Co-infection intestinal fungi and protozoa inimmunocompromised patients with detection of some virulence factors of these organisms by using Polymerase Chain Reaction (PCR).

Baheja Abbes AL-Khalidy*. Ma'ani Naji AL-Shimari**Abbas Ghafil Abbas
*Lecturer College of nursing /Al-Qadissya university,** Lecturer College of medicine/Al-Qadissya university
Email: BAH.1978@yahoo.com
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الخلاصة:

في هذه الدراسة تم الكشف عن بعض عوامل الضراوة للإصابة المشتركة بين الطفيليات والفطريات المعوية في مرضى المناعة المكتسبة باستخدام تقنية تفاعل سلسلة انزيم البلمرة (PCR). تم جمع 100 عينه من البراز من ثلاثة مجاميع تشمل المرضى المصابين بالأورام الخبيثة (35)،والمرضى المصابين بالفشل الكلوي(50) والمرضى المصابين بالفيروس المثبط للمناعة (HIV) (15) خلال الفترة من (5 _تشرين ألثاني 2012 الى 15_مايس 2013) لغرض التحري عن الإصابة المشتركة بالطفيليات والفطريات المعوية.

اوضحت نتائج الدراسة ان اعلى معدل اصابه (66.66%) كانت في مجاميع المرضى المصابين بالفيروس المثبط للمناعة بينما اقل معدل اصابه (57.14%) كانت في مجاميع المرضى المصابين بالأمراض الخبيثة. كما سجلت اعلى اصابه في الفئة العمرية بين (60_75) سنه ، وكان معدل الإصابة في الذكور (90.90%) بينما في الاناث (53.44%) ، لكن احصائيا لا يوجد فرق مهم بين المجموعتين. سجلت *Candida albicans* اعلى معدل انتشار وصل الى (41%) يليها الفطر *Aspergillus fumigatus* (34%) و *Giardia lambelia*(53%) بينما في *Entameba histolytica* (46%). وبينت نتائج الاختبارات الجزيئية ان *Candida albicans* تحتوي جين SAP في تعاقب زوج قاعدي (350) و *Aspergillus fumigatus* تحتوي جين Gliotoxin في تعاقب زوج قاعدي (550)وجين Protease في تعاقب زوج قاعدي(650) كذلك *Giardia lambelia* تحتوي جين Cystein Rich Protein CRP65 في تعاقب زوج قاعدي (460) و *Entameba histolytica* تحتوي جين Le ctin في تعاقب زوج قاعدي(900). بينت نتائج فحص الحساسية للمضادات الفطرية إن هنالك مقاومة عالية للمضادات الفطرية التي استخدمت في هذه الدراسة ما عدا مضاد Amphotericn B الذي استجابت له الفطريات بنسبة (80%).

Abstract:

In this study was undertaken to detect some virulence factors to co-infection of intestinal fungi (*Candida albicans, Aspergillus fumigates)* and parasite (*Giardia lambelia, Entameba histolytica)* in immunocompromised patients by polymerase chain reaction(PCR)technique.

About, 100 specimens of stool were collected from three groups of patients included malignant tumor (35), , renal failure (50) and HIV (15) during period 5 November 2012 to 15 May2013.

The results of this study showed the highest rate infection(66.66%) was in HIV while lowest rate (57.14%) were in malignant tumor patients .So the highest rate were recorded in age group (60-75),then male recorded rate infection(69.04%) while in female (53.44) but statically no significant difference between two groups.*Candida albicans* recorded highest rate prevalence recorded to (41%) followed by *Aspergillus fumigates* (34%) and *Giardia lambelia* (53%) while *Entameba histolytica* (46%).

According to the results of molecular test showed *Candida albicans* contain asp gene in (350 bp) sequence and *Aspergillus fumigatus* contain Gliotoxin gene in (550bp) sequence. So *Giardia lambelia* contain Cystine Rich Protein CRP65gene in (460 bp) sequence and *Entameba histolytica* contain Lectin(*hg*13) gene (900 bp) The results of this study showed the yeast and fungi appeared high resistant to antifungal which used in this study except Amphotericn B which were sensitive to it by rate (80%).

