* كمسبب مرضي بين المرضى في مستشفيات النجف، و اعتبر كل من تقنيتي PCR و CHROMagar طرق مثلى لتشخيص بكتريا *A. baumannii* المتعددة المقاومة للمضادات الحيوية.

Introduction

Acinetobacter baumannii is an important opportunistic pathogen has potential spread among hospitalized patients and persist in the hospital environment (Bergogne-Berezin & Towner, 1996). Currently, *A. baumannii* is becoming an important emerging nosocomial pathogen worldwide and is responsible for 2-10 % of all the Gram-negative infections (Richet, and Fournier, 2006). These microorganisms are known principally for their role as causative agents of nosocomial pneumonia, bacteraemia, urinary tract infection, wound infections, and secondary meningitis (Bergogne- Berezin & Towner, 1996). Although some cases of community acquired infection have been described, the importance of *Acinetobacter* spp. lies mainly in their role in the ability to colonize almost any surface and to acquire antibiotics resistance (Fernandez-Cuenca *et al.,* 2004). *In vitro* studies have also demonstrated that it can cause lethal infections in immunosupressed animals, with a death rate between 75% and 100% (Rodríguez-Hernández *et al.,* 2000). The genus *Acinetobacter* is now defined as Gram-negative nonfermenting coccobacilli, strictly aerobic, no motile, catalase positive, and oxidase negative. *Acinetobacter* spp. generally form smooth and sometimes mucoid colonies on solid media, with a colour ranging from white to pale yellow or greyish-white (Dijkshoorn *et al.,* 2007).

Presently, at least 24 genomic species (DNA group) have been described within the genus *Acinetobacter*. Strains belonging to some of the genomic species are very similar that identification by phenotypic characterisation has always been difficult (Barbe *et al.,* 2004). It has been reported that most of the isolates of clinical origins are closely related to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (*Acb*) complex (Misbah *et al.,* 2005).

As *Acinetobacter* spp. are widespread in nature, typing methods are needed to differentiate strains in epidemiological studies (Towner, 2006). An accurate identification of *Acinetobacter* spp. at the species level is important for the selection of the appropriate therapy because differences in antimicrobial efficacy against strains from different species has been demonstrated (Lin *et al.,* 2008). Differentiation of the genus *Acinetobacter* from other related bacteria is accomplished by a combination of nutritional tests, including most commercially available diagnostic devices and systems. On the other hand, phenotypic identification to the genomic species level is more difficult because commercial identification systems do not separate between the different genospecies.

Biotyping methods are based on biochemical tests and can be used for comparative typing of strains (Dijkshoorn *et al.,* 2007). Among them, the API 20NE is currently used in hospital laboratories and although it is reliable, it usually requires complementation with other biochemical analysis such as growth at 44 ºC to identify *A. baumannii* (Bernards *et al.,* 1996). Nowadays, molecularly identification of *Acinetobacter* spp. using the 16S rDNA-based method and pulsed-field gel electrophoresis random are the most widely accepted methods as large public-domain sequence databases are available for comparison (Misbah *et al.,* 2005). Hence, the proposed aim of this study is to isolate and identify *A. baumannii* isolates from hospital settings in the Najaf city.

Materials and Methods

Isolation and Traditional Identification of Isolates

A total of 520 clinical samples included (lower respiratory secretions (*n*= 220), , urine (*n*= 150), wounds secretions (*n*= 110) and blood (*n*= 40)) were collected from patients in three separate hospitals (Al-Sader Medical City, Al-Hakeem General

Hospital, Al-Furat Teaching Hospital) in Najaf over five months period starting from February to June, 2011. Isolates were recovered from clinical samples after culturing on MacConkey's agar (Himedia, India) and incubated for overnight at 37°C, non lactose fermenting bacteria (colorless or slightly beige) were subcultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative coccobacillary or diplobacillus and negative to oxidase which further identified by the traditional biochemical test according to Holt *et al*. (1994) and MacFaddin (2000).

