DETERMINATION OF URINARY *TRANS, TRANS*-MUCONIC ACID BY GAS CHROMATOGRAPHY IN GASOLINE SERVICE ATTENDANTS

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ABSTRACT: This study aims to develop the reliable method for analysis of urinary *trans, trans*-muconic acid (*t,t*-ma) using methanol in acid medium (HCl) as derivatization reagent, a low cost and less toxic reagent and analysis by GC/FID. The urinary *t,t*-ma and benzoic acid internal standard was extracted, derivatized into dimethyl cis *trans, trans*-muconate and methyl benzoate, and analysis with GC. The method was applied to analysis of urinary *t,t*-ma of 54 gasoline service station attendants. Personal air samples were also collected. Results showed accuracies of urinary *t,t*-ma (1.5-37.5 µg/ml) ranging from 98.0 to 99.6% with less than 2.5% relative standard deviation (RSD). The detection limit of *t,t*-ma in urine was 0.75 µg/ml urine. The average benzene concentration at breathing zone of the gasoline service station attendants was 0.19 ± 0.16 ppm, ranging from non-detectable to 0.65 ppm. The average urinary *trans, trans*-muconic acid was 458.4 ± 446.4 ranging from non-detectable to 2274 µg/g creatinine.

Keywords: urinary *trans, trans*-muconic acid, gas chromatography, gasoline service attendants

INTRODUCTION: Benzene is the smallest and most stable aromatic compound. It is widely distributed as a component of petroleum products and gasoline[1]. The highest levels of occupational exposure to benzene are likely to occur during petroleum refining, the distribution actions of petroleum products such as tank loading, and unloading and filling operations at service stations[2]. Benzene has been shown to be a carcinogen in both animals and humans and is classified by the Environmental Protection Agency and International Agency for Research on Cancer as a human carcinogen[3].

Benzene is primarily metabolized to phenol, hydroquinone and catechol. In humans, approximately 2% of absorbed benzene is excreted as *trans, trans*-muconic acid (*t,t*-ma). In occupational studies, *t,t*-ma was more sensitive than phenol as a biomarker and could be used to reasonably estimate benzene concentrations down to 1 ppm[4]. Other sources of *t,t*-ma have been identified, such as metabolite of sorbic acid, which makes *t,t*-ma a nonspecific biomarker of low-level benzene exposure[5]. However, the results from many studies indicated that *t,t*-ma should be the suitable biomarker for human exposure to benzene[6-8].

There are several methods used for analysis of urinary *t,t*-ma, such as gas chromatography (GC), gas chromatography/mass spectrometry (GC/ MS) and high-performance liquid chromatography (HPLC). GC has the advantage of a lower cost of analysis than HPLC, and matrix interferences can easily be eliminated. However, derivatization is necessary for GC separation of *t,t*-ma. Several methods describe the derivatization process of the *t,t*-ma before analysis by GC, using Tri-Sil BSA in dimethylformamide[9], ethereal diazomethane[10], diazomethane[11] and N,O-bis (trimethylsilyl) trifluoro-acetamide (BSTFA)[12]. The reagents used for derivatization are toxic chemical, carcinogenic and explosive such as diazomethane. The derivatization method used in this study was methanol in acid medium which was considered less toxic. The analytical method for *t,t*-ma is still important, because the National Institute for Occupational Safety and Health (NIOSH) method for analysis of urinary *t,t*-ma has not been made available yet. The purpose of this study was to develop a reliable method for analysis of urinary *t,t*-ma using methanol in an acid medium (HCl) as derivatization reagent. The developed method was used for analysis of post-shift urinary *t,t*-ma of gasoline service station attendants.

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MATERIALS AND METHODS:

Chemicals and reagents

Trans, trans-muconic acid (*t,t*-ma), benzene, hydrochloric acid 37% w/w, methanol (GR Dried, max. 0.005% H_2O) and methanol were of analytical grade purchased from Merck (Darmstadt, Germany). Benzoic acid (internal standard) was from AJAX Company, Australia. Diethyl ether and chloroform were purchased from BDH Co., England.

Instrumentation

The Shimadzu GC-14B gas chromatograph with a DB -1 capillary column (30 m x 0.53 mm I.D), equipped with flame ionization detector and an integrator (Shimadzu C-R7A, Shimadzu, Kyoto, Japan) was used. The carrier gas was helium at a flow-rate of 10 ml/min. The GC condition was isothermal: column, 120°C; injector, 200°C; detector, 200°C.

