CYSTEINE CHALLENGE TEST AS A NOVEL DIAGNOSTIC TOOL TO DISTINGUISH ORAL HALITOSIS

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Running title : Is this case oral or non-oral halitosis?
Key findings: Upper limit for halitosis and cysteine-induced H_2S level can be used to distinguish oral halitosis.

ABSTRACT

Background:
The cysteine challenge test is often used to check the H_2S production capacity of the mouth. Patients with oral halitosis group (n=305) or non-oral halitosis group (n=191) and healthy individuals (control group, n=102) were compared with each other to identify any possible relationship between initial and cysteine-induced oral H_2S concentrations.

Subjects and Method:
Medical records of 598 participants were reviewed retrospectively. Oral H_2S concentrations before (pre-CR) and after cysteine rinse (post-CR) with 5 ml of 20 mmol *L*-cysteine solution for 30 seconds were compared.

Results:
Pre-CR H_2S concentrations were >0.8ppm in 75.1% of oral group patients but less than <0.8 ppm in 87.3% of non-oral group and 86.9% of controls. After cysteine rinse, oral H_2S concentrations exceeded 12 ppm in 72% of oral halitosis patients but were lower in 88% of non-oral group and 99% of controls. While post-CR/pre-CR ratio was >12 in 74.5% of oral group, it was <12 in 81.7% of non-oral group and 83.4% of controls.

Conclusion:
Cysteine challenge test can be used as a diagnostic tool to identify an individual's tendency to produce oral malodor, not only to quantify momentary halitosis level.

Keywords: Halitosis, differential diagnosis, oral malodor, bad breath, hydrogen sulfide.

INTRODUCTION

About 80–90% of halitosis cases fall into the category of oral halitosis which originates from the oral cavity and are due to oral bacteria producing volatiles by breaking down substrates such as amino acids.[1] Primary substance associated with oral malodor in mouth air is composed by volatile sulfurs, mostly consist of hydrogen sulfide (H_2S), organic or nitrogen-based gases. To detect sulfurs in the mouth cavity is used for diagnostic purposes, especially H_2S is thought enough representative for existing halitosis. (Thrane PS, 2010)

Halitosis does not consist of only oral halitosis and also includes airway (type 2), gastroesophageal (type 3), blood-borne (type 4) and subjective (type 5) halitosis, which are distinguishable.[2] All types of halitosis patients first visit dental practitioners with a complaint of oral malodor. Dental practitioners should distinguish oral (type 1) halitosis from other types of halitosis. In particular, clinical features of psychogenic forms of subjective halitosis closely mimic those of oral halitosis and result in loss of dental practitioners' time because such cases can be treated by psychiatrists only.[3] Differential diagnostic protocols are needed to quickly eliminate such patients or even avoid misdiagnosis. There is no specific protocol to distinguish between oral and non-oral halitosis patients.

Kleinberg (2002) has first published the cysteine challenge test for halitosis examination. Oral bacteria act on cysteine substrates, releasing hydrogen sulfide (H_2S) in the oral cavity. H_2S concentration largely depends on cysteine concentration, cysteinase activity of oral microbiota,[4] and ecology or H_2S production capacity of the mouth. Consequently, the H_2S peak after cysteine rinse reflects an individual's oral halitosis production capacity.[5,6]

Pathologic halitosis is usually diagnosed based on the lower threshold; however, upper threshold has not been evaluated as a diagnostic tool to date.

Quantitative measures related to 1) initial, 2) cysteine-induced H_2S concentrations (upper threshold), and 3) increase ratio in H_2S concentrations are needed to discriminate oral halitosis patients from non-oral halitosis patients. In this study, mathematical

relationships between oral H_2S concentrations before and after cysteine rinse were analyzed in oral and non-oral halitosis patients in comparison to healthy subjects with the aim to define these parameters.

