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Clinical and molecular study of *Babesia caballi* in racing horses in Baghdad

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Abstract

The objective of this study was to investigate *Babesia caballi* in horses at three main gatherings of racehorses located in the Baghdad Governorate through clinical examinations, microscopy, and conventional polymerase chain reaction (PCR) assays. The 18S rRNA gene of *B. caballi* was PCR amplified, sequenced, and phylogenetically examined between January and December of 2021. One hundred sixty blood samples were taken from horses of different ages, breeds, and sexes. Prevalence and risk variables for babesiosis were analyzed using chi-square tests and odds ratios. 3 ml of blood was taken from the jugular vein in test tubes containing anticoagulant to detect *Babesia caballi* in blood smears and molecular technology. The outcomes showed that the clinical manifestations of babesiosis comprised pale to icterus, mucus membranes, emaciation, anorexia, and leg swelling, a slight increase in body temperature, heart rate, and respiratory rate. Microscopic observation revealed the presence of *Babesia caballi* in 49 out of 160 (30.625%) horse blood smears. These smears exhibited various morphological stages of *B. caballi* within the red blood cells. The confirmation process of 160 blood samples by PCR to detect 18Sr RNA demonstrated that 91 samples (56.87%) yielded positive findings for the desired product size of 540 base pairs (bp). There was no significant variation in the percentage of infection with *B. caballi* between stallions and mares. Additionally, it is worth noting that no age group exhibited a significant prevalence of infection. However, it is essential to highlight that Arabian horse breeds showed a greater susceptibility to infection at a rate of 63.34%; however, this difference was not statistically significant when compared to Thoroughbred and crossbred horses. This study identified a new genotype of *Babesia caballi* based on phylogenetic analysis of our samples and comparison with data from the International Gene Bank. This genotype, called Clade C, is characterized by a high infection rate and low illness severity.

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Introduction

Babesia caballi are equine hemoprotozoan intra-erythrocyte equine tick-borne parasites sharing piroplasmosis signs with *Theileria equi* (1). They are globally endemic and infect equids (zebras, mules, donkeys, and horses) (2). The organism merozoites take a basophilic 2-5 µm pyriform shape, mostly in pairs or singles inside horse erythrocytes (3). Mode of transmission mainly via *Ixodid* ticks and the genus observed in the north of Iraq (*Hyalomma*, *Boophilus*, and *Rhipicephalus*) (4). Ticks ingest the erythrocyte of infected equine and become infested, then transmit *Babesia caballi* parasites by vector saliva and mechanical means by contaminated instruments, especially needles, surgical devices, or blood transfusions (5). The incubation period of horse babesiosis is 10-30 days. Signs of acute form include high body temperature, tachycardia, anorexia, lethargy, hemolytic anemia, icterus or pale mucus

membrane, dark urine, peripheral edema, tachypnea, and can lead to death (6). In endemic regions, equine babesiosis usually occurs as a carrier with no obvious clinical symptoms (5). Numerous studies described various diagnostic techniques for detecting Equine piroplasmosis. Badawi & Yousif (7) noted that blood smears stained with the Giemsa dye are an inexpensive and commonly used laboratory technique for diagnosing acute infection with *B. caballi*. However, this technique requires skills in cases of chronic or subclinical infection due to the rare presence of parasitemia. According to Bashiruddin *et al*. (8) and Camino *et al*. (9), it has been shown that polymerase chain reaction (PCR) techniques have greater sensitivity in the identification of horse piroplasmosis compared to microscopy, culture, and serological approaches. Equine babesiosis spread worldwide, especially in subtropical and tropical areas, though global limitation for equine movement for trade and equine sports events to avoid economic loss (10) since the risk of transmitted infection from asymptomatic cases comes from endemic regions into non-enzootic countries (11). Equine babesiosis is endemic in Iraq and documented in several provinces (12-15), despite the fact that even though no phylogenetic research reported *Babesia caballi* among racing horse breeds in Baghdad governorate.

As a result, we carried out this study to look into Babesia caballi infection clinically and microscopically in Baghdad racing horses, then molecular sequencing and recording a phylogenetic tree.

Materials and methods

Ethical approve

Approval for this study was obtained from the committee of College of Veterinary Medicine/ University of Baghdad, Iraq. Number 39/PG on 7/1/2021.

