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Detection, Identification & Sequencing of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) among Sudanese Patients

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Abstract MERS-CoV virus is a newly emerged coronaviruses in KSA in 2012; followed by a lot of cases in the Middle East & other European, American & African countries. The goal of this study is to detect, identify & sequencing of MERS-CoV among Sudanese patients suffering from respiratory diseases by using the orf1a with upE gene for the virus detections. Phylogenetic analysis of upE gene of MERS-CoV was done using ViPR. MERS-CoV virus seems to be highly prevalent among Sudanese population especially among patients from Al-Shaab hospital than individuals from the International Khartoum Airport (86.8% & 12.1% vs 66.6% & 9.0%) respectively; this attributed to the fact that Al-Shaab hospital participants were symptomatic, having severe respiratory symptoms (possibly caused by coronaviruses). Phylogenetic analysis of the isolated viruses from Khartoum International Airport participant showed genetic similarity with KSA while Al-Shaab Teaching Hospital individuals showed genetic similarity with Thailand. Viral genomic were deposit in gene bank with those accession N.o: KY794400.1-KY794403.1 and MF133448-MF133457.

Keywords: *MERS-CoV, sequence, Open Reading Frame (ORF), ViPR., RI*

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1. Introduction

Middle East Respiratory Syndrome is one of the newly emerged diseases that have appeared in the last 5 years. It is caused by a coronavirus named as Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV). The actual evolution history suggested that the emergence of the virus is in 2000. The first human case of MERS-CoV infection was reported from Saudi Arabia in 2012. This was followed by few cases and outbreak in KSA. The second outbreak occurred in Korea in 2015 by a recombinant strain called ChinaGD01 [1,2].

A study conducted in Egypt revealed a significantly higher seropositivity rate of MERS-CoV in camels imported from Sudan, Somalia and Ethiopia (Ali *et al*, 2017) [3]. Antibodies against MERS-CoV virus were detected in sera of some camels from nearby countries (Egypt, Kenya, Somalia, Ethiopia, Tunisia and Nigeria). However no primary human MERS cases have been reported to have arisen in African countries. According to the predict 2 modelling and analytical teams; it is expected that there are thousands of undiagnosed MERS cases in Africa with varying numbers by countries and with the potential of high burdens in Kenya, Nigeria, Sudan and Somalia. To our knowledge there was no study that confirmed human MERS-CoV virus infection in Sudan. However the large movement of population to and from Saudi Arabia, other middle eastern countries and south east Asia; are expected to have spread the disease from those countries to Sudan and vice versa.

This study was conducted to determine and to compare the rates of infections with MERS-CoV among two target populations (symptomatic patients at Al-shaab chest hospital and individuals arriving the International Khartoum Airport from the above mentioned countries). Then sequenced of the Sudanese viruses were done to find the possible links between them and the identified viruses.

2. Materials and Methods

This is prospective, cross sectional study was conducted during the period from May 2014 to May 2017. Two target groups were included. Group-A (G-A) were Sudanese individuals arriving the International Khartoum Airport during the study period (most of them pilgrims of

the year 2015) and Group-B (G-B) were Sudanese patients admitted to Al-shaab hospital with symptoms and signs of respiratory infection during the study period. The total number of participants was 200 (167 patients at AL-shaab Chest Hospital and 33 from the International Khartoum Airport). The purpose of the study was explained to each participant and after his/her verbal consent to participate in the study, data were collected using questionnaire. The information requested included; Age, Gender, Marital status, history of travel to Saudi Arabia in addition to medical history. Individual with known underlying diseases such TB, Lung cancer and those who spent less than 14 days in Saudi Arabia or the other countries were excluded.

Sputum samples were collected from each individual whether at the airport or hospital by hospital or medical airport staff according to a protocol of sample collections as follows: the patient was asked to take a deep breath, hold it for 5 seconds, breath it out slowly and then take another one then cough out and the coughed material (sputum) collected into clean, sterile, wide mouthed container then transferred to the laboratory in ice bags for processing.

