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DNA-Based Detection of Ethanol-Producing Microorganisms in Postmortem Blood and Tissues by Polymerase Chain Reaction

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16. Abstract

Forensic investigation of fatal aircraft accidents usually includes the analysis of biological samples for ethanol to establish if alcohol intoxication is a factor in the accidents. The quantitative aspects of ethanol are often complicated by postmortem putrefactive changes, leading to microbial fermentation-mediated production of alcohol and its subsequent redistribution. Without establishing the ethanol origin (antemortem consumption or postmortem production), a precise interpretation of the alcohol analytical results remains a challenge. Therefore, a DNA-based assay was developed using the polymerase chain reaction and microbial DNA primers designed for identifying 3 commonly encountered ethanol-producing microorganisms—*Candida albicans, Proteus vulgaris,* and *Escherichia coli*. The present study focused on examining the applicability of the microbial DNA primers in establishing the existence of postmortem alcohol in samples. The results suggested that species-specific primers could be employed to identify ethanol-producing microorganisms in forensic samples without requiring bacterial cultivation. Continued studies are warranted to define additional primer sequences that are distinctive for ethanol-producing microorganisms.

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INTRODUCTION

Although use of alcoholic beverages in moderation is generally acceptable for recreational purposes, it is irrefutable that alcohol abuse is a social and medical problem of global significance. It is estimated that two-thirds of all adults in the United States are occasional drinkers; about 12% among them are heavy users with risk of becoming alcohol dependent (1). The primary acute effect of ethanol is central nervous system depression, which leads to increased reaction time, diminished fine motor control, and impaired critical faculty (2). The direct hazards of such effect are manifested in the statistics of fatal motor vehicle accidents, with ethanol as a major contributing factor in 50% of all cases (3). The legal limit of blood ethanol for motor vehicle operators in most states is 0.08 or 0.10% (w/v), but it is considerably lower for pilots. According to the Federal Aviation Regulation (FAR) 91.17, no person may operate or attempt to operate an aircraft with a blood ethanol content of 0.04% or greater (4). However, ethanol concentrations exceeding the FAR limit were found in at least 6% of fatal aircraft accident cases during a 2-year (1989-1990) period (5).

Forensic investigation of fatal aircraft accidents usually includes the determination of ethanol concentration in biological samples collected during autopsy from pilots to establish if alcohol intoxication is the cause of the accidents. However, postmortem putrefactive changes—microbial fermentation-mediated production of alcohol and its subsequent redistribution—often complicate the interpretation of determined ethanol levels. Generally, between 12 to 57% of ethanol-positive cases were attributed to postmortem production (3,5). There were other instances in which a significant amount of ethanol was found, but the origin (antemortem ingestion or postmortem production) of the alcohol was undetermined. This situation is emphasized in a study of aviation accidents that occurred during 1989-1990, where no determination could be made regarding the origin of ethanol in 45% of all positive cases (5). The lack of direct evidence for antemortem ingestion, coupled with putrefaction effects, requires interpretation of postmortem ethanol concentrations based on the atypical distribution of this substance in different biological compartments. This normally requires analysis of at least 2 types of specimens, such as blood (or tissues) and vitreous humor, urine, or bile (3,5,6). However, these specimens are often not available. Recently, an altered urinary concentration ratio of 5-hydroxytryptophol (5-HTOL) to 5-hydroxyindole-3-acetic acid (5-HIAA) has been suggested as a possible indicator of ethanol consumption (7,8). At present, this test can be performed on only urine specimens, as the reliability of this ratio has not yet been established in other biological fluids.

In a recent Federal Aviation Administration (FAA)-supported study, Kupfer et al. (9) described the development of species-specific DNA primers for selected microorganisms with ethanol-producing capability. The premise was that the presence of the ethanol producers in putrefying tissues constitutes a possible indication of the microbial origin of the alcohol in the samples. While viability of the microbial species is crucial to the culturing techniques, DNA-based methods allow the identification of the microbial contaminants, whether or not they survive the conditions of environment and/or the specimens' preservation and storage. Here, the polymerase chain reaction (PCR) assay was easily performed, and the results were quantifiable, using oligonucleotide primers for 3 commonly encountered ethanol-producing microorganisms—*Escherichia coli, Proteus vulgaris*, and *Candida albicans*. Detailed information on the development of the primer pairs, along with the protocols for the genomic DNA isolation and amplification, has been previously reported (9).