A gas chromatograph GC-MS Agilent 5973 Network Mass Selective Detector with a HP-1 column (25 m x 0.32 mm I.D., 0.17 µm film thickness), was used for identification of dimethyl *cis, trans*-muconate and methyl benzoate. The carrier gas flow rate was 1.5 ml/min. The GC condition used program rate at initial column temperature of 50°C, initial run time of 1.0 min and ramped rate at 15°C/min to 100°C, and at 20°C/min to 240°C, then maintained at 240°C for 5 min and then ramped rate at 10°C/min to 310°C and maintained for 1 min, injector, 220°C; detector, 280°C.

Spectrophotometer UV–160A, Shimadzu, Kyoto, Japan) equipped with a Shimadzu TB-85 thermobath.

SKC personal air sampling pump (SKC Inc., Eighty Four, Pa., USA).

Preparation of standard solutions

Stock standard solution of *t,t*-ma (0.3 mg/ml) was prepared by dissolving 30 mg of *t,t*-ma in 100 ml methanol. Benzoic acid solution (internal standard) (2.0 mg/ml) was prepared by dissolving 0.2 g of benzoic acid in 100 ml methanol. The derivatization reagent was prepared in dried methanol, 15 ml of 37% hydrochloric acid (HCl) was diluted with methanol to 100 ml.

Optimization procedure for derivatization

A half milliliter of 1.5 mg/ml standard *t,t*-ma in methanol was evaporated to dryness under a compressed air flow at room temperature. The residue was reconstituted with 1 ml derivatizing reagent. The optimal derivatization procedure was investigated by varying the concentrations of derivatization reagent from 5%, 10%, 15% to 20% conc. HCl in methanol, the derivatization temperature in an oven from 60, 70, 80 to 90°C, with durations of 30, 60, 90 to 120 min. The mixture was allowed to cool to room temperature. Two milliliters of distilled water were added and the mixture was extracted with 1 ml chloroform. A 1 µl of chloroform solution was injected into the GC system.

Identification of benzene and benzoic acid derivatives

A half milliliter of 1.5 mg/ml standard *t,t*-ma in methanol and 0.2 ml of 2.0 mg/ml benzoic acid in methanol as an internal standard were placed into screw cap tubes. The solution was evaporated to dryness and reconstituted with 1 ml 15% conc. HCl in methanol and left in an oven at 80°C for 60 min. The mixture was allowed to cool to room temperature. Two milliliters of distilled water were added to the mixture and the mixture was extracted with 1 ml chloroform. A 1 µl of chloroform solution was injected into the GC system. The urinary *t,t*-ma and benzoic acid derivatives were detected and verified by GC/MS.

Sample preparation

Four milliliters of urine sample was pipetted into the screw cap tubes, (If the urine was cloudy, it was centrifuged and the clear portion was used), 0.2 ml internal standard solution was added. The mixture was acidified with 1.5 ml of conc. HCl and extracted twice with 5 ml diethyl ether. Then it was mixed on a vortex mixer about 2 minutes (If the organic phase was not separated from aqueous phase, the mixture was centrifuged at 4,000 rpm for 5 min). The organic phase was transferred to another tube and evaporated to dryness under a compressed air flow at room temperature. The residues were reconstituted with 1 ml derivatization reagent (15% conc. HCl in methanol) and left in an oven at 80°C for 60 min.

The mixture was allowed to cool to room temperature. Two milliliters of distilled water were added to the mixture and the mixture was extracted with 1 ml chloroform. One μl of chloroform solution was injected into the GC system.

Matrix calibration

The calibration curves for *t,t*-ma was obtained from 4 ml urine sample from persons not occupationally exposed to benzene, 0.5 ml of *t,t*-ma working standard solution and 0.2 ml of internal standard solution were added. It was extracted and derivatized according to the method described above. The peak area ratios of *t,t*-ma and internal standard derivatives were plotted against the concentration of *t,t*-ma in the range of 0.94–37.5 μg/ml in urine for four replicate determinations.