2.1. Laboratory Processing and Examination

Each specimen was subjected to RNA extraction using innuPREP Virus DNA/RNA Kit (analytikjena, Germany) and IQeasy Plus Viral DNA/RNA Extraction Kit (iNtRON Biotechnolgy, Inc, Korea) according to the manufacturer's protocol. The extracted RNA stored at-20 C° until used.

2.2. Primers Used

The primers used in this study are shown in Table 1. The MERS-CoV orf1, MERS-CoV upE and pan coronavirus primers were obtained from WHO recommended primers & scientific published papers while MERS-CoV orf1ab and MERS-CoV Spike were designed by the investigator according to NCBI primer design (https://www.ncbi.nlm.nih. gov/tools/primer-blast/index.cgi? LINK-LOC=Blast Home).

PCR amplification was performed for the extracted RNA using the PCR ready premix kits such as; Maxime RT PreMix Kit, HiSenScript TM RH [-]. One-Step RT-PCR Kit, ONE-STEP RT-PCR PreMix Kit, Maxime RT-PCR PreMix Kit (Intron Biotechnology, Korea) according to the protocols shown in Table 2.

2.3. Sequencing

Fourteen randomly selected + ve PCR up-E specimen (7 from each target group) were packaged according to the International Air Transport Association guidelines and shipped with authorized permission to Macrogen Company (Seoul, South Korea; in Netherland branch for standard sequencing according to company protocols using ABI 3730 Genetic Analyser (Applied Biosystems) machine.

2.4. Data Analysis

Data were analyzed by using IBM SPSS statistic (Statistical Package for the Social Sciences) (using Chi Square Test) data editor software program version 23. The chromatogram sequences of the virus were visualized through Finch TV program version 1.4.0. MERS-CoV sequences similarities were searched using nucleotide BLAST. The sequences of the reference MERS-COV strain were retrieved from NCBI (https://www.ncbi.nlm.nih. gov/) and subjected to multiple sequence alignment using BioEdit software version 7.2.5.

Expasy Bioinformatics Resource Portal (http://web. expasy.org/translate/) & ORF Finder (https: //www.ncbi. nlm. nih.gov/orffinder/) were used to translate nucleotide sequences to protein. ViPR (Virus Pathogen Resource) Release Date: Mar 16, 2017 (https: //www.viprbrc.org/brc/ home.spg? decorator=vipr) was used to generate phylogenetic tree & multiple sequence alignment (MSA).

Table 1. Primers used in this study

Primer/probe names	Forward primers	Reverse primers
MERS-COV orf1	CCACTACTCCCATTTCGTCAG	CAGTATGTGTAGTGCGCATATAAGCA
MERS-COV upE	GCAACGCGCGATTCAGTT	GCCTCTACACGGGACCCATA
MERS-COV orf1ab*[1]	GGTTGTTTGCGACCCTTGTC	AGAGCCATAGCCTCCCAAGA
MERS-COV Spike*[1]	CTCCCGTTCTACGCGATCAA	TCCAGCCAACACCTGCTATG
pan coronavirus	ACWCARHTVAAYYTNAARTAYGC	TCRCAYTTDGGRTARTCCCA

W=A/T, N=A/C/T/G, R=A/G. *[1] these primers just used to confirm our results.

Table 2. PCR protocol for MERS-CoV and Pancoronavirus

PCR Condition	RT	Initial denaturation	Denaturation	Annealing	Extension	F-extension
C-DNA	45°C-60 min	95°C-5 min	-	-	-	-
PCR-Steps upE	55°C-20 min	94°C-3 min	94°C-30 sec	55°C-30 sec	72°C-30 sec	72°C-10 min
PCR-Steps upE	45°C-30 min	94°C-5 min	94 °C-30 sec	55°C-30 sec	72°C-1 min	72°C-5 min
PCR-Steps orfa1	55°C-20 min	95°C-5 min	94°C-30 sec	58°C-30 sec	72°C-30 sec	72°C-5 min
MERS-COV orf1ab	45°C-60 min	94°C-5 min	98 °C-30 sec	60.5°C-30 sec	72°C-2 min	72°C-5 min
PCR-Steps Pancoronavirus		95°C-15 min	94°C-30 sec	50°C-20 sec	72°C-1 min	72°C-10min

2.5. Ethical Consideration

The Al-Shaab Chest Hospital & Khartoum International Airport authorities were informed for the purpose of the study and its objectives, before taking their permission, with protections of their privacy in case of specimen collections. Permission to carry out the study was taken from the College of Graduate Studies and Scientific Research The National Ribat University ethical Committee.