Introduction

Parasitic and fungal infection especially the opportunistic ones, are one of the major problems in immunocompromised patients .The frequency of sever systemic parasites and fungal diseases has increased in the last few decades. The clinical use of broad spectrum anti bacterial drugs and immunosuppressive agents after organ transplantation, cancer chemotherapy and advancement in surgery are associated with increasing risk of fungal and parasite infection, (1). Despite the effectiveness of available anti fungal in combating such infection, the emergence of drug resistance to anti fungal and problems of toxicity and poor delivery of drug site in systemic infection, have necessitated a systematic approach to the study of fungal pathogens, host fungi interactions and identification of virulence factors (2).

Because the prevalence of intestinal fungal and parasitic infections is relatively high in immunocompromised patients it is recommended to evaluate these patients for these infections prior to administration of immunosuppressive drugs. It is also suggested to carryout similar studies with a larger sample size and different groups of immunocompromised patents to conform the result of this study. Infection caused by opportunistic agents including Candida Spp. are frequent in diverse pathological states that induce immunodeficiency such as neutropenia, neoplasia,decompensated diabetus mellitus, malnutrition,organ transplantation,and AID.

Aims of study

1. This study is designed this study to determine the prevalence of intestinal fungal and parasitic infections in immunocompromised patients.
2. Detection of some virulence factors in intestinal fungi and parasite.

Martials and Methods:

This study was carried out for 6 months ((from the first of November 2012 till the end of May 2013)) the samples collected from ((Al-Diwinyia teaching hospital, maternity and children hospital and private clinical)) in Al-Diwinyia provine. One hundred patients were included in this study, all those patients sever from diarrhea undergo full history aquestioner and full information were obtained from the patient like age, gender, type of Disease.

Stool samples were taken from each patient, fresh unpreserved stool samples were collected in sterile cappe containers, the specimen container were labeled with name, age, sex of patients examination the stool specimens was divided in to two parts:

A-part 1 of stool was used to detection of protozoa as follow:

1- Direct examination

A- Wet Mount:

Temporary mounts were made in order to diagnose the parasite, with an applicator stick picked up small l drop of fecal sample (size of match head) and mixed with a drop of saline, similarly, small amount of stool was picked up and mixed with a drop of iodine then these mixtures were covered with covers lips (3) The 40x and 100x objectives were used for searching and identification respectively of cysts and the trophozoites of protozoa (4). These two types of wet

mount were used for each of fecal sample (saline, iodine) and some samples stained with methylene blue. The wet mount was used for the initial microscopic examination of stool and to demonstrated amoebic trophoziotes , cysts and can also reveal the presence of RBC and pus cell (3).

B- Concentration Method

This method was done according to (3).

1. 1.10mlof 2% formalin was added to approximately1g of stool and stirred using applicator sticks until get a cloudy suspension.
2. A gause filter was fitted in to a funnel and the funnel was placed on top of the centrifuge tube.
3. The fecal suspension was passed through thefilterintocentrifugetubeuntil7ml mark is reached.
4. The filter was removed and discards with lumpy residue.
5. A volume of 3 ml ethyl acetate was added to stool and mixed well for one minute.
6. Transfer to the centrifugation tube and centrifuge for 1 minute at 1000 xg.
7. The fatty plug was loosed with an applicator stick, and supernatant was poured a way by quickly in verting the tube.
8. The tube was replaced in its rack and the fluid was allowed on the sides of the tube to drain down to the sediment, mixed well and transfer a drop to a slide for examination, also it was made an iodine stain preparation (5).

B –part 2 was used to detection the yeast and fungi as follow:

Direct examination by take 1 drop from suspension of stool and put on slide (and add to it 1 drop from lacto phenol blue to watch yeast and mycelium or conidia of fungus in stool. Culturing 1 drop of suspension by using loop on sabrouad dextrose agar then diagnosis of yeast or fungi which grow on media according to morphological characters such as color, texture, edges of colony. after that by using strait loop take amount from fungal growth and put on slide and add to it 1 drop from lacto phenol blue to exam under microscope to detect nature of hyphae (septa or asepta) and arrangement of conidia.

Molecular technique (PCR)

A-Extracting DNA from Stool :(6).