MATERIALS AND METHODS

Patient selection

A total of 598 subjects who presented to the Halitorium® clinic between 01.01.2018 and 01.01.2021 were included in this study and divided into 3 groups. The oral group comprised 305 patients (mean age 32 years; range, 21-65; 151 females) with oral halitosis. The non-oral group consisted of 191 patients (mean age 37 years; range, 19-66; 102 females) with non-oral halitosis. The control group consisted of 102 volunteers (mean age 35 years; range, 23-44; 61 females) without a complaint of halitosis. Computerized health records of the participants were reviewed retrospectively.

Diagnostic criteria

Self-report or feedback from other persons in social settings[8] or periodontal health scores[9] are the most valuable diagnostic criteria for halitosis. The participants were requested to score their own halitosis on a 5-point scale with anchors of 0 (no odor whatsoever) and 5 (extremely foul odor). The scores for each participant were noted as their halitosis level (HL). All participants were scored for gingival health of all tooth surfaces (including third molars) by the plaque index (PI) and gingival index (GI).

Gingival index (GI) (Loe and Silness 1963) was determined and recorded at 4 gingival sites per tooth using the following criteria: (0) normal gingiva, (1) mild gingivitis without bleeding on probing, (2) moderate gingivitis with bleeding on probing, and (3) severe gingivitis with ulceration and spontaneous bleeding. The sum of the scores from the four

areas of each tooth was divided by 4 to derive the gingival index for that tooth. The mean GI score was obtained after calculating individual GI.

Periodontal examinations were performed by a single examiner for all study subjects. Pocket depth measurements were obtained by using William's periodontal probe. The Pocket depth was measured from the free gingival margin to the base of the pocket. The probe was maintained parallel to the long axis of the tooth. Pocket depth and clinical attachment level were measured for each tooth at 6 sites, namely, mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual. Clinical attachment level was determined by measuring the distance from the cement enamel junction to the gingival margin with a William's periodontal probe. Average probing pocket depth measurements were recorded.

The tongue was imaginatively divided into six sections. Three in the anterior, three in the posterior part of the tongue. Each sextant was categorized by scoring. (Winkel EG, 2008) The resulting WTCI was obtained.

Patients meeting more than 3 of the following criteria were included in the oral group:

1- Suffering from halitosis for at least 2 months,

2- Complaints from the individual’s social environment about his/her oral malodor,

3- Having radiographic evidence of alveolar bone loss,

4- Bleeding from all sites upon probing,

5- Having an average pocket depth of > 6 mm,

6- An HL score >3,

7- Severe caries, severe gingivitis or periodontitis,

8- A tongue coating score >3/6.

Orthopantomograph was taken from each individual. Radiologically and periodontally healthy patients complaining of halitosis (HL>3) were included in the non-oral halitosis group. Radiologically and periodontally healthy subjects without halitosis complaint (HL=0) were enrolled in the control group.

Examination protocol

Prior to the examination, data were recorded for each patient individually including age, sex, medical history, tobacco/alcohol use, and recent medications. Subjects were excluded if they had taken antibiotics or other antimicrobial therapy within 2 weeks prior to the examination, were pregnant, had any malignancy, current menses, or were using alcohol. All measurements were performed in the morning between 08:30 and 11:30 (before lunch) and at least 4 hours after eating or drinking. All patients signed written informed consent in accordance with the World Medical Association Declaration of Helsinki before examination. but no ethics approval needed to calculate patient records.

Each participant's initial oral gas profile was determined by a portable multi-gas detector (IBRID® MX6 C526R311, Industrial Scientific, PA, USA) using a previously described method.[7]

For each subject, baseline organic, NH_3, SO_2, H_2S, H_2 gas levels in oral air were used as individualized control data. Then, the cysteine challenge test was carried out as follows: 5 ml of 20 mM (2.43 g/L) aqueous *L*-Cysteine solution (#1.02839.0100, Merck) was placed in the mouth and held in contact with the dorsal part of the tongue for 30 seconds to generate H_2S in the mouth to challenge oral halitosis.