3. Results

A total of 200 individuals (167 patients from Al-shaab Chest Teaching Hospital and 33 from Khartoum International Airport) were recruited to participate in this study. More than half of them (55.5%) were males and almost two thirds (63%) were married.

The majority of the participants belong to the age groups 21- 40 years and 41- 60 years (34.5%, 34% respectively).

Most of the participants (73.5%) were from Khartoum state in which, (40.5%) were from Khartoum town (Table 3).

Among the participants screened by PCR, MERS-CoV was detected in 167 (83.5%) and Pancoronavirus in 23 (11.6%) (Table 4).

The percentage of MERS-CoV and Pancoronavirus were significantly higher among patients from Al-shaab Teaching Hospital compared to the Airport participants (86.8% vs 66.6%) (12.1% vs 9%) (Table 5).

The percentage of MERS-CoV was almost equal in the age groups; 21-40 years, 41-60 years and 61-80 years (86.9%, 85.3% and 86.6%) respectively (Table 6).

The percentage of MERS-CoV was slightly higher among females (84.3%) than males (82.9%); and Pancoronavirus was twice higher in females (16%) than males (Table 7).

Table 3. Distribution of the studied population according to their residence in Sudan

Residence*	Frequency	Percentage
Khartoum	81	40.5%
Omdurman	33	16.5%
Bahri	33	16.5%
Others	50	25%
Unknown	3	1.5%
Total	200	100%

*Residence from Khartoum state represented (73.5%) when compared with other Sudanese states.

Table 4. Distribution of participants according to types of virus infections

Types of virus	Number tested	Positive for the virus infections	
		Frequency	Percentage %
Pancoronavirus	197	23	11.6%
MERS-CoV (upE)	200	167	83.5%

*Pancoronaviruses includes all coronaviruses (HCoV-NL63, HCoV-229E, FIPV, TGEV, PEDV, HCoV-OC43, BCoV, PHEV, CRCV, MHV, SDAV, SARS-CoV, IBV, TCoV) except MERS-CoV virus.

Table 5. Distribution of infected individuals according to site of collection

Site of collection	No tested	Pancoronavirus + ve		No tested	MERS-CoV + ve	
		Frequency	%		Frequency	%
Airport	33	3	9	33	22	66.6
Al-shaab hospital	164	20	12.1	167	145	86.8
Total	197	23	11.6	200	167	83.5

Table 6. Distribution of infected individuals by coronaviruses according to age group

Age group (years)	No tested	Pancoronavirus + ve		No tested	MERS-CoV + ve	
		Frequency	%		Frequency	%
≤20	44	6	13.6	45	34	75.5
21-40	68	9	13.2	69	60	86.9
41-60	67	6	8.9	68	58	85.3
61-80	15	2	13.3	15	13	86.6
81-100	3	0	0	3	2	66.6
Total	197	23	11.6	200	167	83.5

(P>0.05).

Table 7. Distribution of infected individuals by coronaviruses according to gender

Gender	No tested	Pancoronavirus +ve		No tested	MERS-CoV +ve	
		Frequency	%		Frequency	%
Male	110	9	8.1	111	92	82.8
Female	87	14	16	89	75	84.2
Total	197	23	11.6	200	167	83.5

(P>0.05).

Table 8. Distribution of infected individuals by coronaviruses according to residence in Sudan

Residence	No tested	Pancoronavirus +ve		No tested	MERS-CoV +ve	
		Frequency	%		Frequency	%
Khartoum	80	8	10	81	70	86.4
Omdurman	33	3	9.1	33	28	84.8
Bahri	33	2	6.1	33	28	84.8
Others	48	10	20.8	5	38	76
Unknown	3	0	0	3	3	100
Total	197	23	11.6	200	167	83.5

(P>0.05).