1. 1.5 ml micro centrifuge tube was prepared and 20 µl proteinase K was added, after that about 100-200 mg of the stool sample to the tube was added.
2. 400 µl of SL buffer to the tube was added and mixed by light vortexing for about 30 seconds.
3. The tube was incubated for 10 min at 60 °C.
4. After 10 mins, the mixture was centrifuged at 12,000 rpm for 5 minutes.
5. The supernatant was transferred to a new tube and 400 µl binding buffer was added. The tube was incubated again for 10 min at 60° C.
6. 100 µl isopropanol was added, lightly vortex for about 5 seconds, then the tube was spinet down for 10 seconds to down the liquid clinging to the walls and the tube was led.
7. The binding column was fitted into the 2 µl collection tube. The liquid was transferred into the binding column. Carefully the lid was closed and centrifuged for 1 min at 8,000 rpm.
8. Following centrifugation, the binding column was transferred to a new 2 µl collection tube and washing with 500 µl washing buffer 1 (W1) was added to the column , taking care so that the sides do not get wet ; the lid was closed, and centrifuged for 1 min at 8,000 rpm
9. After centrifugation, the binding column was transferred to a new 2 µl collection tube.500 µl washing buffer 2 (W2) was added , taking care so that sides do not get wet ; the lid was closed , and centrifuged for 1 min at 8,000 rpm.
10. The tube was left to spin down once more at 13,000 rpm for 1 min to completely remove ethanol. Residual

washing buffer 2 left in the binding column can hinder the following steps.
A-The binding column was transferred to a 1.5 µl collection tube, and 200 µl of Elution Buffer was added and let stand for 1 min to allow the buffer to permeate the column.
B-The column eluted by spinning down at 8,000 rpm for 1 min the eluted DNA solution can directly be used, or stored at 4 C° or -20 C° longer storage periods

C- Agarose Gel Electrophoresis The protocol for electrophoresis consists of three steps: (6).

Making the Gel

Two concentrations of Agarose gel were prepared (1%and2%) as needed. The concentration of 1% agarose was used in electrophoresis after DNA purification process, while 2% agarose was used after PCR detection. The agarose gel was prepared by dissolving 0.5gm if the concentration (1%) and 1gm if the concentration (2%) of agarose was dissolved in50ml of TBE1x buffer; the solution was elated on hot plate and stirrer until all agarose particles were completely dissolved, then allowed to cool down at 50 $^{\circ}$C .It was used for electrophoresis, and ethidium bromide stain solution then added to agarose gel (0.5µl).

2-Preparation Casting of the Agarose Gel:

1. The gel tray putted in the electrophoresis chamber in opposite direction.
2. The comb was fixed at one end of the tray form asking well used for loading DNA
3 .The agarose gels was poured gently into the tray, and allowed to solid if at room temperature for30minutes.
4. The gel was assembled to casting tray and the comb was carefully removed and the gel was replaced in an electrophoresis chamber.
5. The chamber was filled withTBE1X electrophoresis buffer until the buffer reached3-5mmoverthe surface of the gel.

3-Loading and Running DNA in Gel Agarose:

1. DNA (8µl) was mixed with (2µl) bromophenol blue (loading buffer) and loaded in the well soft the 1%agarose gel.
2. The cathode was connected to the well side of the unit and the ode to the other side.
3. The gel was run at100Volt until the bromophenol blue tracking dye migrated to the end of the gel in about 45 mints.
4. The DNA was observed by staining the gel with ethidium bromide.UV Trans illuminator was used for the observation of DNA bands, and the gel was photographed using digital camera.

Primers:

Table (1) the specific primers and their sequences:-

Primer		DNA Sequence (5-3)	Product size bp
Cystein rich protein CRP65	F	GGCACTACGAATGACCTA	460
	R	TTGTAGCACCCGCCCATG	

Table (2) the specific primers and their sequences:-

Primer	DNA Sequence(5-3)		Product size bp
SAP2	F	AACAACAACCCACTAGACATCACC	350
	R	TGACCATTAGTAACTGGGAATGCTTTAGGA	