After 3 minutes, emerging oral odor (gas profile) in the mouth was measured and recorded for these 5 gases separately. Only H_2S concentrations were evaluated; no other gases were included in the study due to their decreased production by cysteine rinse.[5,6]

Two oral H_2S concentrations were picked from each patient's records: 1) initially measured concentration, i.e., pre-cysteine rinse (Pre-CR), 2) oral H_2S peak after cysteine rinse, i.e. post-cysteine rinse (Post-CR).

Statistical analysis:

The MedCalc v19.8 software program (MedCalc Belgium) was used for statistical analysis. Data were tested for normal distribution using the Shapiro-Wilk test. One-way ANOVA was performed to compare statistical differences among the mean values of the groups. Multiple comparisons were conducted with Tukey's or Tamhane's T2 post hoc test due to non-homogeneous variances. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

No significant correlation was found between raw data of pre-CR and post-CR values in all groups. However, a significant correlation was observed between pre-CR values and their corresponding post-CR values averaged (mean post-CR/pre-CR) (r=0.97) among all groups ($p<0.001$; $F=51,419$).

In all groups, 20 mM cysteine solution significantly increased H_2S concentration in the mouth. Baseline median H_2S concentration of oral group was found two fold higher than non-oral group where as no statistical difference was found between baseline values of non-oral and control groups. In accordance with this, after cysteine rinse, median value of H_2S concentrations in oral halitosis patients was found two fold higher than non-oral halitosis patients and control group individuals. On the other hand, maximum H_2S levels of oral halitosis group was found 1.6 fold higher than non-oral group, 35.3 fold higher than control group individuals. (Table.1)

Pre-CR oral H_2S concentrations versus post-CR H_2S concentrations for each group are shown in Figure 1.

The mathematical relationship between the pre-CR and mean of post-CR/pre-CR oral H_2S concentrations is depicted and curve fitted (Figure 2). The difference between means of pre-CR H2S concentrations of the non-oral and control group was not statistically significant ($p=0.974$).

Pre-CR values dispersed in a narrow spectrum for non-oral (from 0.4 to 1.3 ppm) and control group (between 0.4 and 1 ppm). Mean pre-CR H_2S value of oral halitosis patients was 1.71 ±1.76 ppm, whereas this value scattered around 0.7 ppm in individuals without oral halitosis (0.67 ±0.18 and 0.66 ± 0.17 ppm in the non-oral and control groups respectively). However, while pre-CR was higher than 0.8 ppm in the majority (75.1%) of oral patients, a considerable percentage (87.3%) of non-oral group and 86.9% of controls showed lower values (Figure 3)

Post-CR H_2S concentrations were above 12 ppm in 72% of oral group but 88% of non-oral group and 99% of controls exhibited lower values.

Post-CR / Pre-CR ratio was greater than 12 in 74.5% of oral group, whereas it was lower than 12 in 81.7% of non-oral group, and 83.4% of control group (Fig.3- A,B,C)

Most frequently detected pre-and post-CR H_2S concentrations are listed in Table 2.