Animal groups

One hundred and sixty racehorses included (60 Arabian horses, 25 Thoroughbred horses, and 75 Crossbred horses), both sexes and ages from 2- more than 11 years old. These horses are located in Baghdad Governorate (AlAmeria Equestrian Club, Alzwraa Zoo, and Iraqi equestrian school). The study was done for an entire year and covered all seasons from January 2021 to December 2021.

Clinical examination

Each horse was examined clinically for symptoms, especially babesiosis, respiratory rate, heartbeats, and rectal temperature.

Samples collection

Two tubes of 3 ml vacutainer blood with EDTA anticoagulant were used to aspirate samples of each horse blood from the jugular vein, and one drop was withdrawn and placed onto a suitable slide for microscopic examination of blood smears; the tubes were subsequently frozen for PCR technique.

Molecular genetic assay

Extraction of DNA: (Promega, USA, ReliaPrep™ Blood gDNA Miniprep System) set with malefactor instructions were applied on horse blood for extracting genomic DNA, then DNA purity and concentration evaluated by NanoDrop Thermo Fisher Scientific company, USA instructions were determine ranged 1.6-1.9 at 260/280 nm (16).

PCR protocol

A set of primers, built from the 18S ribosomal RNA genes was used for PCR are Bec-UF2: 5' TCGAA GACGA TCAGA TACCG TCG 3', Cab-R: 5' CTCGT TCATGA TTTAG AATTG CT 3' to amplify 540 base-pairs of *B. caballi* DNA fragments (17). A thermocycling protocol starts initial denaturation with ten minutes of heating to 94°C. The second step is forty cycles comprising denaturation with one minute of heating at 94°C and one minute of annealing at 55.9°C, then extension with heating for one minute at 72°C, the third step, final extension with 10 minutes of heating at 72°C, then holding at 4°C for a couple of hours. DNA fragments that were amplified were most visualized through UV light after electrophoresed with (Promega, USA, Diamond™ Nucleic Acid Dye) on agarose gel (1.5%) (18).

Sequencing and phylogenetic analyses

Positive yields were sent to sequenced by (Macrogen lab, Korea) by employing the forward Bec-UF2 and Cab-R reverse primers of the 18S rRNA gene of *B. caballi*, and the results were analyzed via BLAST of the NCBI database (19,20); and recorded in international Gene bank as Iraq reference accession number. Statistical analysis: The results data were examined by using the program Statistical Package for the Social Sciences (SPSS) version 26.0 (IBM Corp., Chicago, USA). Significant results levels (at levels $P \leq 0.05$) indicated a potential risk of infection (21). Ethical approval: The Department of Internal and Preventive Veterinary Medicine/Committee of Veterinary College Number 39/PG approved the study's protocol on 7 January 2021.

Results

Clinical signs

Babesia caballi infection in horses varies according to the level of and duration of infection between acute, chronic, subacute phase, and carrier status. The clinical manifestations among 91 infected horses were recorded in (Table 1), such as icterus, which was the main sign, pale mucus membrane (MM), anorexia with emaciation, and leg swelling. Clinical examination revealed a mean rectal temperature of 37.76ºC ranging from 36.9-38.9ºC^{o} (no horse suffered real fever), but a remarkable increase in means of

Heart rate of 40.3 bpm (21 - 60 bpm) and found a respiratory rate of 16.72 (8 - 36 breath/minute) with only 5 animals had polypnoea of the infected horses. Tick infestation was shooed in a single one.

Table 1: Clinical Singes Related to Infection with *Babesia caballi*

Clinical signs	(No.) %
Anorexia	(8) 8.79%
Depression	(2) 2.2%
Diarrhea	(1) 1.1%
Emaciation	(7) 7.69%
Heart rate Arrhythmia	(2) 2.2%
Hemoglobinuria	(2) 2.2%
Icterus	(14) 15.38%
Legs Swelling	(7) 7.69%
Pale Mucus Membrane	(13) 14.29%
Pain Colic	(1) 1.1%
Petechial hemorrhage in 3rd eyelid	(3) 3.3%
Poor Performance	(1)1.1%

Microscopic examination

Babesia caballi was observed Microscopically in 49 cases in about 30.625% of total 160 horses sample that showed different development stages of *B. caballi*, as typical large pairs of pear shapes inside red blood cell, or as singles and sometimes oval shape and may small in size and may be seen extracellular (Figure 1).