Results and Discussion

The result of this study showed that the highest infection rate (35%) was

recorded in renal failure patients followed by malignant tumor (30%) and lowest infection rate (10%) was recorded in HIV patients as in table (1). The patients were classified according to age were varied between (8-75) year, the results listed in table (2) showed the highest rate (90.90%) infection were in age group (60-75) years followed by (68.75%) in age group (36-59) year and (65.21%) in age group(8-15) years. So the results summarized in table (3) revealed to that the rate infection in male (57%) we higher than in female which were (53%). But statically ,the results in table(2) and 3 showed that no significant difference in infection between age groups so male and female($p<0.05$) this could be explained on the basis of that all patients in this study suffers from loose of defenses systems that help to protect them any infection. So the results in table(4) showed that 9(31.03%) from male patients infected with parasite only 6(20.68%) infected with fungi and 14(48.27%) infected with parasite and fungi while in female there were 8(25.80%). Infected with parasite and 7 (22.58%) infected with fungi and 16(51.61 %) infected parasite and fungi and no significant difference between two group. The results of microscopic examination showed *Giardia lamblia*, *Entamoeba histolytic*a in its cyst form, so *candida albicans* yeast and some fungi such as *Aspergillus spp.*

Table 1: Distribution of infection according to type of disease:

Type of disease	Number of patients	Number of infected	Percentage
Malignant tumor	35	20	57.14%
Renal failure	50	30	60%
Human immune virus	15	10	66.66%
Total	100	60	100

Table2:Distribution of parasite and fungal infection according to the age:

Age groups	Number of patient	Number of infected with fungi and parasite	Percentage %	Non-infected	Percentage %
8-15	23	15	65.21%	5	45.45%
16-22	24	12	50%	8	72.72%
23-35	26	12	46.15%	12	50%
36-59	16	11	68.75%	14	53.84%
60-75	11	10	90.90%	8	34.82%
Total	100				

x^2 cal =4.66 df=8 p.v = 0.1333

table 3 : distribution of parasite and fungal infection according to the gender:

Sex	Number of patients	Infected	Percentage %	Non-infected	Percentage %
Male	42	29	69.04	13	30.95
Female	58	31	53.44	27	46.55
Total	100	60		40	

p .v = 0.2983 **x^2 cal = 2.47** **df =2**

Table4: distribution of parasite and fungal infection according to the type of microorganism:

Sex	Number of infected	Infected with parasite	Percentage %	fungi Infected with	Percentage %	Infected with parasite and fungi	Percentage %
Male	29	9	31.03	6	20.68	14	48.27
Female	31	8	25.80	7	22.58	16	51.61
Total	60	17	28.33	13	21.66	30	50

x^2 cal = 0.852 **df = 3** **x^2 tab = 1.42** **p.v = 0.836**

Table 5 : distribution of fungal infection:

Fungi	Number	Percentage %
Candida albican	21	41%
Aspergillus fumigates	17	34%
Aspergillus flavus	5	11%
Total		

Table 6: distribution of parasite infection:

Parasite	Number	Percentage %
Entambe .histolytica	23	46%
Giardia lambela	24	54%
Total	47	

The result in table(5) revealed to that the highest prevalence (41%) of *Candida albicans* below by *Aspergillus fumigatus* (34%) and (11%) for each *Aspergillus flavus*.

(10). The yeast *Candida albicans* is a ubiquitous communal and an important opportunistic human pathogen causing superficial infection as well as invasive fungal disease in immunocompromised patients

(9). Systemic candidiasis is associated with long hospital stays and mortality rate of 18 to 70 %, and it is an itragenic infection encountered among certain groups of hospital patients who carry more yeasts in mouth and gastro-intestinal tract than the normal population, the highest number of yeast occurs in patient, after surgical treated with antibiotics or steroids in immunocompromised patient, after surgical procedures such as organ transplant or heart surgery (8)

In this study *A.fumigatus* had high prevalence rate (34%).This fungus had many virulence factors which completes the infection in patients, *A.famigatus* activated the complement system in normal human serum, with result at deposition of C3b and C3b on the surface of the organism and generation of potent chemotactic factor (probably C5a) .Hyphae activate complement via both alternative and classical pathway, perhaps a reflection of

the air poor ability to stimulate antibody production. In vitro, *A. fumigatus* produce inhibitors of the alternative pathway of complement.

