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RESEARCH ARTICLE

Oral bacteria detection among children with cancer in a tertiary teaching hospital in Kuala Lumpur, Malaysia

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ABSTRACT

This study sought to determine the prevalence of pathogenic and non-pathogenic bacteria in the oral cavities of children with cancer. There were 68 paediatric patients with cancer who were included in this study. Oral swab samples from the dorsum of tongues and mouth floors of these patients were subjected to culture, staining, and molecular methods to detect the bacteria. The overall prevalence of gram-positive and gram-negative bacteria was 79.4% (54/68; 95% CI = 68.4 – 87.3) and 25% (17/68; 95% CI = 16.2 – 36.4), respectively. *Streptococcus salivarius* and *Streptococcus parasanguinis* were the predominant pathogenic gram-positive bacteria, while *Neisseria subflava* and *Neisseria perflava* were the most common pathogenic gram-negative bacteria. The results revealed that the number of bacteria isolates recovered in patients receiving cancer treatment was higher (55.9%) than those who had not received treatment (16.2%). Therefore, more isolated pathogenic bacteria were observed post-therapy (54.4%). Pathogenic organisms can have significant implications on patient health. Awareness of the types of bacteria inhabiting the oral cavity is essential to predict and prevent dental problems, and their associated systemic complications. Findings on the diversity of oral microflora can also provide a better understanding of the aetiology of oral diseases in paediatric patients receiving cancer treatment.

Keywords: Oral bacteria; paediatric; cancer; gram-staining; molecular method.

INTRODUCTION

The oral cavity is the second most complex microbiota in the body after the gut (Verma *et al*., 2018; Zhang *et al*., 2018; Kitamoto *et al*., 2020). Nearly 800 microbial species have been identified, mainly bacteria, fungi, viruses, and parasites (Sampaio-Maia *et al*., 2016; Verma *et al*., 2018; Zhang *et al*., 2018). In healthy individuals, these microbial communities serve as commensals that can influence the host immune system to maintain homeostasis (Mira *et al*., 2017). However, if the balance is disrupted, such as in the immuno-compromised host, the oral cavity will be colonized by potentially pathogenic species, leading to various diseases (Idris *et al*., 2017). Some of these pathogens may be responsible for life-threatening infections.

Despite significant advances in treatment and supportive care, cancer predisposes patients to serious infections (Villafuerte *et al*., 2018; Daugëlaitë *et al*., 2019; Nivoix *et al*., 2020). Cancer itself or chemotherapy can disrupt the intricate balance between commensal bacteria and the host defence mechanism, resulting in the spread of potentially life-threatening infections leading to ruptures in the oral mucosal tissues (Villafuerte *et al*., 2018; Bunetel *et al*., 2019), resulting in severe oral mucositis (Hong *et al*., 2019), oral candidiasis (Bertolini & Dongari-Bagtzoglou, 2019), gingivitis (Curtis *et al*., 2020), viral mucosal eruptions, and cellulitis (Crescente *et al*., 2018; Miranda-Silva *et al*., 2020). The oral microflora may subsequently be replaced by potentially pathogenic organisms like *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida* species (Kong *et al*., 2015; Bunetel *et al*., 2019).

Within the oral cavity, bacterial populations result from the dynamic relationship between pathogens and commensals (Bowen *et al*., 2018). Oral bacteria have been implicated in causing bacterial endocarditis (Abranches *et al*., 2018), aspiration pneumonia (Maraki & Papadakis, 2015), osteomyelitis in children (Castellazzi *et al*., 2016), preterm low birth weight (Ye *et al*., 2020), coronary heart disease (Liu *et al*., 2020; Priyamvara et *al*., 2020), and cerebral infarction (Patrakka *et al*., 2019). The incidence of bacteremia following dental procedures has been well documented (Cahill *et al*., 2017; Fernández *et al*., 2018). Oral mucositis has been

identified in several studies as an independent risk factor for bacteraemia and systemic infections, involving anaerobic bacteria and other species generally not found in the oral cavity (Lecomte *et al*., 2017).

