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GENOTYPING AND DNA SEQUENCING DIVERGENCE OF *TOXOPLASMA GONDII* STRAINS ASSOCIATED WITH RABBIT *TOXOPLASMOSIS*, IRAQ

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ABSTRACT

The aim of this study is to determine the genetic patterns and sequencing of *Toxoplasma gondii* (*T. gondii*) isolated from humans and inoculated into rabbits to determine the effect of these strains on animals and the changes that occur in these strains. Therefore, DNA sequencing is necessary to apply with genetic developing for microorganisms to determine the relation between different organisms and how they are developed. Thirty-eight samples of congenital Toxoplasmosis were collected from the placenta of women who have had abortion and after their development were inoculated into rabbits. Seventeen samples of whole blood from rabbits were investigated. Iraqi human *T. gondii* were infected in 38 samples of rabbits, 17 samples out of 38 underwent gene sequence work from period 15th September 2015 to 10th may 2016. Data from 38 samples using the Surface Antigen Glycoproteins (SAG2) gene locus according to geno typing indicated that 22 out of 38 was types II, while 8 samples were types I and 8 showed types III. The results showed that there was a significant effect by the congenital Toxoplasmosis, which was developed in the bodies of rabbits, different strains of I, II, III, taken from the placenta of women who have had abortion because they were infected with *T. gondii*.

KEYWORDS: ***Toxoplasma gondii*, Rabbits, Surface Antigen Glycoproteins, Genotyping and DNA sequencing.**

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INTRODUCTION

Toxoplasma gondii (T. gondii) is considered to be a part of the greatest common intracellular parasitic agent, causing infection in human and other animals. Studies estimated the epidemiology of *T. gondii* which infects high population in the world.[1] Mammals such as human suffer from high- risk factor to be infected by *T. gondii* which is a particular intracellular parasite; this parasite can infect different hosts including birds, cattle, and humans, and also marine mammals. Pathogenesis of *T. gondii* is considered an essential and highly epidemic in the world.[2-4] *T. gondii* has a complicated life cycle with two stages and therefore needs to infect different hosts and requires a unique regulatory mechanism to complete its stages of life such as modifications of phosphorylation and some transcription proteins included in the immune defense. [5]Moreover, some studies revealed that there is a different genetic diversity from the first isolated parasite and they also indicated the incidence of other complex population structure of Toxoplasma with greater genetic diversity from other isolated countries[6].Genetically, strains of *Toxoplasma gondii* were divided into three clonal types and these strains were considered the causative agents of human toxoplasmosis in the world. Parasites with type I are extremely virulent in animals such as mice, whereas parasites with types II remain predominant in human infection and those of type III are lesser destructive, and these were established in latent infection in murine model.[7-8] The overproduction in certain acute inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukins considered as a markers activation associated with the large virulence factors of type I parasites in rodents. [9-10] Moreover, heart and kidney disease patients used rabbit meat as a special diet for their health; rabbit meat has lower fat and cholesterol levels than other meats.[11-12] However, one important route for infecting the human being with *T. gondii* is due to severe consumption of undercooked rabbit.[13] Transmission of infection to the human depends on the prior infection of rabbits with oocysts *of T. gondii* present in the contaminated food or water from cat feces. Then the parasites pass through transplacental infection to the fetus. [14-15] "A Genetics main factor such as a deoxyribonucleic acid or a DNA molecule consisting of a monosaccharide sugar (named with deoxyribose), a phosphate group, and four kinds of nitrogen-containing nucleobases, namely, cytosine (C), guanine (G), adenine (A), and thymine (T)". Biological information and genetic instructions at the molecular level depends on the type and the order of these nucleobases in a DNA molecule. Medical rules for DNA applications were useful in some approach from obtained DNA sequences. Genomic results such as a DNA sequencing provide blueprints for the life.[16-19] Forensic DNA[20], medical DNA[21]and other Clinical application regarding initial diagnosis and management in addition to considering the mechanisms of genetically related diseases can remain dependant.[18-19] There are three types of antigens responsible for the invasion process by this parasite to the tissue. These are mediated via major tachyzoite surface antigens which have different molecular weight "SAG1 (30 kDa), SAG2 (22 kDa) and SAG3 (43 kDa)".[22-24]However, antigens were preserved between different strains of T. gondiihas for medical importance such asvaccination process.[25] These surface antigens of *T. gondii* variant varies according to the molecular weight, virulence and site stage of the parasite. Surface Antigen Glycoproteins (SAG1) is the first portion which deals with the first interaction and contributes between the mechanism of host cell and parasite attachment. Free tachyzoites contains this type of antigen on their surfaces on specific location.[24,26] Previous studies have indicated the medical importance of SAG1 as useful immunological stimuli in rodents through inducing a humoral immune response. This reactions were applied in DNA vaccination; in addition, SAG1 gene plasmid encoding enhanced the mechanism of defense against interaction with the infectious of *T. gondii* RH strain.[22,27]Additionally, SAG2 was considered the second surface antigen, identified as a route of invasion as an attachment ligand.[28] once again this type displays perfect antigenicity and immunogenicity.[29] Consequently, several studies confirmed the immunogenicity of this portion in BALB/c mice by using recombinant SAG2.[30-32]In this study, we have studied the identification of somatic or germline DNA variants in *T. gondii*. However, polymerase chain reaction (PCR) has played a key role in these variant identification studies. Our targeted SAG2 provides knowledge of focused SAG which can help us gain greater scope to our study, less time consumption time, and more dependable results. Information of DNA sequences developed should be necessary for basic biological investigation besides several practical parts such as medical diagnosis and biotechnology. Identification of new genes is helpful if it is associated with