The increased of risks of infection with *A.fumigatus* in persons receiving high doses of corticosteroids is generally to be a result of impairment of macrophage and perhaps T-cell function recently (6) demonstrated that pharmacological doses of the corticosteroid hydrocortisone modestly enhanced the growth of *A.fumigatus* in culture of interest is whether Aspargillus species have specific steroid-binding proteins analogous to those that have been found on other fungi. *Aspargilli* are ubiquitous in the environment, and frequent exposure to the fungus via inhalation of air borne conidia (which are an ideal size for alveolar deposition) undoubtedly occurs or during eating of contaminated food with Candida of this fungi, and reach to intestine. In the suspitable host conidia germinate in to hyphae the invasive form of disease, which is usually seen in individuals who are severally immunocompromised as a result of neutropenia, therapy with high doses of corticosteroid or late-stage AIDS, or HIV. Isolated *Aspargillus fumigates* form patient suffers lack of immune defencence is very dangerous problem , where this fungus produce secondary metabolism which call (mycotoxin) such as Gliotoxin and these compounds cause many effects on all body system especially digestive system such as necrosis digestive tract and in liver (7).

According to the result of study *Giardia lambelia* infection is more prevalent (46%). This result agrees with (12). Who reported (40%), prevalence of *E. histolytica* in immunocompromised patients. So had prevalent (0.5%) in these patients. *E.histolytica* had the ability to invade the intestinal mucosa that induce ulcer and lead to bloody stool, and found this parasite in intestinal of immunocompromised patients lead to more effects.(13)So Giardia causes many effect in patients as enteric infection with Giardia spp. is responsible for decrease absorption of electrolytes glucose and fluid, at least as part because of diffuse of epithelial microvillus shortening which may be combined or not with villous atrophy(14).

Another study also explain that the distribution of functional integrity of intestinal mucosal cells lead to disturbance in the fluxes of water and electrolytes, together these abnormalities lead to diarrhea , also changes in cells proliferation rate effect the number of absorptive epithelial cells (enterocytes) villous surface area and perhaps the rate at which cells mature as they migrate from crypts base to villous crest in turn the number and maturity of cells in villa determine the extent and the activity of striated brush border .Further more microvillus –glycocalyx complex effect the potential for incorporation of membrane-associated with hydrolytic enzymes and various transporter molecules.One of the pathological changes in experimental giardiasis in inhibition of the activities of several digestive enzymes, including sucrose and maltose, during the acute phase of the infection (15).

A-Results obtained from amplified primers of virulence factors in fungi:

1- *SAP* gene in *candida albicans:*

The results of this technique revealed that the amplified DNA has (350 bp) for *SAP* in 18/21, (85.71%) *Candida albicans* isolates this study searched for *SAP* in *candida albicans* and importance of identification of this proteins in pathogenises of candidiasis, this proteins play important roles in the association of secreted proteolytic activity with virulence in*C.albicans* is a story that has developed over the past 40 years. From the earliest biochemical work which tacitly assumed that this major fungal pathogen secreted a single proteinase

enzyme to the genomic revelation of a family of 10 genes encoding secreted aspartyl proteinases (*Saps*), the presumption has been that externally secreted *C. albicans* hydrolases of broad substrate specificity ought to contribute to the pathogenesis of disease. Many excellent studies, including reverse genetics and *in vivo* expression technologies, have contributed to detailed knowledge of the roles of the various *SAP* gene products in the pathogenesis of superficial and disseminated *Candida* infections (11)