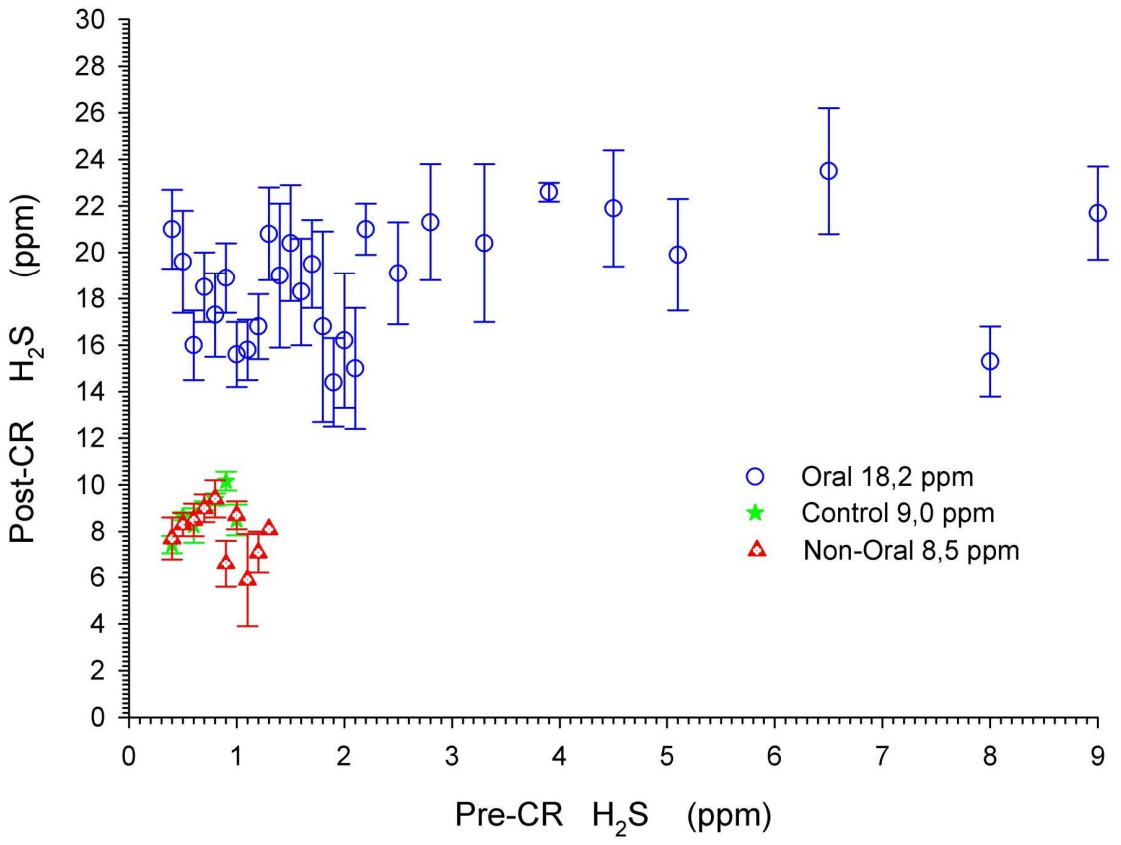

Fig. 1 *Changes in oral H_2S gas concentration values after 20 mM cysteine rinse for 30 seconds. Pre-CR values represent the initial value of H_2S in the mouth of oral and non-oral halitosis patients, and control subjects. Each data point represents the mean ± standard error of the mean (SEM).*