diseases, phenotypes and identifies possible drug target.

MATERIALS AND METHODS

Rabbits and experimental pathogens

Parasite

The amniotic fluids samples for *T. gondii* isolation were obtained from aborted females " who attended Hospitals for delivery (Baghdad Teaching Hospital and Al-Ilweia Hospital, Baghdad, Iraq)".Our samples were collected according to experimental study by Al-taie and Abdulla, 2000[33].The direct microscopic examination were made for parasite detection through wet mount preparation. Two samples were prepared on every slide having a drop of sediment, first slide sample examined directly by microscope at 40X and the other slide stained with 10% Giemsa stain. Suspensions were kept with 3 ml of preservative Ringer saline until next experimental work.[34]For *in vitro* maintenance of parasites, the tachyzoites were grown in RPMI supplemented with 10% fetal calf serum at 37 ˚C in a 5% CO_2 condition within MBDK cell monolayers and then the parasites have been kept in preservative Ringer saline and sent for sequencing and genotyping.

Rabbits

Thirty-eight of rabbits weighing between 2.5 and 4 kg and three to thirty-six - months- old female rabbits were prepared during *in vivo* study. These rabbits were obtained from the Animal House, College of Medicine, Baghdad University. All the animal experiments were performed according to the institutional ethical committee approval and within the scientific standards.

Toxoplasma gondii parasites and infection model

All rabbits were injected with human *T. gondii* intravenously. Specific tissues, heart, and other tissues were collected from infectious rabbits after killing. These tissues were crushed to small pieces and were digested by acid pepsin solution, and then suspended in a clean, dry beaker, stirred by a magnetic stirrer for 30 minutes and then left at room temperature.[35] The digested materials were filtered through several layers of sterile gauze; the filtrated suspension was placed in a clean test tube and then filtrate separated by centrifuge instrument using 3000 rpm for 15 minutes.[33] Supernatant was discarded, and pellets were washed with the same volume of Ringer saline. The process was repeated three times, then the solution was re-suspended by 3 ml of Ringer saline until use.[34]Virulent *T. gondii* RH strain of *T. gondii* followed by using serial passaging number in rabbits followed by the standard method.[36]

Isolation of local strain of T. gondii for the strain in rabbits

The *Toxoplasma* strains were isolated from different infected places such as heart, diaphragm, liver and other tissues. The samples were collected and processed by method followed by Abdel-Rahman et al. 2005[37].The infectious local strain of *T. gondii* were harvested from bio-assay samples and maintained by serial passage in rabbits. In brief, the prepared solution suspension was centrifuged on 3000 rpm for 15 minutes then (0.9%) sterile normal saline was added to the sediment to volume of 10 ml and the centrifugation was repeated. Three times this process was repeated. then 10 ml of sterile suspension was collected using two types of sterile antibiotics (100 µg) of Streptomycin and 1000 IU of Penicillin per 1 ml of inoculum.[38] Numbers of rabbits as described above were injected by intraperitoneal (IP) route with 0.2 ml of *T.* genetic sequence of the human *T. gondii*,[39] Parasites isolation was obtained after 7- 10 days after infection.[40]