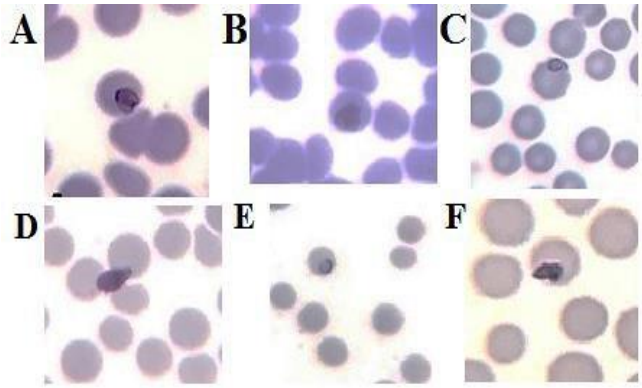

Figure 1: Different forms and sizes of *Babesia caballi* in horse blood smears stained with Giemsa stain (A, B: typical large pairs of pear shape inside RBC; C: single pear shape inside RBC; D: single pear shape outside RBC; E: single oval shape inside RBC; F: small pairs of pear shape inside RBC:(light microscope X100).

Results of PCR assay

The PCR technique used for confirming the presence of 18Sr RNA in 160 blood samples from horses found that 91 (56.87%) of the samples tested positive for the target product size 540 bp (Figure 2). There was a significant association between microscopic and PCR results, but the superiority of molecular results with a significant increase at (P≤0.05) (Chi-square(X2) =22.4; df = 1; P value = 0.00000221374).

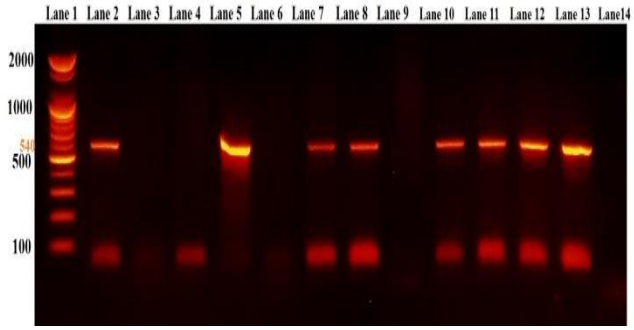

Figure 2: Lane 1= 100bp Ladder, Lane2,5,7,8,10,11,12,13 = Positive Amplicons of *Babesia caballi* 18S ribosomal RNA genes at 540bp stained (Diamond™ Nucleic Acid Dye), Lane 14= control negative sample, and Lane 3,4,6,9= negative PCR results.

Sequenced positive products of *Babesia caballi* and twelve of them were recorded in international Gene bank the National Center for Biotechnology Information (NCBI) under accession number: ON328303.1, ON328304.1, ON328305.1, ON328306.1, ON328307.1, ON328308.1, ON328309.1, ON328310.1, ON328311.1, ON328312.1, ON328313.1, and ON328314.1. The phylogenetic tree (Figure 3) shows two significant groups of *Babesia caballi* genotypes. All Iraqi *B. Caballi* isolates appeared highest. Similarly, 97.49 - 98.99% sequence identity to the first group (Turkey, middle of Iraq, India, and Egypt isolates) with 98 - 100% site coverage and compared with the second group showed 90.73 - 97.49% Similarly to (Malaysia, China, Kazakhstan, north of Iraq, Brazil, Cuba) with 65% site coverage.

Relative risk factors infection of racehorses affected with *B. caballi* according to age, sex, and breeds

Relative risk factors in horse sexes to infection with *B. caballi* did not reveal significant variation between stallions and mares despite male infection percentage higher than (1.48 odds ratio) females (Table 2). Furthermore, there was no significant overwhelming of any age group than others and probability of infection (2.26 odds ratio). Moreover, the Arabian horse breed showed higher susceptibility to infection, 63.34%, comparable to Thoroughbred and Crossbred horses.