Knowledge of the type of bacteria that inhabit the oral cavity is essential in predicting and preventing dental diseases and the associated systemic complications caused by them (Chimenos-Küstner *et al*., 2017; Graves *et al*., 2019). It is known that opportunistic pathogens are widespread among patients with cancer. However, knowledge of the micro-organisms and their associations with clinical manifestation is scarce. Therefore, this study was carried out to determine the prevalence of oral bacteria present in the oral cavities of children with cancer. The molecular approach to determine specific species levels will aid in more accurate diagnosis and treatment. In addition, such information will provide invaluable insights into the epidemiology of these infections, thus facilitating the understanding of the clinical diseases, diagnoses, and management.

MATERIALS AND METHODS

Study Design and Participants

This study was approved by the Medical Ethics Committee of the University of Malaya Medical Centre (MREC ID NO: 2019528-7454). Convenience sampling was conducted in the Paediatric Oncology Unit, University of Malaya Medical Centre from August 2019 to September 2020. Participants were between the ages of 1 year to 18 years and diagnosed with cancer. They were recruited from the oncology ward and from the paediatric oncology daycare unit. They had not taken antibiotics in the previous month. Written consent was obtained from parents or relatives after verbal information was provided. The participants were divided into two groups: (1) patients who had not received cancer therapy (chemo-therapy or radiotherapy), and (2) patients who were on cancer therapy. Demographic information such as age, gender, diagnosis, and date of cancer therapy was retrieved from their hospital records. All information obtained in the study was kept confidential and accessible only to researchers involved in this study.

Sample Collection and Staining

Samples were collected by gently rubbing a sterile cotton swab on the dorsum of the tongues and the floor of the mouths using Stuart with charcoal media Transport Swab (Labchem, Malaysia). The swabs were sent to the Emerging Pathogen Laboratory 2, Department of Parasitology, Faculty of Medicine, University of Malaya, within 2 hours of collection. The swabbed samples were plated on Columbia blood agar with 5% sheep blood (Isolab, Malaysia) and incubated aerobically at 37°C for 3-5 days. Distinct colonies (2-3 colonies of apparently similar morphology) were selected from mixed growth and streaked separately on a new blood agar plate until a pure culture was obtained. Single colonies obtained from the pure culture were characterized grossly by colour and shape, and by microscopy.

Gram staining was performed on a single colony from the pure culture using a gram-stain kit according to the manufacturer's instructions (Condalab, Madrid, Spain). A single colony smeared on sterile distilled water was fixed on a glass slide by flaming briefly. Next, the smears were stained for 1 minute with crystal violet and iodine solutions, briefly rinsed with a decolourizer for 10 seconds, and stained with safranin solution. Each step was accompanied by washing with sterile water. The slides were air-dried and observed under a light microscope at high magnification (x100) with immersion oil. The bacteria colonies were graded as gram-positive if they were purplish-blue in colour or gram-negative when a red colour was observed.

DNA Extraction

Based on the manufacturer's instructions, the genomic DNA from the pure cultures were extracted using GF-1 spin Bacterial DNA extraction mini kit (Vivantis Technologies, Malaysia). Approximately 10 to 15 colonies selected from the pure cultures were re-suspended in buffer R1 and incubated at 37°C for 20 min with a cell lysis agent. The pellet obtained was subjected to protein denaturation and homogenization for complete cell lysis. The DNA was eluted with an elution buffer to a final volume of 50µL. The extracted DNA was stored at -20°C until required for amplification by Polymerase Chain Reaction (PCR).

Amplification of 16S rDNA by PCR

16S rDNA genes were amplified using a set of universal primers: 27f: 5'-AGA GTT TGA TCA TGG CTC AG and 149r: 5'-TAC GGC TAC CTT GTT ACG ACT T (Philip *et al*., 2009). The PCR was performed in a 20 µL reaction mixture containing 10 µL of the master mix (Genet Bio, South Korea), 2 µL each of forward and reverse primers, 4 µL of distilled water, and 2 µL of the DNA template. Both negative (reagent mixture without template DNA) and positive controls were included in each PCR set. The amplification steps were initially denatured at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 40 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified DNA was analysed by gel electrophoresis in 1.5% (w/v) agarose gel stained with SYBR® Safe DNA (Invitrogen, Auckland, New Zealand) to visualize the amplified PCR product under UV illumination.