Antigens Preparation

Tachyzoites of RH strain were prepared in the form of soluble crude antigens ;The parasite was isolated from rabbit following the method reported by[41]Daryani et al. 2003[41]. Consequently, tachyzoites 5 x 10^6 per ml were frequently frozen and melted for rupturing the wall of parasite, centrifuged after sonication at 12,000 rpm for 45 minutes in cold condition at 4°C. The supernatant was then collected and the protein contents were determined by the method of gigley et al.[42] Isolated protein were considered as a crude antigen and was kept at deep freeze at -20°C until use.

Extraction and DNA Amplification

Extraction and isolation of *T. gondii* DNA from rabbit peritoneal exudates infected tissues by the procedure given in the extraction procedure of the kit of the DNA extraction kit providedby the manufacturer (Promega, USA). Amplification of DNA was completed via Nested Polymerase Chain Reaction (nPCR)

Genotypes of Toxoplasma gondii determination

Restriction of fragment length polymorphisms method was used for this purpose.Endonucleases

enzyme of two types were applied in the splitting process and they were "Sau3A1enzyme which digest the 3rd allele at 5' end (type III) and a Hhal enzyme which digest the 2nd allele at 3' end (type II)". While, Type I strain will be referred if the fragmentation or splitting doesn't induced by using any of the above two enzymes

Preparation for Toxoplasma antigens

After destroying the vegetative phase of the parasite a soluble antigen was prepare, Using recurrently freezing and defrosting method, final volume of 5 x 10^6 per ml was made up and again centrifuged on 12,000 rpm to 45 minutes at 4°C, and the samples were stored at at -20°C until use.[45]

Preparation of sample for Sequencing and loading

Since the detection of molecular methods about separate DNA molecules sequencing were not sensitive enough, *in vitro* method of cloning steps were carried out for DNA amplification.[46]PCR products were applied on agarose gel plate which were submerged in 1X TBE solution. A voltage of 95 volts for 40 minutes were passed for base pairs determination.[47] The procedure of sequence reaction made by the application of the instructions issued by the manufacturer were performed by V3.1 cycle dye of sequencing kit. (Perkin-Elmer, Foster City, CA) number 4336817.

RESULTS

Phylogenetic analysis of rabbit Toxoplasma gondii strains established on SAG2 sequencing

Samples were made to run out and assessed for sequencing analysis by using PCR reaction. The bands of Figure 1 represented the differentiation of strains of genotypes for all group of the parasite as I, II, and III. Fig 1 profile indicated the SAG2 amplicons formed a single product of approximately 1000 bp in length on plate of agarose gel for all 17 *T. gondii* strains. All the isolated amplicons were sequenced respectively. Fig 1 demonstrated that Phylogenetic investigation of *T. gondii* strains constructed on SAG2 sequencing, and the bands obtained from this Figure gave the results of sequencing according to the following parameters. Band number 4- related to the markers of molecular weight paralleled to 100 bp ladder (fermintus). While the band's number 1 & 2 related to the 3' end of SAG2 cut with HhaI, Type II. Bands of 3 & 5 considered with the 3' end in the cut of SAG2, Type I. Finally, 6 & 7 bands demonstrated the 5' end of SAG2 digested with Sau3AI, Type III as shown below in Fig 1respectively