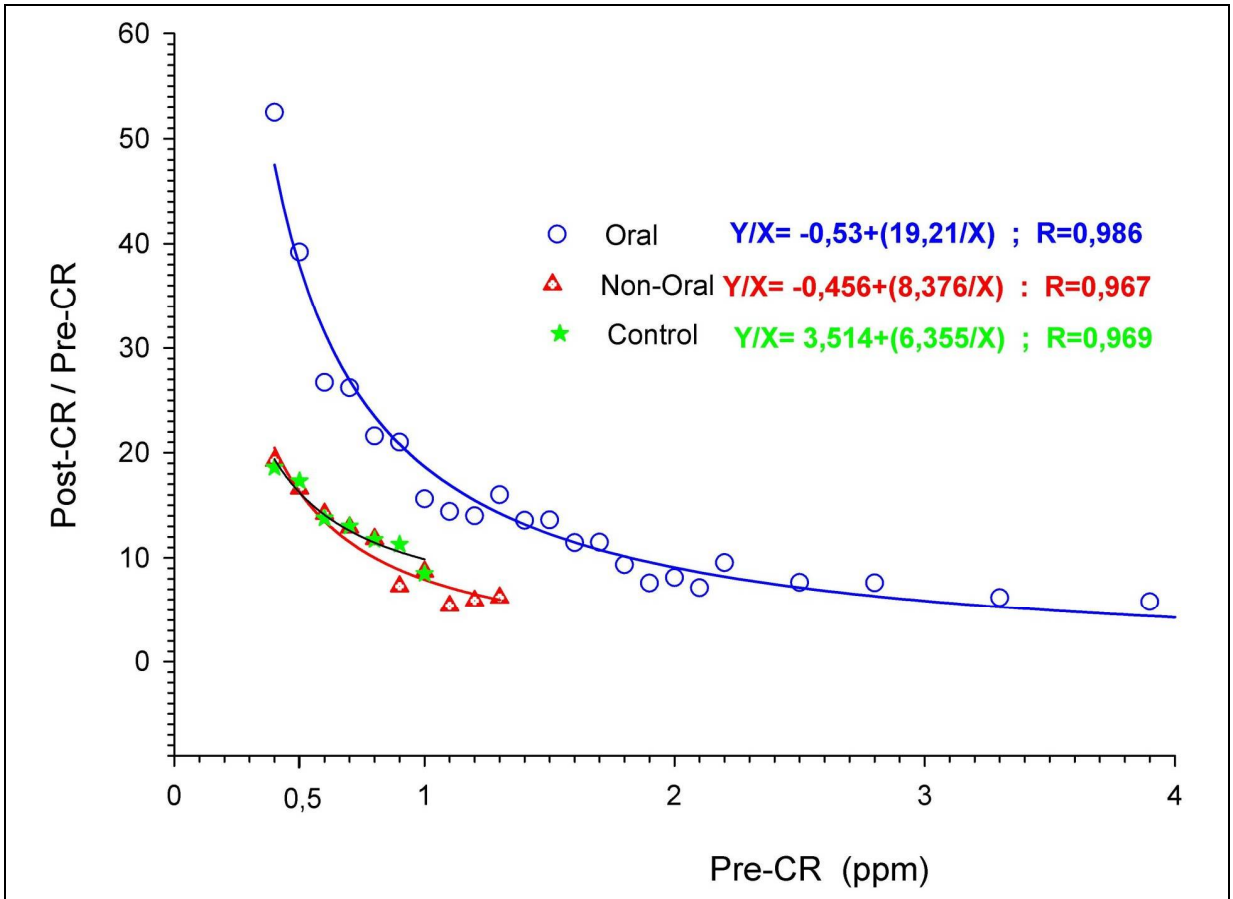

Fig.2 *Relation between pre-CR and mean post-CR/pre-CR oral H_2S gas concentrations is shown. Each data point represents the mean ± standard error of the mean (SEM).*