The PCR product was subjected to DNA commercial sequencing. Sequence chromatograms were viewed using Sequence Scanner version 1.0 program (Applied Biosystems, Waltham, MA, USA). Both forward and reverse nucleotide sequences were manually aligned using the BioEdit Sequence Alignment Editor version 7.0.9 program. Sequences were blasted to the National Centre for Biotechnology Information (NCBI) databases using Basic Local Alignment Search Tool (BLAST). The bacterial species were identified based on 98% similarity to the 16S RNA sequence database. Cross-reference was also made to the Human Oral Micro-biome Database (HOMD).

Statistical Analyses

Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 25.0, SPSS Inc, Chicago, III, USA). Categorical variables were presented as frequency (percentage) and 95% confidence interval (95% CI). Where appropriate, a Chi-square test or Fisher's exact test was performed to determine any differences among the groups. A *p*-value of less than 0.05 was considered significant.

RESULTS

There were 68 individuals recruited for this study. There were 41 (60.3%) male and 27 (39.7%) females, respectively. Forty seven (69.1%) were Malay, 19 (28%) Chinese, and 2 (2.9%) Indian (Table 1). Median age was 7 years (range: 1 year to 18 years). Of the 64 patients with complete datasets, most were diagnosed with acute leukaemia (66%). Eight (11.8%) had bone cancer, 7 (10.3%) had central nervous system malignancies, 3 had germ cell tumours and lymphoma (4.4%),

2 (2.9%) had Langerhans cell histiocytosis, and 1 (1.5%) had nasopharyngeal carcinoma. Of the 56 patients with complete datasets, the majority were actively undergoing cancer therapy (n=43, 63.2%), while 13 (19.1%) were newly-diagnosed patients who had not received cancer treatment.

The gram staining showed that the overall prevalence of gram-positive and gram-negative bacteria was 79.4% (54/68; 95% CI = 68.4 – 87.3) and 25% (17/68; 95% CI = 16.2 – 36.4), respectively. Table 2 shows the prevalence of gram-positive and gram-negative aerobic bacteria from the oral cavities of paediatric patients based on pathogenicity. Gram-positive bacteria was most common (57.4%; 95% CI = 45.5 – 68.4) followed by both gram-positive and gram-negative aerobic bacteria (22.1%; 95% CI = 13.9 – 33.3) and gram-negative bacteria alone (2.9%; 95% CI = 0.8 – 10.1). A total of 51 (75%; 95% CI = 63.6 – 83.8) isolates were reported as pathogenic bacteria. Of these, 55.9% (95% CI = 44.1 – 67.1) were identified as gram-positive while 2.9% (95% CI = 0.8 – 10.1) were gram-negative. Both gram-positive and negative bacteria were observed in 20.6% (95% CI = 12.7 – 31.6). Meanwhile, 7.4% (95% CI = 3.2 – 16.1) isolates were recorded as non-pathogenic species. Of these, 1.5% (95% CI = 0.3 – 7.9) were identified as gram-positive while both gram-positive and negative bacteria were observed in 1.5% (95% CI = 0.3 – 7.9) of these patients.

A total of 102 bacterial isolates were recovered from the 58 paediatric patients in this study (Table 3). Of these, 58 (56.9%; 95% CI = 47.1 – 66.1) were gram-positive and 2 (2%; 95% CI = 0.5 – 6.9) were gram-negative bacteria. Additionally,

Table 1. Demographic characteristics of participants (N=68)

Characteristics	n (%)
Gender	
Male	41 (60.3)
Female	27 (39.7)
Age in years (median range)	7 (1-18)
Age Group (Years)	
1-6	30 (44.1)
7-12	24 (35.3)
13-18	14 (20.6)
Race	
Malay	47 (69.1)
Chinese	19 (28)
Indian	2 (2.9)
Type of Cancer[a]	
Leukaemia	40 (66)
Bone	8 (11.8)
Central Nervous System	7 (10.3)
Germ Cell	3 (4.4)
Lymphoma	3 (4.4)
Langerhans Cell Histiocytosis	2 (2.9)
Nasopharynx	1 (1.5)
Cancer Treatment[b]	
Before treatment	13 (19.1)
After treatment	43 (63.2)

N=Total number of participants; n=Total number of participants per variable. A total of 52 and 45 patient information according to cancer type ([a]) and therapy ([b]), respectively was used for the analysis.