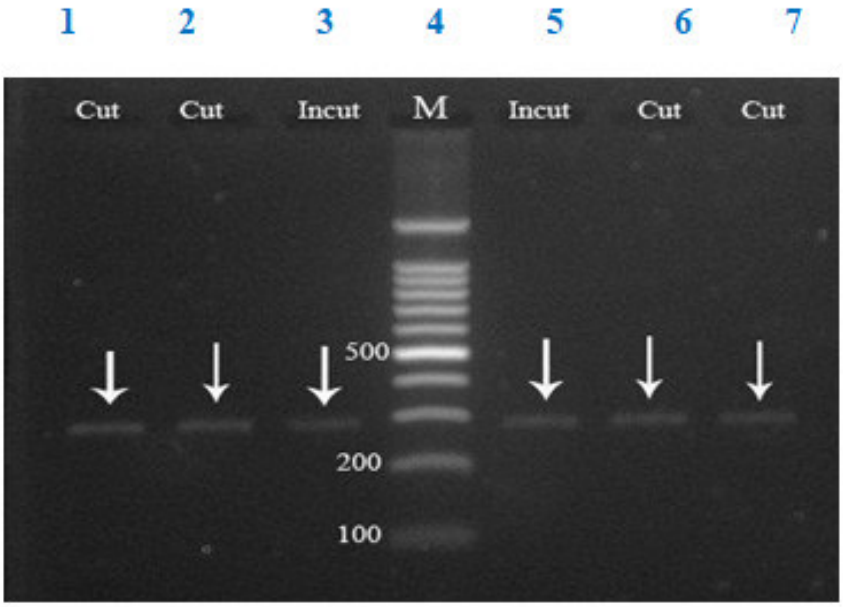


Figure 1

Restriction digestion of SAG2 T. gondii amplified PCR products: Lanes are labelled as follows: Lane 4- Molecular weight markers (fermintus), Lanes 1 & 2 The 3' end of SAG2 cut with HhaI, Type II, Lanes 3 & 5 The 3' end in cut of SAG2, Type I, Lanes 6 & 7 The 5' end of SAG2 digested with Sau3AI, Type III.

Results (Fig 2) indicated that examination of sequencing polymorphisms in the SAG2 gene between two clonal genotypes (strains RH/GTI,PRU/QHO/PTG,CTG) exposed the presence of polymorphic targeting sites. The bands of PCR technique showed that band no. 1 reverse the molecular weight markers resembled 100 bp ladder (fermintus). The bands in numbers 2 and 3 relates to the 3' end of SAG2 cut with HhaI, Type II. While band in no. 4 correlated with the 3' end un cut of SAG2, Type I. Finally, bands no. 5 and 6 related to the 5' end of SAG2 digested with Sau3AI, Type III (Figure 2).

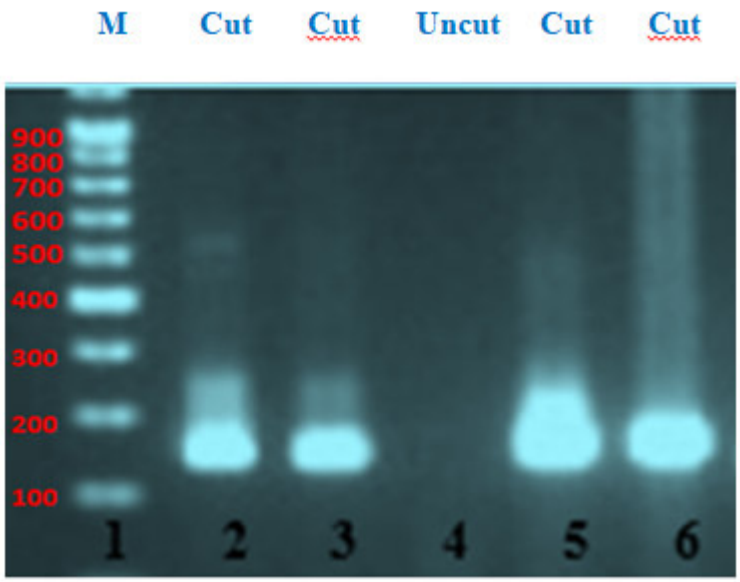
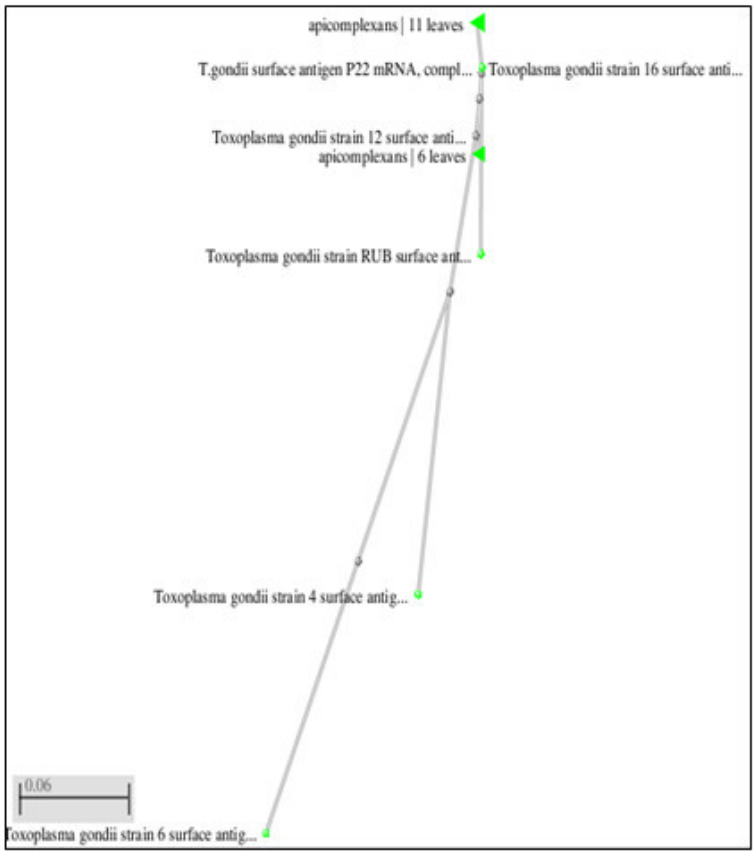