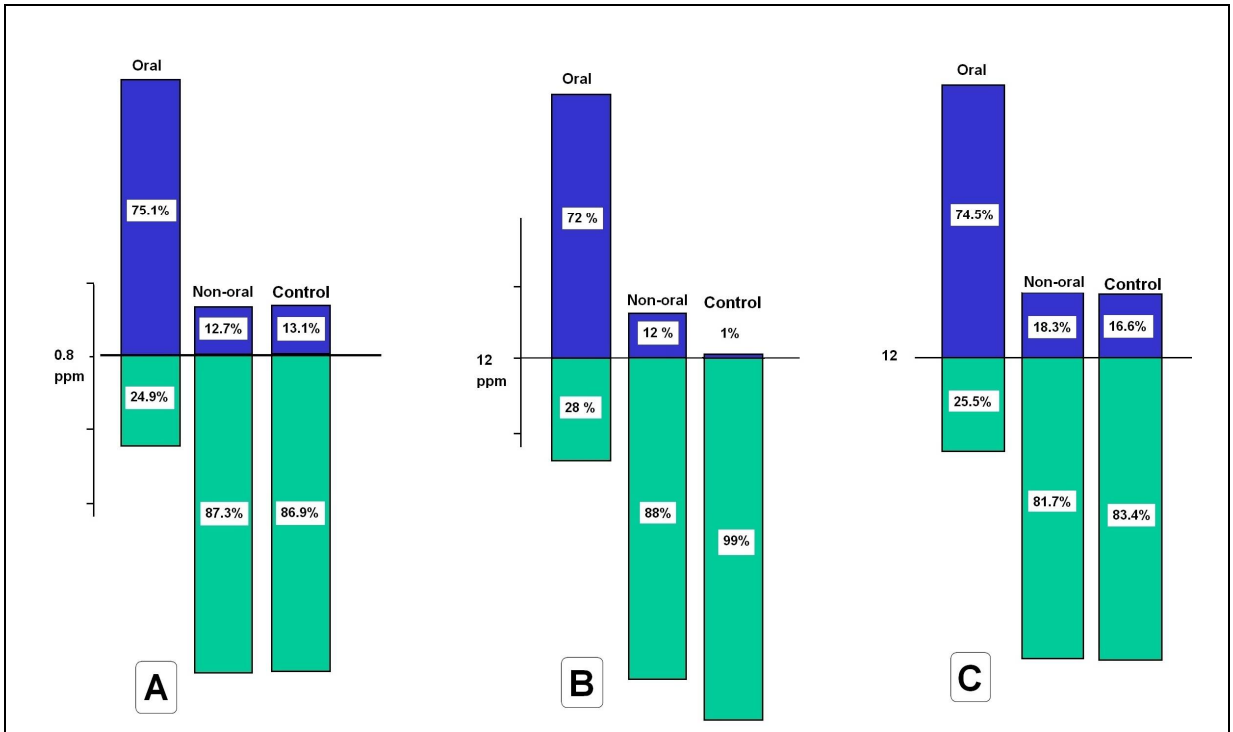

Fig.3 *Oral H_2S concentrations were above 0.8 ppm in a significant percentage of individuals complaining of halitosis (A), their H2S concentrations exceeded 12 ppm after cysteine rinse, (B) with a*

12-fold increase (C). Main percentage of non-oral and control group individuals remained under these limits.

Table 1. Distribution of changes in oral H_2S concentrations before/after cysteine rinse in each group

Data	Oral group (N=305)		Non-oral group (N=191)		Control group (N=102)	
	Pre-CR	Post-CR	Pre-CR	Post-CR	Pre-CR	Post-CR
Min	0.4	8.1	0.4	1.5	0.4	6.1
Max	9	35.3	1.3	21.6	1	12.3
Mean	1.71	18.24	0.67	8.52	0.66	9
SD	1.76	7.15	0.18	3.73	0.17	1.08
SEM	0.1	0.4	0.01	0.23	0.01	0.16
Median	1.2	17.7	0.6	9	0.7	9

DISCUSSION

To establish any possible relationship between initial and cysteine-induced H_2S concentrations, oral or non-oral Halitosis patients and healthy individuals were compared with each other.

In this experimental setup, Post-CR oral H_2S level represents maximum halitosis level that can be produced by that person (designated as upper threshold), whereas pos-CR / pre-CR ratio represents H_2S production capacity of the same person.

On cysteine challenge:

Cysteine has been widely accepted as a challenge test and used in many studies for quick assessment of anti-malodor agents[5,6] or effectiveness of tooth or tongue brushing, chewing gum.[4] Usually, 8.23 mmol of *L*-cysteine rinse for 1 minute, or 10 mmol,[10] 6 mmol (pH 7.2)[11] or 6 mmol (pH 7.1) for 20 seconds,[12,13] 6 mmol, 30 seconds[4] or 0.06 mmol (pH 7.2) for 30 seconds have been used in the cysteine challenge test.