Table 3. Prevalence of aerobic bacterial species in the oral cavity of paediatric patients

Bacterial species	n (%)	95% CI
Gram-positive		
Pathogenic		
Streptococcus salivarius	20 (19.6)	13.1 – 28.4
Streptococcus parasanguinis	11 (10.8)	6.1 – 18.3
Rothia mucilaginosa	4 (3.9)	1.5 – 9.7
Staphylococcus aureus	4 (3.9)	1.5 – 9.7
Streptococcus australis	4 (3.9)	1.5 – 9.7
Streptococcus mitis	4 (3.9)	1.5 – 9.7
Actinomyces oris	2 (2)	0.5 – 6.9
Streptococcus gordonii	2 (2)	0.5 – 6.9
Corynebacterium argentoratense	1 (1)	0.2 – 5.3
Corynebacterium durum	1 (1)	0.2 – 5.3
Streptococcus anginosus	1 (1)	0.2 – 5.3
Streptococcus cristatus	1 (1)	0.2 – 5.3
Streptococcus infantis	1 (1)	0.2 – 5.3
Streptococcus oralis	1 (1)	0.2 – 5.3
Non-pathogenic		
Streptococcus sanguinis	1 (1)	0.2 – 5.3
Overall	**58 (56.9)**	**47.1 – 66.1**
Gram-negative		
Pathogenic		
Acinetobacter baumannii	1 (1)	0.2 – 5.3
Neisseria subflava	1 (1)	0.2 – 5.3
Overall	**2 (2)**	**0.5 – 6.9**
Both		
Pathogenic		
Streptococcus salivarius	11 (10.8)	6.1 – 18.3
Streptococcus parasanguinis	5 (4.9)	2.1 – 11
Neisseria subflava	4 (3.9)	1.5 – 9.7
Neisseria perflava	4 (3.9)	1.5 – 9.7
Neisseria mucosa	3 (2.9)	1.0 – 8.3
Streptococcus infantis	3 (2.9)	1.0 – 8.3
Neisseria flava	2 (2)	0.5 – 6.9
Acinetobacter baumannii	2 (2)	0.5 – 6.9
Streptococcus mitis	2 (2)	0.5 - 6.9
Actinomyces oris	1 (1)	0.2 – 5.3
Actinomyces naeslundii	1 (1)	0.2 – 5.3
Klebsiella pneumoniae	1 (1)	0.2 – 5.3
Rothia aeria	1 (1)	0.2 – 5.3
Rothia mucilaginosa	1 (1)	0.2 – 5.3
Non-pathogenic		
Streptococcus sanguinis	1 (1)	0.2 – 5.3
Overall	**42 (41.2)**	**32.1 – 50.9**

n = Total number of patients per bacterial species.

Table 2. Prevalence of gram-positive and gram-negative bacteria isolated from the oral cavity of paediatric patients

	Pathogenic		Non-pathogenic		Total	
	n (%)	95% CI	n (%)	95% CI	N (%)	95% CI
Gram-positive	38 (55.9)	44.1 – 67.1	1 (1.5)	0.3 – 7.9	39 (57.4)	45.5 – 68.4
Gram-negative	2 (2.9)	0.8 – 10.1	0	–	2 (2.9)	0.8 – 10.1
Both	14 (20.6)	12.7 – 31.6	1 (1.5)	0.3 – 7.9	15 (22.1)	13.9 – 33.3
Total	51 (75)	63.6 – 83.8	5 (7.4)	3.2 – 16.1	56 (82.4)	71.6 – 89.6

N=Total number of participants; n=Total number of participants per variable.