API 20 Multi Test System

The identified *Acinetobacter* spp. isolates were confirmed by API 20E and API20 NE multi-test systems (BioMerieux ,France). These tests were used according to manufacturer's protocol for *Enterobacteriaceae* and non enteric bacteria. Wells of biochemical test were inoculated with overnight 0.5 McFarland bacterial suspension and incubated at 37°C for 24 hrs. The results were read after addition of reagents, as 7 digit number that identify by API 20 analytical index. .

PCR Amplification

DNA was extracted from the isolates by salting out method according to Pospiech and Neumann. (1995). DNA extract was used as template for the polymerase chain reaction (PCR) amplification. Amplification reaction mixtures (25 µl) containing 12.5 µl of GoTaq® Green master mix 2X (Promega, USA), 2.5 µl (10 µM) for each forward (AGAGTTTGATCCTGGCTCAG) and reverse (TACCAGGGTATCTAATCCTGTT) (Misbah *et al.,* 2005) gene primers (Kapa, USA), 5 µl of DNA template (100 µg) and 2.5 µl of PCR grad water (Promega) Amplification of the 16S ribosomal RNA was performed in a DNA thermal cycler, GeneAmp 9700, Thermal Cycler (Applied Biosystem, Singapore) with the following cycling programme: Initial denaturation at 95ºC for 3 min, and 30 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 5 min. The resulting PCR amplicons were examined by electrophoresis and visualization by gel documentation system (BioDocAnalyze Live; Biometra biomedizinische Analytic GmbH, Germany) on 2%agarose gels containing ethidium bromide.

CHROMagar *Acinetobacter*/MDR Technique

The supplements were prepared according to the manufacturer recommendations by dissolving 100 mg/ml of supplement, separately, in sterile D.W., vortexed, homogenized and added in proportions of 10 ml/l of final melted orientation CHROMagar *Acinetobacter*/MDR (CHROMagar, France) after cooled at 45°C, then poured in plates and used freshly.

Antimicrobial susceptibility testing

Isolates were plated on Mueller–Hinton agar and their susceptibilities to different antibiotics were tested by disk diffusion method according to the Clinical and Laboratory Standard Institutes guidelines (2010).

Results

A total of 335 non-repeat Gram negative bacilli isolates were collected from the 520 clinical samples. Based on the morphological characterization and biochemical behavior, 23 (6.8%) isolates were identified as suspected *A. baumannii*, which detected as non-lactose fermenter and oxidase negative coccobacilli (appeared as pale

or beige colonies on MacConkey agar). They were isolated from patients with lower respiratory tract (5.1%), urinary tract (9.5%) and wound (6.2%) infections (Table 1).

Table (1): Gram negative bacteria isolated from clinical samples

Clinical sample	No. of samples	No. of Gram negative bacilli isolates	No. (%) of suspected *A. baumannii*
Lower respiratory secretion	220	135	7(5.18%)
Urine	150	115	11 (9.5%)
Wound secretion	110	80	5 (6.2%)
Blood	40	5	0(0%)
Total	520	335	23 (6.8%)

However, the frequency of the *A. baumannii* isolates and their sites of isolation are listed in Table (2). It was found that 10 (43.4%) isolates were specified as *A. baumannii* at very good identification level by API 20E system. While, 12 (52.1%) of *A. baumannii* isolates were verified by using API 20NE system at excellent identification level (Figure 1).

In the same manner, out of 23 *Acinetobacter* spp. suspected isolates, the results of PCR using *A. baumannii* 16S rRNA gene confirmed that 12 (52.1%) of isolates were harbored positive gene bands (Figure 2). Adversely, table (3) shows a clear decrement in the percentage of isolation 6/12 (50%), when using CHROMagar *Acinetobacter*/MDR as a specific medium (Figure 3). Consequently, this medium was good indicator to multi-drug resistant isolates (resistant to more than 3 antibiotic classes).