Table 2: Predisposing racehorse age, sex and breeds to *Babesia caballi* infection

Risk factors		Total Horses (160)	Infected horses (n)	Percentage (%)	Confidence interval 95%
Sex	Stallions	69	43	62.3188%	(1.4816)
	Mares	91	48	52.747%	0.783 to 2.803
Age	2 years age	22	11	50%	(2.2652) 0.7817 to 6.5637
	3 years age	24	14	58.33%	
	4 years age	32	16	50%	
	5 years age	25	12	48%	
	6 -10 years age	34	23	67.65%	
	<11 years of age	23	14	60.87%	
Breed	Arabian Horses	60	38	63.34%	(1.5944) 0.7969 to 3.1899
	Thoroughbred	25	14	56 %	
	Crossbreds	75	39	52 %	

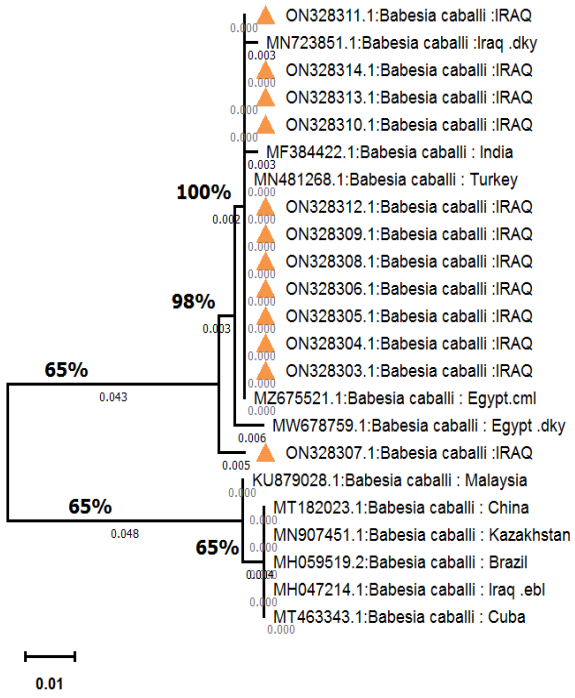

Figure 3: The scheme in a Phylogenetic tree using a part sequence of *Babesia caballi* isolates in the gene called 18S ribosomal RNA. Orang triangles referred to sequenced Iraqi samples in the present study. The rest represent isolates from GenBank.

Discussion

The occurrence of the peracute form is uncommon, and the acute form is manifested by emaciation with decreased performance, a loss of appetite, icterus or paleness of the mucus membrane, and leg swelling. However, the frequent form is asymptomatic, subclinical, or chronic carrier over a long period (22). The following signs were noticed among 91 molecular-positive horses in our investigation: icterus, paleness, anorexia, swelling legs, Petechial hemorrhage in the 3rd eyelid, depression, heart rate arrhythmia, and hemoglobinuria. Additionally, a single case of diarrhea and another displaying colic pain were noted. Interestingly, these symptoms were not evident in the majority of affected horses. lameness is initially diagnosed by history, clinical examination, but mainly due to tendon injury (23). This revelation indicates that carriers and subclinical infection were the predominant cases. The resample results were obtained in Mosul (24,25).

Hailat *et al.* (26) clarified presence of highly positive piroplasmosis results without obvious clinical signs may go back to subclinical infection and carrier state, but exercise converts carrier horses to clinical cases; on the other hand, linked signs as icterus 85.5% mucus membrane as well as legs swelling 62.5% to chronic infection in Drought horses of Basrah (13).

Average mean rectal temperature of 37.76 ranged from 36.9-38.9 (no horse suffered from hyperthermia with unique signs of fever) that, agreement with 37.88ºC of (13) and disagreement with 40.8ºC (12), but a remarkable increase in means of Heart rate 40.3 (21-60 beat/minute) that agreement with another Iraqi survey (12,13); and moderate respiration rate 16.72 (8-36 breath/minute) with Five infected horses had polypnoea, have reflected presence the Carriers states in most cases due to chronic anemia and decrease oxygen delivery.

Only a single tick infestation occurred with a history of bringing this horse from a farm; this low tick infestation was due to housing variances and daily grooming activity. Aziz & Al-Barwary (14) found the same issue in a North Iraq study.