Individuals residing in areas other than Khartoum state showed higher frequency of MERS-CoV (100%) followed by residence in Khartoum town (86.4%) (Table 8).

Fourteen of our samples were deposited in Gene Bank with those accessions N.o: KY794400.1-KY794403.1 and MF133448-MF133457.

4. Phylogenetic Analysis

Fourteen MERS-CoV strains (7 from each of the two groups) were randomly selected and sent for sequencing then development of Phylogenetic tree was done.

Phylogenetic tree for International Airport Viruses showed, 20-SUDAN-airport 2015 & 33-SUDAN-airport 2015 had the same origin; 16-SUDAN-airport 2015. 1-SUDAN-airport 2015 was closed to Hu/Artawiyah -KSA (G.B accession no. KX154694.1) then MERS-COV isolate KFMC-7|KSA-hospital outbreak (KT121581.1) (Figure 1).

While phylogenetic tree for Al-Shaab Chest Hospital Viruses showed 56-SUDAN-Al-Shaab 2015 & 126-SUDAN -Al-Shaab 2015 had the same origin but 126-SUDAN -Al-Shaab 2015 was close to 125-SUDAN-Al-Shaab 2015; 36-SUDAN-Al-Shaab 2015 & 156-SUDAN-Al-Shaab 2015 had the same origin and closed to Thailand human MERS-CoV (G.B accession no. KT225476.2), MERS-COV Hu/Kharj-KSA (G.B accession no KT806053.1) and MERS-COV strain ChinaGD0 (G.B accession no KT006149.2) (Figure 2).