Method validation

A half of milliliter of known *t,t*-ma solution in methanol (12, 75 and 300 μg/ml) and 0.2 ml benzoic acid solution (2.0 mg/ml) were added to 4 ml pooled urine; the analysis was described above together with quality control samples prepared at concentrations of 1.5, 9.38, and 37.5 μg/ml *t,t*-ma in urine. The within- and between-day precisions, and the percent recovery of concentrations, were calculated.

Field applications

Sampling of atmospheric benzene

Atmospheric benzene concentrations were sampling from 54 gasoline service station attendants working in 12 gasoline service stations located in Bangkok area and Nonthaburi province. The gasoline service station attendants were 47 male and 7 female; most of them (88.9%) were under 30 years old. The majority of them (55.6%) had duration of employment between 1-6 months. According to NIOSH Method 1501[13], personal air sampling pumps and activated charcoal tubes (SKC-226-09) (SKC Inc., Eighty Four, Pa., USA) were used with an airflow rate of 0.15 l/min for full shift ranging 8–12 hours in workday.

Urine samples from gasoline service station attendants

Urine samples from gasoline service station attendants were also collected in 20 ml bottles at end of shift work. The samples were stored at room temperature not more than 48 hours or frozen in a refrigerator at –35°C until analysis. The samples were brought to room temperature before analysis.

Sample analysis

Atmospheric benzene samples were analyzed following the NIOSH method number 1501[13]. The urine samples were analyzed together with quality control samples as described above. Urinary creatinine determination was performed by the kinetic Jaffe method[14].

RESULTS:

Optimization procedure for derivatization

For the derivatization procedure, the results (Fig.1) showed that the peak area of *t,t*-ma derivative was greatest at the concentration of 15% conc. HCl in methanol, in contrast, the concentrations of derivatization reagent lower than 15% may not show a completed derivatization process. Hence, the concentration of 15% conc. HCl in methanol was chosen for the derivatization procedure.

The peak area of *t,t*-ma derivative was greatest at the derivatization temperature of 80°C (Fig. 2). The duration of *t,t*-ma derivative forming was varied from 30 to 120 min for the maximum completion of *t,t*-ma derivative. The peak area of *t,t*-ma derivative was greatest at the derivatization period of 60 minutes after that the peak area dropped gradually (Fig. 3). Therefore, the optimal conditions for the derivatization process of this study were derivatized with 15% conc. HCl in methanol in the oven at 80°C for 60 minutes.

Identification of benzene and benzoic acid derivatives

The *t,t*-ma and benzoic acid derivatives were identified by GC/MS as dimethyl cis *trans, trans*-muconate or 2, 4-hexadienedioic acid, dimethyl ester ($C_8H_{10}O_4$) (Fig. 4) and methyl benzoate ($C_8H_8O_2$), respectively.

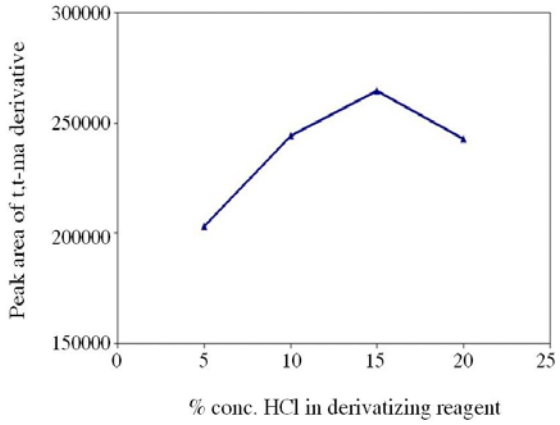

Fig. 1 The peak area of *t,t*-ma derivative at various concentrations of conc. HCl in methanol (%)

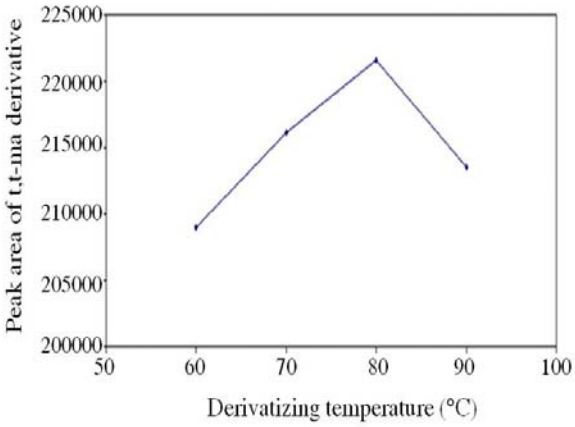

Fig. 2 The peak area of *t,t*-ma derivative at various derivatization temperature (°C)