Table 4. Distribution of gram-positive and gram-negative bacteria based on the demographic characteristic (N = 58)

Characteristics	Overall (%)	Gram-positive		Gram-negative		Both	
		n (%)	P-value	n (%)	P-value	n (%)	P-value
Gender							
Male	33 (48.5%)	21 (30.9)	0.24	2 (2.9)	0.507	10 (14.7)	0.48
Female	23 (33.8%)	18 (26.5)		–		5 (7.4)	
Age Group (Years)							
Less than 7	31 (45.6)	17 (25.0)	0.007*	2 (2.9)	0.497	12 (17.6)	0.03*
More than 7	25 (36.8)	22 (32.4)		–		3 (4.4)	
Race							
Malay	41 (60.3%)	30 (44.1)	0.23	2 (3.4)	0.660	8 (11.8)	0.11
Chinese	15 (22.1%)	8 (11.8)		–		7 (10.3)	
Indian	2 (2.9%)	1 (1.5)		–		–	
Type of Cancer							
Leukaemia	34 (50.0)	22 (32.4)	0.15	2 (2.9)	0.981	10 (14.7)	0.13
Bone	7 (10.3)	6 (8.8)		–		1 (1.5)	
CNS	4 (5.9)	3 (4.4)		–		1 (1.5)	
Germ Cell	3 (4.4)	3 (4.4)		–		–	
Lymphoma	1 (1.5)	0		–		1 (1.5)	
Langerhans Cell Histiocytosis	2 (2.9)	0		–		2 (2.9)	
Nasopharynx	1 (1.5)	1 (1.5)		–		–	
Cancer Treatment							
Before therapy	11 (16.2)	6 (8.8)	0.022*	1 (1.5)	0.402	4 (5.9)	0.71
After therapy	38 (55.9)	27 (39.7)		1 (1.5)		10 (14.7)	

N=Total number of bacterial isolates; n=Total number of bacterial isolates per variable.
* Significant difference, $p < 0.05$, based on Chi Square Test.

42 (41.2%; 95% CI = 32.1 – 50.9) had other bacteria species isolated. As seen in Table 3, the *Streptococci* and *Neisseria* species were the most frequently isolated gram-positive and gram-negative bacteria, respectively. Of the pathogenic gram-positive bacteria, *Staphylococcus aureus* (2%; 95% CI = 0.5 – 6.9) was reported among these patients while *Klebsiella pneumoniae* (1%; 95% CI = 0.2 – 5.3) was detected in the pathogenic gram-negative bacteria. Table 3 also provides details on other bacterial species reported in this study.

Based on age groups, there was a significant difference between patients above 7 years (32.4%) infected with gram-positive bacteria compared to those who were younger (25%) ($p = 0.007$) (Table 4). Additionally, 12 (17.6%) of the patients below the age of 7 years had both gram-positive and gram-negative bacteria compared to 3 (4.4%) in the older age group ($p = 0.03$). In general, the number of bacteria isolates recovered were significantly higher in patients who had received cancer treatment (55.9%) compared to those who had not (16.2%) ($p < 0.05$). Similarly, the number of patients with gram-positive bacteria were significantly higher among those receiving treatment (39.7%) compared to those before treatment (8.8%) ($p = 0.022$). In addition, more bacteria with both types of gram stains were observed among patients receiving cancer treatment (14.7%). *Streptococcus salivarius* and *Streptococcus parasanguinis* had the highest prevalence (22.5% vs 3.9% and 9.8% vs 3.9%, respectively) in patients who had received cancer therapy than those who had not received treatment. Furthermore, other bacterial species such as *Corynebacterium durum, Rothia mucilaginosa, S. aureus, Acinetobacter baumannii, Klebsiella pneumoniae*, and viridans streptococci like *Streptococcus anginosus, Streptococcus australis, Streptococcus cristatus, Streptococcus gordonii, Streptococcus infantis*, and *Streptococcus oralis* were more common in those who had cancer therapy (data not shown).

DISCUSSION

This study describes the prevalence of bacteria in the oral cavities of children with cancer. Gram-positive bacteria were most common, with viridans streptococci being the most frequently isolated organism. Similar findings were reported by Sixou *et al*. (1998). However, another study found that gram-negative bacteria were commonly isolated (52.6%), followed by gram-positive bacteria (45%) (Tang *et al*., 2020). Furthermore, among paediatric patients with acute leukaemia gram-negative bacteria, gram-positive bacteria, and fungi constituted 56.3%, 42.3%, and 2.4% of oral cavity isolates, respectively (Kuo *et al*., 2017). Major gram-negative bacteria observed included *K. pneumonia*, *Pseudomonas aeruginosa*, and *Eschericia coli* (Kuo *et al*., 2017).