Microscopically, results of *Babesia caballi* in Geimsa stained horse blood smears were observed in 49 cases as single or paired pyriform with different sizes from a total of 160 horses. PCR results revealed a significant increase in positive results at level ($P \leq 0.05$) than microscopic results with (91 horses). The superiority of the PCR technique agrees with (15,27) since the highly sensitive technique of

polymerase chain reaction compared with imperfect microscopic examination to diagnosing piroplasmosis, especially in subclinical and chronic carrier infection (28). Furthermore, our study conducted a microscopic examination and PCR analysis, which yielded a high percentage of *B. caballi* compared to other studies. Specifically, our results showed a prevalence of 30.625% and 56.875%. In contrast, studies conducted in Erbil city, north Iraq (29), Baghdad draft horses (15), Iran (30), and Turkey (31) reported lower percentages ranging from 1.7% to 30.62% by microscopic examination and 5.83% to 56.9 by PCR analysis. Notably, other researchers have reported PCR percentages that are significantly lower than those found in our study. For instance, in France (32), the prevalence was 6.3%, while in Jordan (33) and Egypt (28), it was 7.3% and 12.5%, respectively.

The Overwhelming microscopic and molecular results shown by this study against other investigations may be reared to the genetic diversity of *Babesia caballi* that was improved by sequencing and phylogenetic analysis, as improper treatment by horse breeders without superintendence of veterinary staff that facilitated carrier state of *Babesia caballi*.

There are three known 18S rRNA genotypes of *B. caballi* (34). Nehra *et al.* (35) collected the results from Genbanks for rRNA gene sequences of *Babesia caballi* isolates from worldwide until year 2021 and found *B. caballi* clades (A and B) and subclades (A1 and A2) with several subclades of clade B. All sequenced isolates of our work (ON328303.1 ON328304.1, ON328305.1, ON328306.1, ON328307.1, ON328308.1, ON328309.1, ON328310.1, ON328311.1, ON328312.1, ON328313.1, and ON328314.1) categorized in the new clade (clade C) shared identity 97.49 - 98.99% (98 - 100% Site coverage) by phylogenetic analysis with other researchers' results (MN723851.1) Iraq donkeys, (MN481268.1 -MN481271) Turkey, (MF384422) India, (MW678759.1) Egypt donkeys, (MZ675521.1) Egypt camels; on other hand they displayed 90.73 - 97.49% (65% site coverage) nucleotide identity to clade A1 (MN907451) Kazakhstan, (MH059519.2) Brazil, (MH047214.1) Erbil Iraq, (KU879028) Malaysia, (MT182023.1) China, (MT463343.1) Cuba. This group of new clades may explain the high infection rate with *Babesia caballi* comparable to global research due to genotype diversity.

Sex of horses had non-significant variation between stallions and mares (1.48 odds ratio) despite stallions having a higher percentage of infection with *Babesia caballi* 62.32% than mares 52.75%, some surveys like us found non-significant sex variation as (14,15,28,30,36). In contrast, others found significant for females (37) and others for males (33).

Horse age groups (after excluding foals under two years of age) in our data had an equal effect (2.26 odds ratio) on *Babesia caballi* infection, with the highest percentage occurring in the 6 -10-year-old age group 67.65%. These results lined with (15,29,30,36-38), but (37-39) estimate positive *Babesia caballi* decreased with age, though (28) Concerned horses less than five years ago had the highest prevalence, in adverse (33) having high risk with increased age.

Breed as a risk factor for *Babesia caballi* infection was statistically non-significant (1.59 odds ratio); this result agrees with the North Iraq study (14); on the other hand, the Arabian horse breed showed higher susceptibility to infection 63.34% comparable to Thoroughbred and Crossbreds horses. Bartolomé Del Pino *et al.* (37) found significant differences among equine breeds. Evaluation *Babesia caballi* infection according to breeds mostly interfere with Local or imported horses, and the purpose of keeping it subject to many other factors illustrated by many researchers, such as in which area more vector ticks occur (40), the dietary scheme applied for racehorses needs on both levels: growth and intensive sports training (41), presence different diseases especially viral infections were raise risk awareness interfered with animal welfare (42,43), different climatic place and the types of diagnostic tests with control programs in each area, Sport horses in enzootic regions suffering increase the possibility of infection due to sport activities and hard training (40,44).