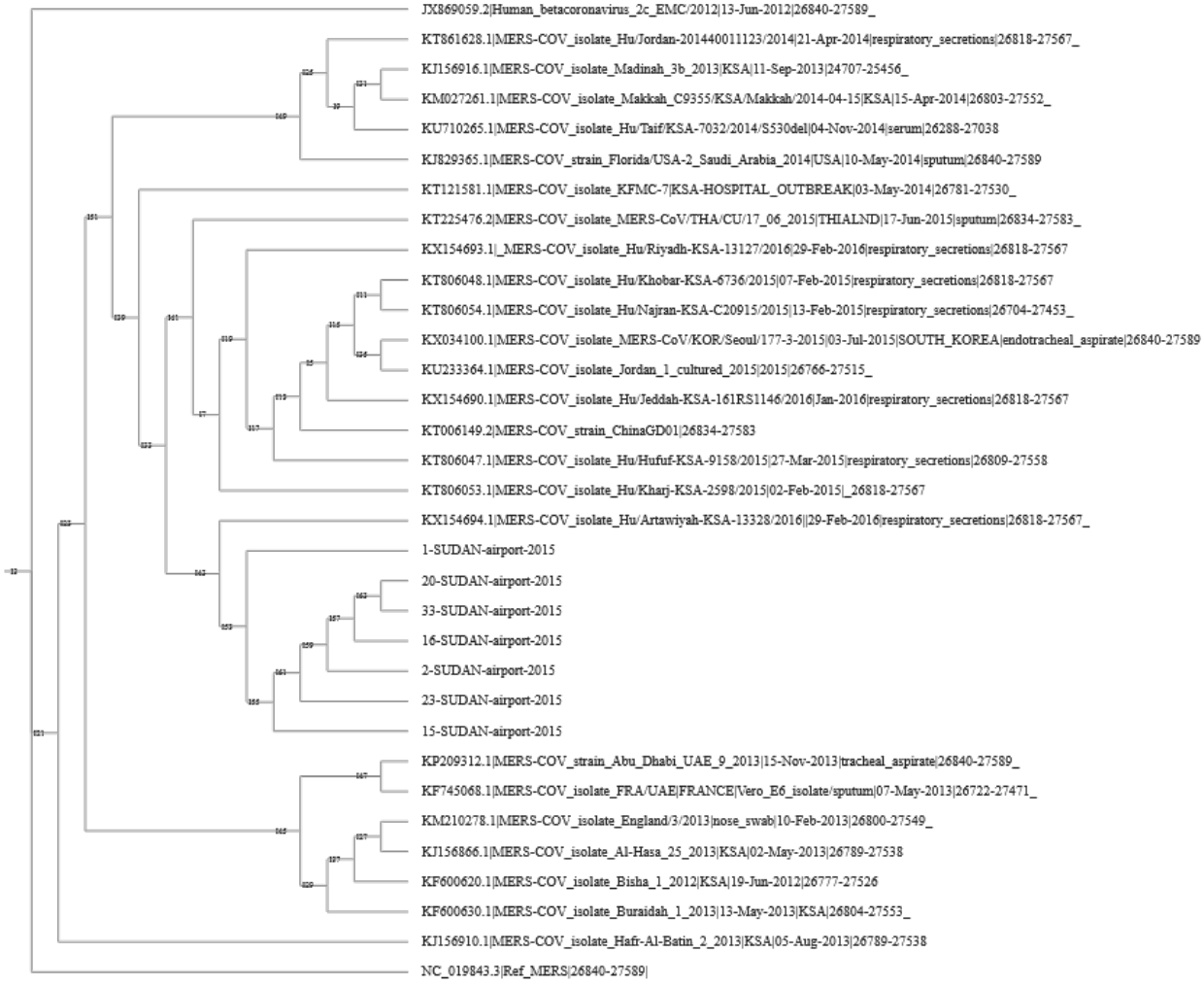

Figure 1. It illustrate phylogenetic tree of G-A Sudanese MERS-CoV (Khartoum international airport individuals) when comparing with other human MERS-CoV reference genes from NCBI Gene Bank.