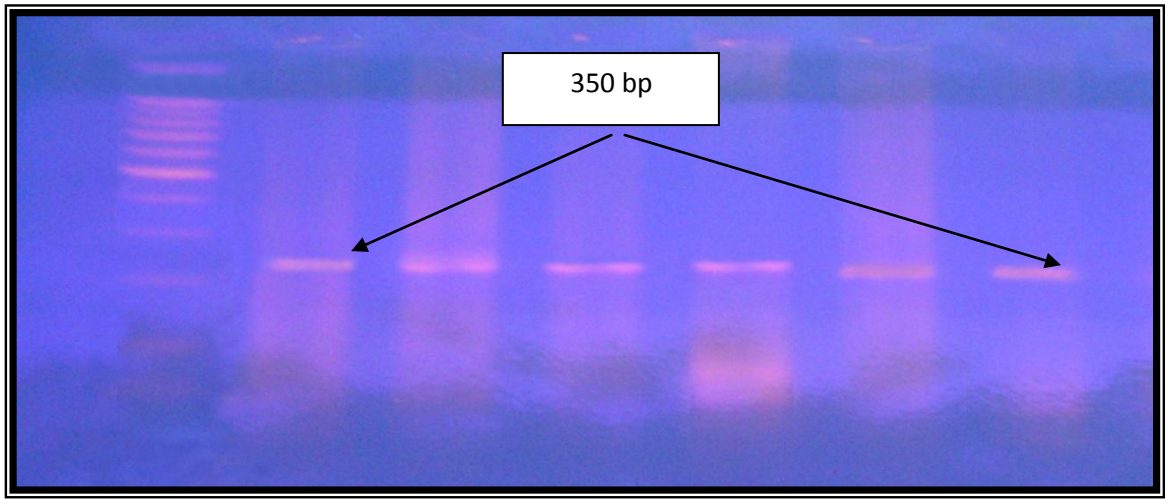


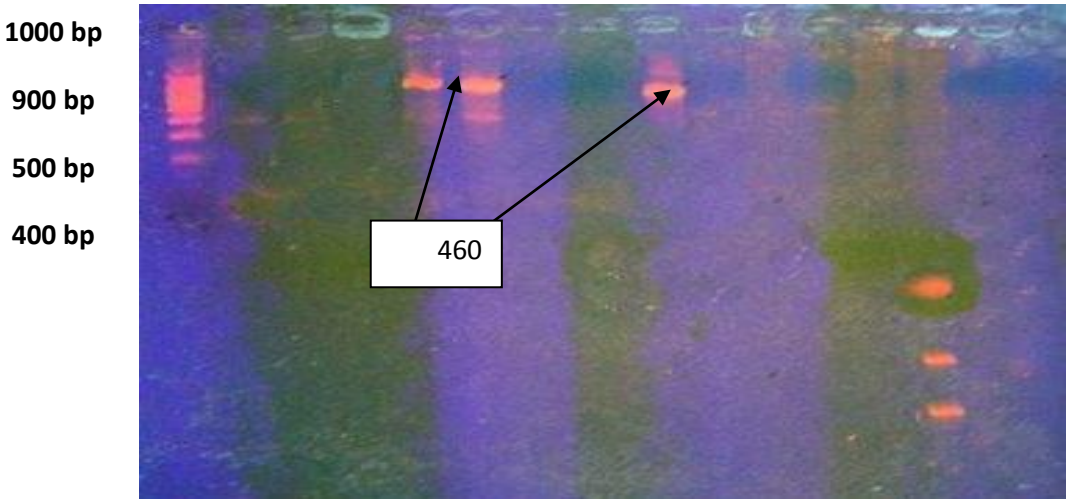
Fig (1) SAP gene in *Candida albicans*

2-Cysteine Rich Protein 65 (CRP 65):

Giardial Cystein Rich Protein is a protein with high content of the amino acid Cystein; this protein has multiple properties like heavy metal resistances, metronidazole resistance, bile salt resistance and toxin like protein activity. (16). The first three characteristics may depend on the chemical structure of Cystein which has a thiol group (SH) which is an active or unstable group ,the hydrogen can bind to hydroxyl group to form water leaving the free sulfur (S) in the medium which may bind to the heavy metals like Mg +2 ,Ca+2, Cd+2 and Zn ,the binding of sulfur to the heavy metals prevent the small intestine from take these metals and lead to metal malabsorbtion, this result agree with (19).On the other hand, the binding of free (S) with zinc may play an important role in trophozoite survival and colonization in the small intestine because zinc binding by CRP can lead to inhibition of a number of intestinal enzymes, and this result agrees with (18).The resistant to metronidazole may come from binding of giardial CRP by its free sulfur to this common anti giardial drug or Giardia appear to have defective pyruvate :ferredoxinoxidoreductase (PFO) activity transfers electrons to Giardia ferredoxin (Fd) with simultaneous reduction of metronidazole, this result agrees with (19).

The free sulfur also may binds with bile salts preventing lipid metabolism in the small intestine resulting in steatorrhea. The fourth

characteristic of CRP is toxin like protein activity. This property depends on the whole protein, by this activity Giardia may form a tube helps it to enter anther organ like bile duct in cases of heavy infection with giardiasis and when the nutrients of host small intestine become not enough for the parasite .Many evidenced recorded that Giardia has been seen in the bile duct, this result agree with (17).



Fig(2) CRP56 gene in *Giardia lamblia*

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