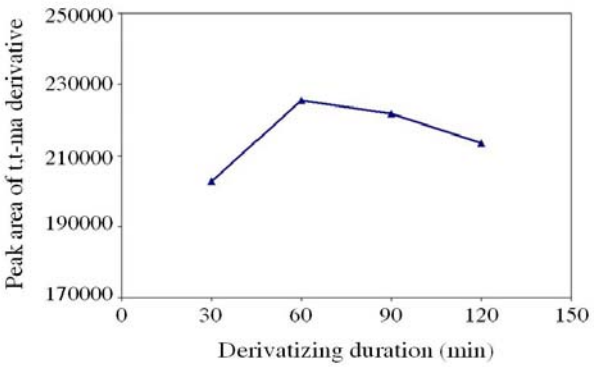

Fig. 3 The peak area of *t,t*-ma derivative at various derivatization duration (min)

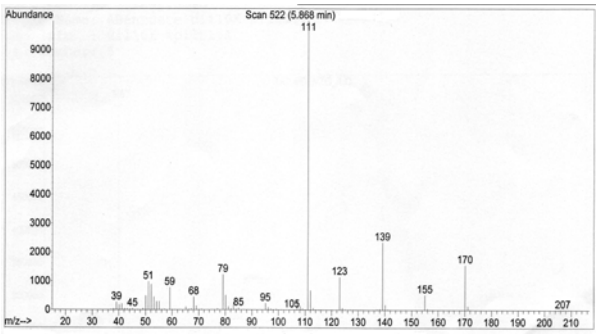

Fig. 4 Mass Spectra of dimethyl *trans,trans*-muconate (*trans,trans*-muconic acid dimethyl ester) obtained by GC/MS analysis

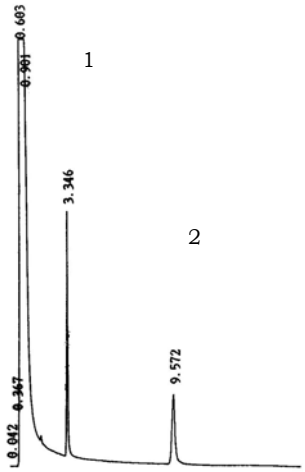

Fig. 5 Chromatogram of standard benzoic acid and *t,t*-ma as methyl benzoate (internal standard (1) and dimethyl cis *trans,trans*-muconate)(2)

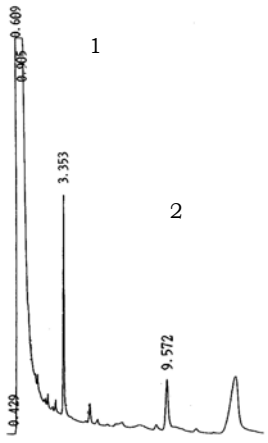

Fig.6 Chromatogram of methyl benzoate (internal standard) (1) and dimethyl cis *trans,trans*-muconate (2) in urine of a gasoline service station attendant.

Chromatogram of standard t,t-ma and benzoic acid derivatives

Fig. 5 shows the peaks of benzoic acid and *t,t*-ma derivatives. They were separated completely within 10 minutes. Apart from the solvent peak, the first peak is methyl benzoate (internal standard) and the second one, dietmhyl cis *trans, trans* muconate (*t,t*-ma derivative) at the retention times of 3.34 and 9.57 min, respectively.

Matrix calibration

The matrix calibration curve of *t,t*-ma was studied in the range of 0.94–37.5 µg/ml of *t,t*-ma within the expected range found in the urine samples. The calibration curve gave a linear relationship, y = 0.0098X–0.0074; where y = peak area ratio of *t,t*-ma and benzoic acid derivatives and x = *t,t*-ma concentration. The slope of the linear correlation was 0.0098 with y intercept of -0.0074. The correlation coefficient of the calibration curve of *t,t*-ma derivative in urine was 0.999 (n=4). This method could detect individual *t,t*-ma at concentration of 0.75 µg/ml.