Figure 2

Phylogenetic determination of T. gondii strains established on SAG2 sequencing: 1-Molecular weight markers correspond to 100 bp ladder (fermintus). 2 & 3 The 3' end of SAG2 cut with HhaI, Type II. 4The 3' end un cut of SAG2, Type I. 5 & 6 The 5' end of SAG2 digested with Sau3AI, Type III.

Isolated of Toxoplasma SAG2 gene sequencing from rabbits infected strains

Through the phylogenetic reconstruction established on SAG2 sequencing results of all 17 strains, we got the phylogram (Figure 3). Therefore, Phylogenetic examination exposed 3 significant groups, which resembled standard genotypes (I, II, III) correspondingly, and the Phylogram map of 17 *T. gondii* strains illustrated by investigation for whole sequencing of the SAG2 genomic region displayed correspondence among all these sequesters.



"Sequencing ID: tpe [N7144984498, 1] Length: 6937759 Number of Matches: 1 Range 1: 4761662 to 4761831 Gen Bank Graphic".

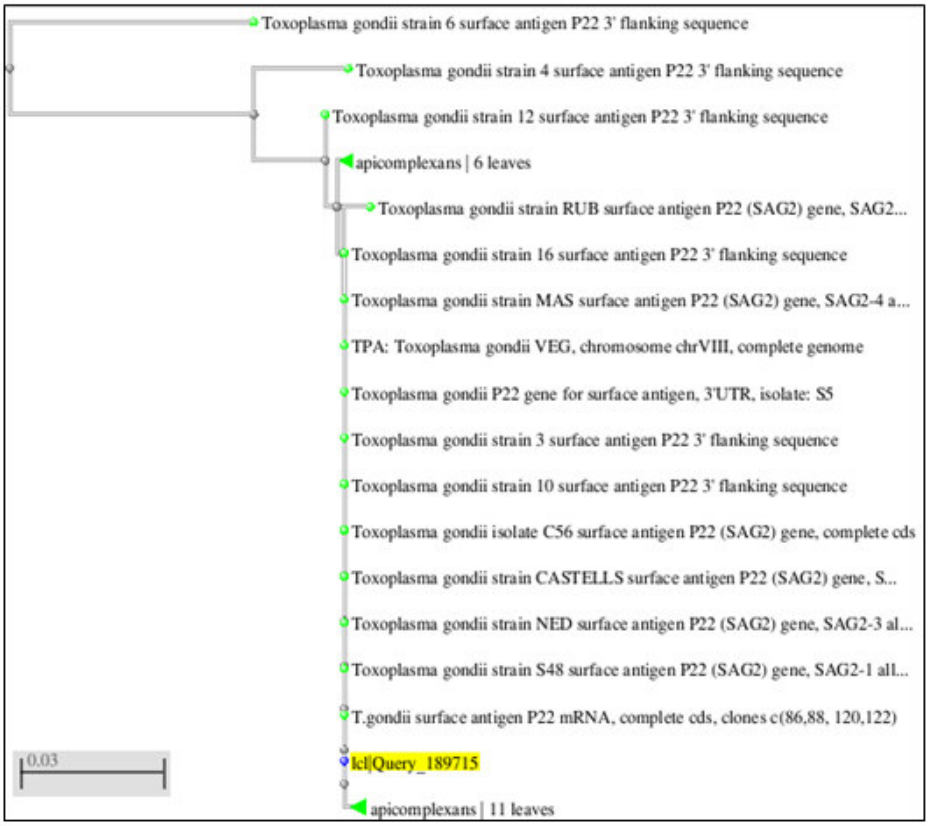


Figure 3

Phylogram map of SAG2 genomic region from 17 Toxoplasma gondii strains defined through investigation with whole sequencing: The genetic variance of SAG2 gene shown 3 significant groups (indicated as I, II, and III). Tree stated made by "Bayesian inference (BI) and maximum parsimony (MP) analysis".

DISCUSSION

Toxoplasmosis is considered a major problem of medical health in the Middle East countries. This disease is caused by an intracellular parasite. These pathogens infect several animal types. Domestic cats in particular and this *Toxoplasma* can transfer the infection to humans .[3,48-50] The epidemiology, genetics population, and phylogeny of *T. gondii* were analysed and identified following the detection of these pathogens in the animal. For this, the *T. gondii* infection in rabbits were studied to detect and determine the Iraqi rabbit strain. The results of this study showed a classification of *Toxoplasma* isolated from rabbit tissues in Iraq. Genetic analysis based on sequencing contribute to display full genetic diversity including small insertions, deletions and single nucleotide polymorphisms which are used to diagnose other specific relationships between isolated *T. gondii* phylogenetic.[51] Genetic application studies showed some strategies for escaping the *Toxoplasma* from the immune defense. These mechanisms involved by various immune components of pathogen and immunization studies indicated the improvement of innate and adaptive response through plasmid applied for encoding antigenic proteins of invading pathogens.