A study in Malaysia noted that in adults receiving chemotherapy with febrile neutropenia, 60% of the bacteria isolated was the gram-negative *Enterobacteriaceae* (Baskaran *et al.,* 2007). In contrast, a study in Lebanon noted that gram-negative bacteria were responsible for 78.8% (22/33) of bloodstream infections compared to 33.3% (11/33) caused by gram-positive bacteria (Kanafani *et al*., 2007). The study reported that the dominant gram-negative bacteria were *P. aeruginosa* and *E. coli.* A possible explanation for these findings was the relatively low proportion of indwelling catheters (Raad *et al*., 2007). The different predominant bacteria reported in children compared to adults emphasize variations in the oral flora between them. It is important to note that the paediatric oral flora is more diverse than in adults (Villafuerte *et al*., 2018). Their response to treatment regimens may explain the differences in the quality of their oral microflora.

We observed that there were more bacterial species reported in the post-cancer therapy group than in the pre-cancer therapy group. The isolation count for opportunistic bacteria among those receiving cancer therapy was higher compared to pre-cancer treatment. Pathogenic bacteria such as *S. aureus* and *K. pneumoniae* were also isolated in this study in patients who had received cancer therapy. However, our results revealed no significant change in the isolation frequency of aerobic bacteria before and after receiving cancer therapies. The varying bacterial counts between the two groups may be due to the relative differences in the number of patients. A study on the oral microflora within dental plaques of patients with cancer reported that 57% had gram-positive cocci, whilst 43% of the bacterial flora were periodontal pathogenic species (Vozza *et al*., 2015). In contrast, Napeñas *et al*. (2007) reported no clear pattern regarding quantitative and qualitative oral flora changes among patients with cancer. Their study found that the most frequent gram-negative bacteria were from the *Enterobacteriaceae* family, *Pseudomonas* sp. and *E. coli.* Meanwhile, the most common gram-positive species isolated were *Staphylococcus* sp. and *Streptococcus* sp. (Joel *et al*., 2007).

S. salivarius and *S. parasanguinis* were the predominant bacterial isolates post-cancer therapy in our study. Similar findings were reported in a mutant and non-mutant streptococci survey in patients receiving radiotherapy (Tong *et al*., 2003). The study reported *S. mitis, S. salivarius,* and Lactobacilli as predominant isolates post-irradiation, with decreased *S. sanguinis* counts. A possible explanation is the increased acidic oral environment due to reduced salivary flow (Schubert & Izutsu, 1987) thus altering normal oral microflora. Lactobacilli are known to grow well in acidic environment. Hence, a change in the environment may cause aciduric bacteria to survive and inhibit non-aciduric bacteria such as *S. sanguinis* (Schubert & Izutsu, 1987). Therefore, the higher abundance of *S. mitis* and *S. salivarius* after radiotherapy would suggest a more acidified oral environment, increasing the risks of developing caries (Schubert & Izutsu, 1987).

We acknowledged several limitations of this study. Firstly, the exclusion of anaerobic bacteria may preclude a better understanding of the oral microflora present in immunocompromised patients. Secondly, at the time of sampling, oral bacteria may have undergone selection after disease manifestation, with some bacteria inhibited and others thriving, resulting in divergent conclusions in various studies (Wang *et al*., 2014). While the traditional culture method is less costly, it is recommended that all cultivable bacteria be included in the analysis to fully understand the patterns of oral microflora and how they affect one another. Furthermore, there were lack of certain clinical information, such as the types of cytotoxic drugs used, periodontal examination, and other predisposing factors. Such data would be essential to determine how the micro-organisms can affect the clinical condition of children with cancer.

CONCLUSION

This study demonstrated the species of pathogenic bacteria present in the oral cavities of paediatric patients with cancer. These bacteria can affect the diversity of oral microflora and may result in systemic infectious diseases. The presence of non-pathogenic bacteria may be beneficial in maintaining species balance in the oral cavity. The findings from this study can facilitate the better understanding of oral diseases in children with cancer before and after treatment.

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Conflict of Interest

All authors have no conflict of interest concerning the work reported in this paper.

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