Conclusion

This study focused on detecting equine haemo protozoan *Babesia caballi* infection by molecular method for the first time in Racehorses of Baghdad city in the middle of Iraq. We recognized that *Babesia caballi* seems to be highly endemic in the region of our study, and most infections occurred as carrier state or subclinical disease. We observed new *Babesia caballi* after phylogenetic analysis of our samples and compared it with data from the international Gene bank, which we illustrated as Clade C. The outcomes of the whole revision might have a significant impact on how horse Babesiosis is distributed, monitored, treated, and controlled, which causes decreased performance of racehorses and financial losses in the horse field.

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Conflicts of interest

No conflicts of interest have been declared.

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دراسة سريرية وجزيئية للإصابة بطفيلي البابيزيا كبالي في خيول السباق في بغداد

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الخلاصة

تهدف هذه الدراسة الى التقصي عن داء الكمثريات في الخيول المتواجدة في أكبر ثلاث تجمعات لإسطبلات خيول السباق في العاصمة بغداد من خلال الفحوص السريرية والاختبارات المجهرية والجزيئية باستخدام تفاعل البلمرة المتسلسل التقليدي، إذ تم تضخيم جين 18S rRNA لطفيلي البابيزيا كبالي باستخدام تقنية تفاعل السلسة المتبلمرة ومن ثم تسجيل تسلسل القطع الجينية وتحليل شجرة النشوء والتطور. للفترة ما بين كانون الثاني وكانون الأول من عام ٢٠٢١. تم جمع مئة وستين عينة دم الخيول من مختلف الأعمار والسلالات والجنس. حللت النتائج إحصائيا متغيرات انتشار ومخاطر داء البابيزيا باستخدام اختبارات مربع كاي ونسب الأرجحية. ما بين كانون الثاني ٢٠٢١ وكانون الأول ٢٠٢١، تم أخذ عينات دم من ١٦٠ حصانًا من مختلف الأعمار والسلالات والجنس. إحصائياً، تم تحليل نسبة انتشار وعوامل الخطر المهيئة للإصابة بمرض الكمثريات باستخدام اختبارات مربع كاي ونسب الأرجحية. جمعت ثلاث مليلترات من دم الوريد الوداجي في أنابيب اختبار حاوية على مانع التخثر لاستخدامها في الكشف عن طفيلي البابيزيا كبالي في المسحات الدموية والاختبارات الجزيئية. تمثلت العلامات السريرية في الخيول المصابة بداء الكمثريات بشحوب واصفرار الأغشية المخاطية، الهزال وفقدان الشهية، مع تورم الأرجل. لوحظ ارتفاع طفيف في درجة حرارة الجسم، وزيادة في معدل ضربات القلب، ومعدل التنفس. أظهر الفحص المجهري عن وجود طفيليات البابيزيا كبالي في ٤٩ مسحة من دم الخيول من أصل ١٦٠ (٣٠,٦٢٥٪). أظهرت هذه المسحات مظاهر مختلفة لطفيلي البابيزيا كبالي داخل كريات الدم الحمراء. بينت الفحوص التوكيدية باستخدام تفاعل البلمرة المتسلسل للكشف عن جين 18S rRNAوالتي أجريت على ١٦٠ عينة دم، أسفرت عن ٩١ نتيجة إيجابية (٥٦,٨٧٪) بالحصول على الحجم الجيني المستهدف والمقدر بـ ٤٥٠ زوج قاعدي. لم يكن هناك اختلاف معنوي في نسبة الإصابة ـ البابيزيا كبالي بين الفحول والأفراس. بالإضافة إلى ذلك، تجدر الإشارة إلى أنه لم تظهر أي فئة عمرية انتشار إصابة كبيرة بالعدوى. من الجدير بالذكر، أظهرت سلالة الخيول العربية أعلى استعداد للإصابة وبنسبة ٦٣,٣٤٪ مقارنة بالخيول الهجينة الأصيلة والخيول المختلطة الأنساب، بالرغم من إن هذه النسبة لم تكن ذات دلالة إحصائية معنوية. كشفت هذه الدراسة عن الاصابة بفئة جديدة من كمثريات البايزيا كبالي بعد إجراء تحليل النشوء والتطور لعيناتنا ومقارنتها ببيانات البنك الدولي للجينات. هذه الفئة والتي أشرنا اليها بمصطلح كلاد سي، تميزت بمعدل إصابة مرتفع وعلامات مرضية منخفضة الشدة.

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