[52,53] Understanding the Iraqi prevalence of Toxoplasmosis, is essential and the outcomes of polymerase chain reaction (PCR) technique analysis displayed the molecular weight of SAG2 protein was approximately 1000 bp in length on an agarose gel. The infection in pregnant women by *T. gondii* varies in prevalence considerably between countries, in particular, Middle East people. However, from the previous studies, it was unclear to understand the epidemiological information on *T. gondii* in pregnant women and related animal model in Iraq. The present study showed that 38 samples of rabbits were infected by Iraqi human *T. gondii*, 17 samples out of 38 underwent gene sequence work. Again, results obtained by PCR demonstrated that genotyping using the surface antigen glycoproteins (SAG2) gene locus described as 22 isolates existed as type II, 8 existed as types I and 8 existed as types III. The results obtained from SAG2 analysis indicated that high avidity or percentage of type II, and equal with fewer incidences of I and III in *T. gondii* strain. The data agreement with numerous recent studies on different animal models, established that similar findings were demonstrated in mice inoculated to identify the prevalence of SAG2 *Toxoplasmosis* in Iraq for all groups of genotyping according to the standard classification of *T. gondii* as I, II and III respectively.[54] Furthermore, two recent studies reported that the immunogenicity functional of SAG2 in stimulation of classical and alternative immune pathways also decided the role of this antigen protein as a tool used in the development of a vaccine for protecting from such disease.[55,56] In conclusion, this study has aimed to analyse that in the future the ability of SAG2 protein can be used as a specific serological indicator to detect immunoglobulin classes formed against *T. gondii* by different samples such as serum, saliva, and biopsy and can also be considered as a vaccine for protection against Toxoplasmosis.

CONCLUSION

This study recommends that the work of the technique of gene expression could be performed by real-time and micro-RNA for people with congenital infection and to determine the mechanism of their effect on human and animal cells. Consequently, identification of novel genes is helpful if it is associated with diseases, phenotypes and identifies target of possible drug. Therefore, DNA sequencing is necessary to determine the relation between different organisms and how they are developed.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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