Kleinberg (2002) applied successive cysteine challenges for 20-min periods using 5 ml of 6 mmol cysteine rinse for 30 seconds. Initially, ~80 ppb H_2S concentration increased to ~1.7 ppm (21.2 folds) after cysteine rinse, increased to ~0.5 ppm (6.25-fold) at 20 minutes after brushing whole tongue, increased up to ~1.2 ppm (15-fold) at 20 minutes after Peridex

mouthwash, and increased to ~280 ppb (3.5-fold) at 20 minutes after %1 $NaClO_2$ treatment.[4] However, all of the subjects in their study were healthy individuals and cysteine concentrations used was low (6 mmol). In the present study, 20 mmol cysteine was used in order to better identify any increase in oral H_2S concentrations; as a result, 1.5-35.3 ppm H_2S peaks (Table 1) were obtained depending on oral ecology of individual subjects. Cysteine-induced H_2S level depends on cysteine concentration or oral microbiota[4,14] as an ecological determinant of that individual. The increase in H_2S is greater for those with oral halitosis because the tongue biofilm has high numbers of anaerobic bacteria. The increase in H_2S production is not as marked if the halitosis is due to other (non-oral) reasons. Some individuals who showed high cysteinase activity produced extremely high H_2S concentrations due to their oral ecology having a capacity to produce greater malodor.[5,6] Cysteine challenge provide quantitative information about an individual's H_2S production capacity.

Lower and upper thresholds for pathologic halitosis:

The main goal of the study was to determine thresholds for 1) Pre-CR, 2) Post-CR H_2S concentrations and 3) their ratio for oral and non-oral halitosis patients.

First of all, it is neither meaningful nor sufficiently distinctive to set a lower limit for pathological halitosis in principle. It is also highly controversial and problematic. In the literature, different concentrations for oral H_2S were proposed as a lower limit for pathologic halitosis: 75 ppb,[15] 100 ppb,[16] 110 ppb,[17] 125 ppb,[18] 150 ppb,[19] 250 ppb,[20] 700 ppb[5,6], 1.05 ± 0.97 ppm,[21] 890 or 1000 ppb H_2S.[22] In this study, pathologic halitosis limit was calculated as 0.8 ppm. Even though this threshold is consistent with the literature, to diagnose oral halitosis using this limit solely would not be acceptable because H_2S level in the human mouth is highly unstable.[23] In fact, initial H_2S values of T1 group varied over a wide range (Table 1 and Fig 3A). Misdiagnosis is always possible if lower threshold for pathologic halitosis is used alone. Currently, clinicians rely mostly on minimum oral H_2S concentration to make diagnosis of pathologic halitosis. Traditionally, cases are diagnosed as oral

halitosis, if initial H_2S concentrations exceed a prespecified value (lower limit for pathologic halitosis). However, an upper limit can be proposed since it is also a sufficiently descriptive parameter for the diagnosis of oral halitosis.

It is well known that some patients persistently complain of severe halitosis even when a low oral H_2S level is detected in their mouth. These patients are assumed to have non-oral halitosis. Most dental practitioners consult such patients with other doctors to find out any potential reason for non-oral halitosis. However, these patients may have just had a low gas value momentarily, due to fluctuations in their oral H_2S concentrations. In such cases, oral H_2S concentration sharply increases after cysteine rinse, forming the basis for the diagnosis (data not shown). Although they may have a low oral H_2S level initially, extremely high levels may be detected immediately after cysteine challenge compared to those of healthy individuals. Called “cryptic halitosis”. In order to diagnose such patients, defining an upper limit of halitosis may be useful. The results of the present study showed that oral halitosis patients produced > 12 ppm H_2S after cysteine rinse, while other individuals did not (Figure 3). If the maximum halitosis level is above this threshold, the patient may still have oral halitosis despite a low level of H_2S initially detected in the mouth. This may represent a new diagnostic criterion for the oral halitosis.

Statistically, pre- and post-CR H_2S concentrations of oral group were highly significant ($p<0.001$), and these data led us to consider how H_2S responses to cysteine challenge in the mouths of oral halitosis patients and healthy people can be tabulated.

Momentary halitometric measurements fluctuate throughout the day even as often as every two minutes; [23] for this reason, one-time measurement would not be sufficient to make a diagnosis. Oral gas concentration at the measurement date and minute may not accurately reflect a patient’s tendency to produce halitosis. The characteristics of elevated oral H_2S concentration after cysteine rinse and the post/pre-CR ratio are more accurate parameters for a particular case. These parameters reflect the ability of oral bacteria in the mouth to produce malodor.[4,14] The fold increase in oral H_2S concentrations after cysteine rinse can be

used to ascertain oral halitosis at other times apart from momentary halitometric examination.