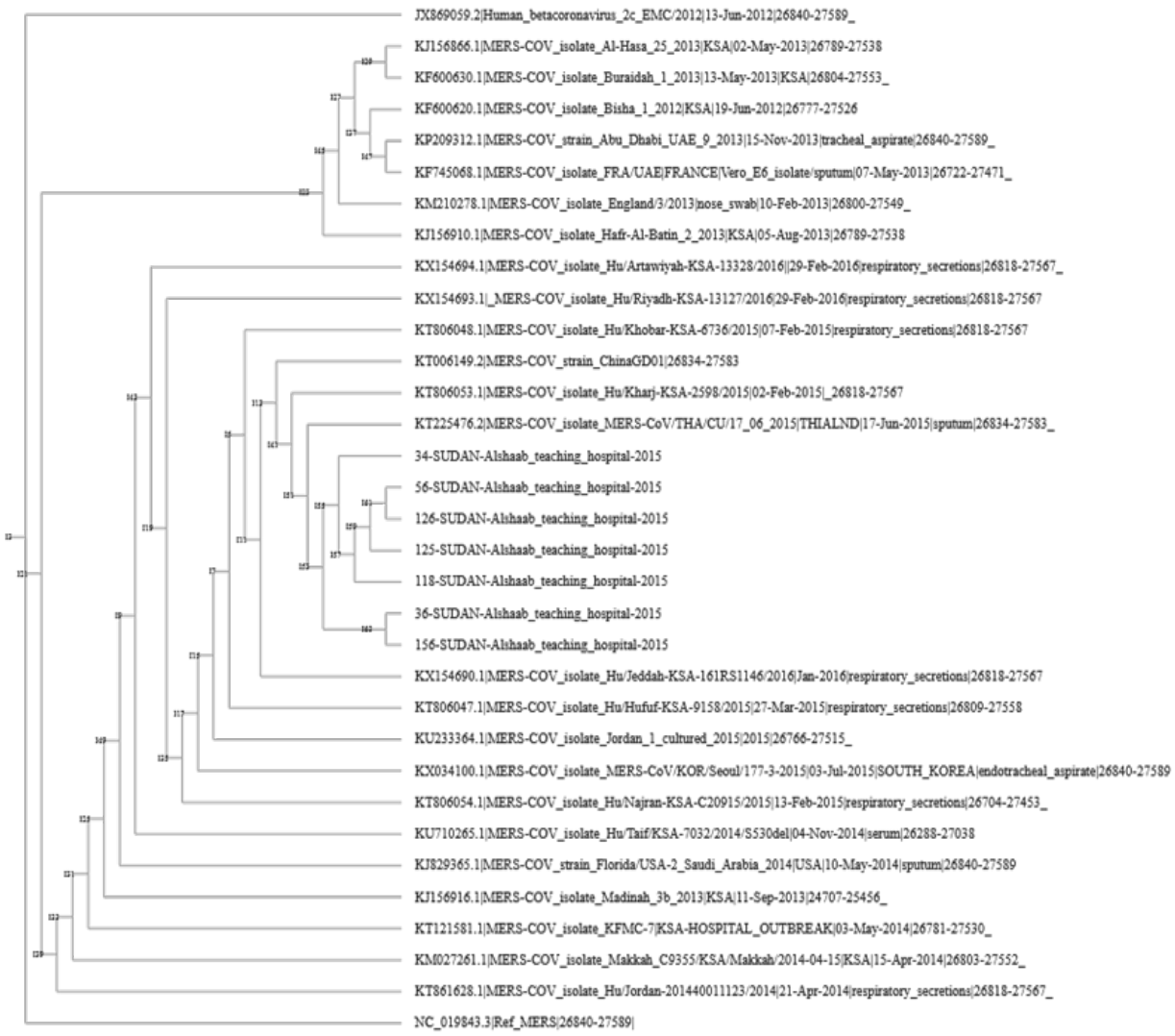

Figure 2. It illustrate phylogenetic tree of G-B Sudanese MERS-CoV (Alshaab teaching hospital patients) when comparing with other human MERS-CoV reference genes from NCBI Gene Bank.

5. Discussion

In this study, the detection rate of MERS-CoV virus was (83.5%) and Pancoronavirus (11.6%).

The detection rates of MERS-CoV and Pancoronaviruses were significantly higher among patients from Al-Shaab Hospital than individuals from the International Khartoum Airport (86.8% & 12.1% vs 66.6% & 9.0%) respectively. The high frequencies of MERS-CoV and Pancoronaviruses among AL-Shaab Hospital relative to Khartoum Airport candidate can be attributed to the fact that all Al-Shaab Hospital participant, were symptomatic having severe respiratory symptoms (possibly caused by coronaviruses). The rate of coronavirus infection was almost consistent with the studies done in Sao Paulo in Brazil which reported prevalence of Pancoronavirus to be (11.5%) (Cabeça *et al*, 2013) [4]. Previous studies conducted among pilgrims of 2015 and immunocompromised children revealed lower percentages of Pancoronaviruses (7% & 5.7%, respectively) compared to our study with no detection of MERS-CoV (Burel, Gerna *et al*) [5,6]. Other studies showed higher percentage of Pancoronaviruses than our results like the study conducted by Moës and his colleagues (25%) [7]. Mahony *et al* in 2005 reported lower prevalence of Pancoronavirus. The variation in the rate of Pancoronavirus infection is due to the different study groups (Mahony) [8].

In this study the percentage of MERS-CoV was insignificantly higher, higher in females than males (84.3% vs 82.9%). In disagreement with our results is the WHO report which stated that males are more infected with MERS-CoV than females. Assiri *et al* 2013 also reported higher rate of MERS-CoV among males than females.

Phylogenetic analysis of MERS-CoV up-E revealed that the Sudanese strains of MERS-CoV detected in both groups (Al-Shaab Hospital & Khartoum Airport) were very close to each other's.

The MERS-CoV strains detected in Khartoum Airport individuals were close to MERS-CoV strains of KSA, while the Al-Shaab Hospital strains were close to Thailand MERS-CoV strains. The similarity in MERS-CoV strains of Sudan, KSA and Thailand can be explained by movement of citizens between these countries for many reasons, such as education, pilgrimage, trade and other activities.

Some of our isolate after sequencing where registered in the gene bank (N.o: KY794400.1-KY794403.1 and MF133448-MF133457).

6. Conclusion

The results of this study showed high prevalence of MERS-CoV, despite this it is not paid attention to MERS-CoV by the health care providers and therefore misdiagnosed. There is considerable percentage of Pancoronavirus. More in-depth studies are recommended.

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