Table (2): Number and source of *A. baumannii* isolates identified by multi-test systems

Clinical sample	No. (%) of suspected *Acinetobacter* spp.	No. (%) of *A. baumannii* isolates detected by multi-test systems	
		API 20E*	**API 20NE***
Lower respiratory secretion	7	3(42.8%)	4(57.1%)
Urine	11	6(54.5%)	6(54.5%)
Wound secretion	5	1(20%)	2(40%)
Total	23	10(43.4%)	12 (52.1%)*

***E:enterobacteriaceae, NE: non enteric bacteria; L.S.D. (0.05) = 4.031**

(A)

(B)

Figure (1): Multi-tests systems for identifying of *A. baumannii* isolates. (A) very good identification level of *A. baumannii* by API 20E system with 7 digit number (0004042) according the analytical profile index. (B): Identification of *A. baumannii* by API 20NE.

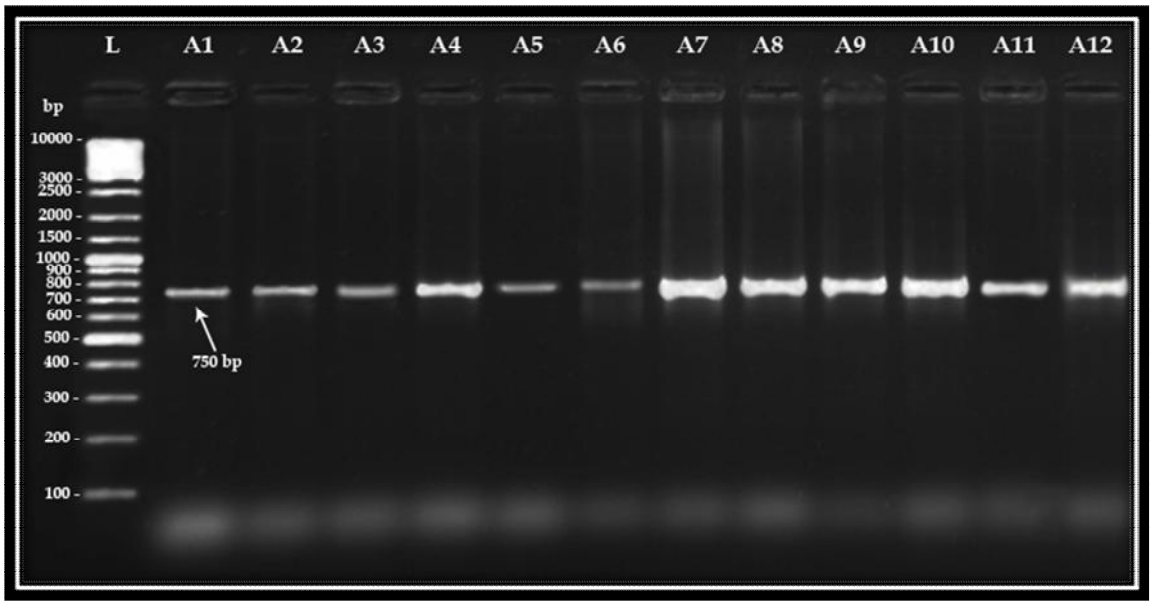

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *A. baumannii* isolates and amplified with primers of 16S rRNA gene. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l0000 bp ladder), Lane (A1-A12) show positive result with positive bands of 750 bp.

Table (3) : Antibiotic susceptibility profiles of multi-drug resistant *A. baumannii* isolates

Isolate code number	Growth on CHROMagar *Acinetobacter*/ MDR medium	Antibiotic resistant pattern by disk diffusion method
A1	-	CTX, CAZ, ATM, FEP, TE, PRL, TIC
A2	-	CTX, FOX, FEP, TEP, CIP, PRL
A3	+	Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV , TE, PRL, TIC, PY
A4	+	Ac, CTX, CI, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, CIP, GT, LEV , PRL, TIC, PY
A5	-	CTX, CAZ, ATM, FEP, PRL, TIC, PY
A6	+	CTX, CAZ, ATM, FOX, FEP, TOB, AK, CN, TEP, TE, PRL, TIC, PY
A7	-	CTX, CAZ, ATM, FEP, TIC, PY
A8	-	CTX, CAZ, ATM, TIC, PY
A9	+	AC, CTX, CI, CAZ, ATM, FOX, IPM, MEM, FEP, TOB, AK, CN, TEP, CIP, GT, TE, PRL, TIC, PY
A10	+	AC, CTX, CI, CAZ, ATM, FOX, IPM, MEM, FEP, TOB, AK, CN, TEP, CIP, GT, LEV ,TE, PRL, TIC, PY
A11	-	CTX, CAZ, ATM, PRL, TIC, PY
A12	+	CTX, CAZ, ATM, FOX, MEM, FEP, TOB, AK, CN, TEP, CIP, TE, PRL, TIC, PY