Although it was not a prespecified goal, the study showed that many of the pre-requirements for halitosis examination (e.g., fasting for several hours before the test, avoiding medication or brushing) can be neglected or loosened with the use of the post-CR / pre-CR ratio and this can be an advantage for patients. Furthermore, calibration or little measurement errors of gas detectors used for halitosis examination can be partly ignored because errors and their occurrence rates will remain constant when the readings are obtained with the same device. This needs to be confirmed with further experiments.

Table 2. Guidance chart for evaluating H_2S concentrations read before and after cysteine rinse

Pre-CR (ppm)	Post-CR (ppm)	Post-CR / Pre-CR ratio	Possible Diagnosis
0.4	19.0	47.5	oral halitosis
	8.2	20.5	non-oral halitosis
0.6	18.9	31.5	oral
	8.1	13.5	non-oral
0.8	18.8	23.5	oral
	8.0	10.0	non-oral
1.0	18.7	18.7	oral
	7.9	7.9	non-oral
1.2	18.6	15.5	oral
	7.8	6.5	non-oral
1.3	19.2	14.8	oral
	7.8	6.0	non-oral
1.5	18.4	12.3	oral
	7.7	5.1	non-oral

One more goal, based on the findings of our study, a tabulated diagnostic guidance was developed to assist clinicians in distinguishing oral halitosis cases. By using Table 2, dental practitioners can easily identify whether the case clinically seems closer to oral or non-oral halitosis. Data for healthy individuals are not shown in Table 2 because the difference between post-CR / pre-CR ratios for non-oral group and controls was not statistically significant.

In order to validate the data presented in Table 2, a small sample of patient records from oral group were revisited to check consistency with the data in Table 2. One patient randomly selected from oral group was examined. She had severe bone loss, gingival bleeding, >6 mm periodontal pockets, HL score=5, and a tongue coating score of 5 / 6. Her

pre-CR, post-CR and post/pre-CR ratio were 1.3 ppm, 19.6 ppm and 15, respectively. These figures corroborate the diagnosis of oral halitosis and closely match the data in Table 2. The medical records of another patient with pre-CR, post-CR and a post/pre-CR ratio of 0.6, 17.7, 29.5 respectively were consistent with Table 2 as well. He was diagnosed with oral halitosis. The records of 14 patients randomly chosen from oral group and 21 from non-oral group were also checked, showing a good correlation with Table 2 data (data not shown).

There are some limitations of this study. The multi-gas detector used contains 5 electrochemical sensors. Despite the presence of more than 700 [19] or 3481 [22] gases in the human breath, H_2S and sulfur-containing gases are principal odorants in oral malodor. Only H_2S gas was taken into consideration. Gases other than H_2S were not included in the study because other gases in the mouth were reduced after cysteine rinse while H_2S increased.[5,6]

The lower limit of the H_2S sensor is 0.4 ppm. H_2S concentrations under this level could not be read; however, this did not affect the results.

Conclusion

Under selected conditions, relationships between pre- and post-cysteine rinse oral H_2S concentrations were defined. It can be concluded that cysteine challenge test can be used as a new diagnostic tool to support clinical findings based on the following criteria or Table 2.

If

- pre-CR oral H_2S concentration is greater than 0.8 ppm,
- post-CR oral H_2S concentration is above 12 ppm, and
- post-CR / pre-CR oral H_2S ratio is greater than 12,

then the patient can be diagnosed as having oral halitosis.

If:

- pre-CR oral H_2S concentration is less than 0.8 ppm,
- post-CR oral H_2S concentration is less than 12 ppm, and

- post-CR / pre-CR oral H_2S ratio is lower than 12

then the patient can be diagnosed as having non-oral halitosis.

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Conflict of interest:

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