Method validation

The accuracy of the method for analysis of *t,t*-ma in urine (1.5-37.5 µg/ml) was very high ranging from 98.5 to 100.1%. The inter-day RSDs (100 x SD/mean) were in the range of 2.06 to 2.45 (Table 1). The method was sufficiently accurate and precise to detect *t,t*-ma from workers exposure to benzene.

Atmospheric benzene concentration

Results revealed that average benzene concentration at breathing zone sampling of 54 gasoline service station attendants was 0.19 ± 0.16 ppm ranging from non-detectable to 0.65 ppm. Most of workers (94.4%) were exposed to benzene concentrations lower than the Threshold Limit Value-Time-Weighted Average of 0.5 ppm, recommended by the American Conference of Governmental Industrial Hygienists (ACGIH)[15]. Only 3 of them were exposed to benzene slightly higher than 0.5 ppm. They were working in different gasoline service stations.

Urinary t, t-ma

Fig. 6 shows the peaks of *t,t*-ma and benzoic acid in urine of a gasoline service station attendant. The first peak was methyl benzoate (benzoic acid derivative; internal standard) and the second one was dimethyl cis *trans, trans* muconate (*t,t*-ma derivative) at the retention times of 3.34 and 9.57 min, respectively.

The average *t,t*-ma of 54 gasoline service station attendants were 0.86 ± 0.58 µg/ml urine ranging from non-detectable to 2.66 µg/ml urine or 458.4 ± 446.4 ranging from non-detectable to 2274 µg/g creatinine. Most of the attendants (70.37%) had *t,t*-ma levels below the biological exposure indices of ACGIH, 500 µg/g creatinine with an average of 260.8 ± 180.6 µg/g creatinine. In contrast, 29.6% had the *t,t*-ma level higher than 500 µg/g creatinine at the average of 927.7 ± 538.4 µg/g creatinine**.**

The relationship between atmospheric benzene concentration and urinary *trans, trans*-muconic acid among the gasoline service station attendants

Data from only 43 gasoline service station attendants were used for determination of relationship between the atmospheric benzene concentration and urinary *t,t*-ma acid, because 11 attendants had non detectable atmospheric benzene concentration or urinary *t,t*-ma acid or either. The relationship between benzene exposure and urinary *t,t*-ma acid were calculated by the Spearman's rank correlation. The scatter diagram between benzene exposure and urinary *t,t*-ma acid is shown in Fig. 7. The correlation coefficient of benzene concentration (ppm) and urinary *t,t*-ma acid was 0.047 at the p-value of 0.766 indicating the benzene exposure did not have linear correlation with the urinary *t,t*-ma acid.

DISCUSSION:

The occupational benzene exposure can be determined by monitoring benzene vapor exposure in the air. The urinary *t,t*-ma can also be used as biological exposure indices for benzene exposure in workers.

Derivatization procedure

This study developed the method for analysis of *t,t*-ma by methyl esterification with methanol in acid medium (HCl). It is simple to prepare with low cost and less toxic reagent. For the derivatization procedure, the optimum derivatization reagent was 15% conc HCl in dried methanol. The peak area of

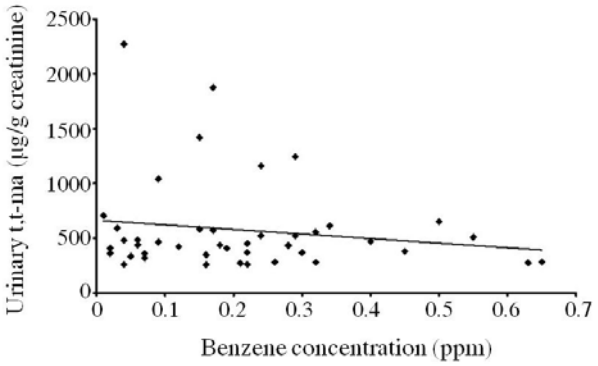

Fig. 7 Scatter diagram of benzene concentration (ppm) and urinary *t,t*-ma (µg/g creatinine)

the derivatives was highest at the concentration of 15% conc HCl in methanol, in contrast, the concentrations of derivatization reagent lower than 15% may not show a completed derivation process. The *t,t*-ma derivatization in this study was different from several previous studies using trimethylsilyl ester[9], ethereal diazomethane[10], diazomethane[11] and BSTFA[12] as derivatization reagent. The derivatization method used in this study was less toxic when compared to other methods.