PY, Carbenicillin; PRL, Piperacillin; TIC, Ticarcillin; AC, Amoxi-clav; CFX, Cefexime; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CI, Ceftriaxone; FEP, Cefepime; IMP, Imipenem; MEM, Meropenem; ATM, Aztreonam; AK, Amikacin; CN, Gantamycin; TOB, Tobramycin;;CIP, Ciprofloxacin; LEV, Levofloxacin; GT, Gatifloxacin; TE, Tetracyclin; TEP, Trimethoprime

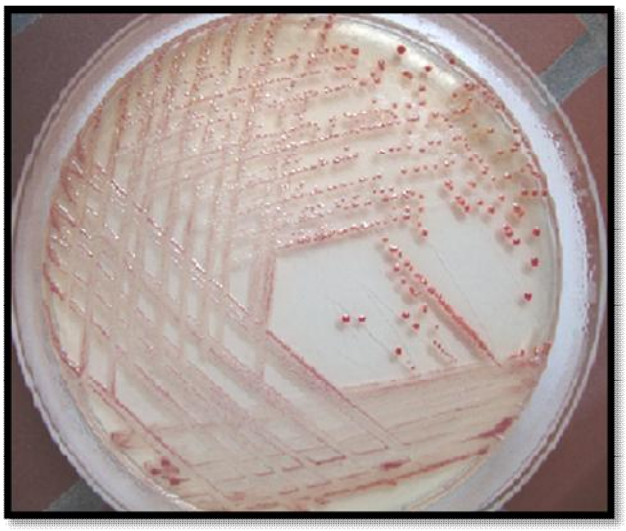

Figure (3): Identification of *A. baumannii* isolates using CHROMagar *Acinetobacter*/MDR medium. Colonies appeared as metallic reddish after incubation at 37°C for 24hr.

Discussion

In this investigation, the bacterial isolates obtained as a pure and predominant growth from clinical samples were only considered for the present study. All these bacteria were identified based on colonial morphology, and comparison of the biochemical characteristics with standard description in Bergeys manual of

determinative bacteriology (Holt *et al*., 1994) and MacFaddin (2000). However, culture morphology and biochemical characteristics of the isolates that they behave as a typical *A. baumannii*, when they screened with additional biochemical test .

One purpose of this study was to evaluate the dissemination of *A. baumannii* and to ascertain the detection rate of this microorganism from the Gram-negative pathogens in patients with significant infections. Moreover, Table (1) revealed that among 335 Gram-negative bacteria only 23 (6.8%) isolates were identified as suspected *A. baumannii*. So, in a large study of 584 *Acinetobacter* isolates realized by Seifert *et al.* (Seifert *et al.,* 1993), over 70% of the strains were classified as *A. baumannii*. However, Fontana *et al.* (2008) found that the *A. baumannii* was the most frequent pathogen (7%) among Gram negative bacteria isolated from patients of intensive care units in France hospitals. Although, it has always been considered a microorganism of low virulence, recently, suggest that sometimes it can be highly pathogenic and cause invasive diseases (Joly-Guillou, 2005). However, The members of this genus are normal inhabitants of human skin, throat, respiratory and intestinal tract of hospitalized patients; other reservoirs include the medical equipment within the hospital environment as well as the patients and staff (Towner, 2006).

This study indicated that most common isolates were isolated from patients with urinary tract infection (9.5%), then wound (6.2%) and lower respiratory tract (5.1%) infections (Table 1). The dissemination of *A. baumannii* in clinical and samples may be due to its ability to cause different nosocomial infections and resistance to a wide range of antibiotics. Also, some authors claim that the source of infection perhaps from endogenous routes rather than the exogenous routes via sink taps and hands of hospital personnel (Thomson *et al.,* 2004). In addition to that *A. baumannii* survives desiccation better than other *Acinetobacter* spp. with its ability to form biofilm that involved in cell attachment on epithelial cell and smooth surfaces of medication instruments like urinary catheters and lung tubes.(Musa *et al.,* 1990 ; Peleg *et al.,* 2008) The present study revealed that no significant differences ($P < 0.05$) between *A. baumannii* isolates were specified by API 20E system10 (43.4%) and *A. baumannii* isolates were verified by using API 20NE system 12 (52.1%) (Table 2). This may be resulted from the identification of *Acinetobacter* spp. to the species level by the conventional identification tests remains difficult in the clinical microbiology laboratory (Monnet and Freney, 1994). Therefore, in this study the multi test API 20E system was used to confirm species identification and to avoid the variability in findings of manual biochemical tests. However, These phenotypic characteristics included in commercial identification systems with 20 biochemical tests (API 20E and NE) are not considered a sufficiently reliable identification method (Bergogne-Berezin, 2001). Worth mentioning, in this study the variability in identification of *A. baumannii* may not because the insufficiently of the tests systems, but due to some properties of *A. baumannii*. Obviously, it has typical Gram-negative cell wall is difficult to distain and occasionally can be confused with Gram-positive cocci, together with variations in cell size and arrangement (Allen and Hartman, 2000) .

On the other hand, phenotypic identification to the *Acinetobacter* genomic species level is more difficult because commercial identification systems do not separate between the different genospecies (Bergogne-Berezin and Towner, 1996). Recently, more precise and accurate identification requires DNA-based methods, which are increasingly used. Moreover, this study used the PCR technique as an accurate tool for identification depending on16S rRNA gene as housekeeping gene for *A. baumannii*. However, the results of PCR had been confirmed that the 12 (52.1%) of (API 20 NE positive) isolates were harbored positive gene bands (Figure 3). PCR

results was in accordance the identification by API 20 NE system (Table 2). Perhaps, the biotyping methods are based on biochemical tests can be used for comparative typing of strains (Peleg *et al*., 2008). Among them, the API 20NE is currently used in hospital laboratories and although it is reliable, it usually requires complementation with other biochemical analysis such as growth at 44 ºC to identify *A. baumannii* (Bernards *et al.,* 1996)

An accurate identification of *Acinetobacter* spp. at the species level is important for the selection of the appropriate therapy because differences in antimicrobial efficacy against clinically important strains from different species. Therefore, CHROMagar *Acinetobacter*/MDR medium (Figure 2) was also evaluated for detection of *A. baumannii* isolates. Only 6 (50%) of isolates able to gave overnight heavy growth onto this medium (Table 3). The decrement in percentage of identification by this test may because the high selectivity of this medium to multi-drug resistant isolates, with inhibition of antibiotic susceptible *A. baumannii* isolates. However, because the originality of this method, there is no available studies previously used this technique. Table (3) also revealed that 50% of isolates to resist the major antibiotic classes.

Multi-drug resistance (MDR) clinically, is the ability of disease causing microorganism to withstand a wide variety of antimicrobial compounds (Mooij, 2009). Hence, a strain is considered a MDR if an isolate is resistant to representatives of three or more classes of antibiotics. However, transference of resistance determinants by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons between and across different bacterial species are important factors that can contribute to the increase in multi-resistant strains (Livermore, 2007). Based on this observation, the ability of *A. baumannii* isolates for fully resistant to antibiotics could be caused by either; predominate exposure of present isolates to suboptimal levels of antibiotic, prolong use of broad-spectrum antibiotics, exposure to isolates carrying resistant genes, lack of hygiene in clinical environments and usage of antibiotics in foods and agriculture. but the occurrence of alarming MDR *A. baumannii* isolates may be threat to arise of extensive and pan-drug resistant (XDR and PDR) isolates in the future.

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