Matrix calibration

The calibration curve of *t,t*-ma ranged 0.94–37.5 µg/ml in urine due to the expected range found in the urine samples. The calibration curve gave a linear relationship, y=0.0098X–0.0074; where y=peak area ratio of *t,t*-ma and internal standard derivatives and x=*t,t*-ma concentration. When comparing the detection limit of this method with the previous studies[10-11], one finds that the other method could detect *t,t*-ma down to the level of 29 ng/ml urine. Thus, the detection limit of this method should be improved to detect efficiently low concentration to reach the range due to ecological exposure.

Method validation

The accuracy of the method for analysis of *t,t*-ma ranged 1.50–37.50 µg/ml was 96.8-99.0% and 98.5–100.1% for intra-day and inter-day assay, respectively. The RSDs were less than 2.5%. The method was sufficiently accurate and precise to detect *t,t*-ma from workers exposure to benzene. When compared with other methods; the accuracy and reproducibility were slightly better than the other methods. The method with Tri-Sil BSA derivatization method and analysis with GC/MS gave accuracy of 104.7%[9]; the other one presented the accuracy exceeded 90%[16]. The precision of analysis of *t,t*-ma was found to be 9.7% RSD[10].

Atmospheric benzene concentration

The average benzene concentration at breathing zone of 54 gasoline service station attendants was 0.19 ± 0.16 ppm ranging from non-detectable to 0.65 ppm. The benzene concen-trations of gasoline service attendants in this study were slightly greater than those of the previous studies, 0.14-0.24[6], 0.02-0.2 ppm[17], 0.02-0.29 ppm[18], 0.01-3.5 ppm[19], and 0.03-0.11 ppm[20].

The variation of benzene concentration among individuals may be due to several factors, including working postures of station attendants when refueling, variation of environmental factors such as wind direction, wind speed, relative humidity and environmental temperature and individual working activities such as reloading gasoline from the truck, and refueling the automobiles. Although the main duties of the gasoline service station attendants were refueling the automobile, other factors should be considered, such as the difference in the volume of gasoline handled and the number of automobiles serviced each day. Some of attendants might have to help with the reloading gasoline. The personal hygiene of attendants was also important; some might contact with gasoline during refueling which lead to high exposure.

Urinary *t,t*-ma

The average *t,t*-ma of gasoline service station attendants were 458.4 ± 446.4 µg/g creatinine ranging from non detectable to 2274 µg/g creatinine. The levels of *t,t*-ma were slightly greater than the previous studies[21] which found average *t,t*-ma of gasoline service station attendants to be 0.20 ± 0.02 mg/g creatinine in some cases, probably due to the difference in hygiene of the subjects and their individual benzene exposure. The other study found that the urinary *t,t*-ma concentration using high pressure liquid chromatography averaged 4.00 ± 12.49 mg/g creatinine in gas station attendants in Bangkok[22].

The relationship between atmospheric benzene concentration and urinary *trans*, *trans*-muconic acid among the gasoline service station attendants.

The atmospheric benzene concentration was not a linearly related with urinary *t,t*-ma concentrations in the gasoline service station attendants. The fact that *t,t*-ma is excreted with a half-life of five hours[23], while the benzene level from breathing zone sampling was assessed over the 8-12 hours work shift, might have an effect on the observed lack of correlation. Furthermore, non-occupational sources of benzene exposure might increase urinary *t,t*-ma in some workers, such as sorbic acid which was used widely as food preservative. Also, benzene concentration in breathing zone sample is an evaluation of inhalation exposure, while the urinary *t,t*-ma is the estimate uptake of benzene in all routes of entry. The difference in entry routes of benzene among individuals may be due to absorption through skin and food contamination.

All gasoline service station attendants should be notified about health risks of benzene exposure from gasoline so that they may protect themselves as much as possible by avoiding direct contact with gasoline from every route of exposure, not only by inhalation but also by ingestion and absorption. They should perform their activities using good hygiene, keeping their hands and clothes clear from the spillage of gasoline, washing hands before meals, standing in the upwind direction when refueling automobiles, and also using personal protective equipment such as gloves and disposable masks.

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