The objective of the present study was to examine the applicability of the aforementioned microbial DNA primers in establishing the existence of postmortem ethanol. The question was whether a PCR-based assay using species-specific primers could

effectively determine the origin of ethanol present in certain forensic samples. Therefore, 3 batches of specimens with indisputable ethanol interpretation, based on both typical and atypical distribution criteria, were selected for the PCR analysis.

MATERIALS AND METHODS

Materials

For alcohol analysis, all reagents were of analytical grade and solvents were of chromatographic grade. The reagents and solvents employed in the DNA analysis were of molecular biology grade or sterilized by autoclaving. These reagents, solvents, standards, internal standards, and other necessary supplies were obtained from commercial sources. The QIAamp® Tissue Kit for DNA isolation was obtained from QIAGEN, Inc., Valencia, CA.

Description of Postmortem Forensic Samples

The forensic specimens were collected during autopsy and placed in the FAA's TOX-BOX evidence containers. The boxes containing samples were shipped with ice packs for analysis to the FAA's Civil Aeromedical Institute (Oklahoma City, OK) by an air carrier service for next-day delivery. Blood was submitted in 10-mL vacutainers, containing 10 mg of sodium fluoride and 20 mg of potassium oxalate. Tissue specimens were submitted in sterile plastic bags with no added preservatives. Using standard laboratory procedures, these samples were analyzed for drugs, alcohols, carboxyhemoglobin, and cyanide, and stored at –20°C. Alcohols were identified and quantified using headspace gas chromatography with *t*-butanol as the internal standard. All cases with a blood ethanol concentration equal to or greater than 0.04% (40 mg/dL) were considered positive. Other volatiles were screened at a limit of detection of 1 mg/dL.

The criteria for determining postmortem alcohol production were based on the absence of ethanol in the vitreous humor and/or urine and positive ethanol in the blood or tissues. In accordance with the results of the alcohol analysis, the specimens were divided into 3 batches. Batch 1 consisted of 23 blood and tissue samples wherein no ethanol was detected, Batch 2 was composed of 24 blood and tissue samples where the presence of ethanol was attributed to consumption, and Batch 3 was composed of 22 samples containing ethanol due to postmortem production. Solid tissues or tissue fluids, including brain, heart, kidney, liver, lung, and muscle, were used, depending on the specimen availability.

Microbial Cell Culture and Concentration Determination

Isolates of *Escherichia coli* (strain ATCC 8739), *Proteus vulgaris* (University of Oklahoma strain 10052), and *Candida albicans* (strain ATCC 10231) were used for the preparation of stock culture for each species. The microbial cells were obtained from overnight broth culture and maintained at –70°C in the presence of 17% glycerol. To estimate the concentration of the microbes in a sample, a calibration curve was constructed for each species to correlate the colony-forming units (cfu) and the absorbance at 600 nm of the cell suspension in 10 mM $MgSO_4$. The bacteria, *E. coli* and *P. vulgaris,* were grown in L-broth (1% peptone, 0.5% yeast extract, and 0.5% NaCl in water), while *C. albicans* was cultured in modified Saubouraud's medium (1% neopeptone and 2% dextrose in water). Each 10-mL broth culture was incubated at 37°C with aeration to late log phase. The microbial cells were collected by centrifugation and resuspended in 10 mL of sterile 10 mM $MgSO_4$ solution. The cell suspensions were sonicated (300 Ultrasonik, NEY Barkmeyer Division, Yucaipa, CA) at 50% power for 60 sec to separate the cell clumps. A serial dilution was prepared for each cell suspension; a 100-µL sample was removed from each series, and it was streaked on the appropriate agar plate for a colony plate count following 24-hour incubation at 37°C. The *E. coli* and *P. vulgaris* cells were spread on L-broth agar and MacConkey's agar, respectively. *C. albicans* cells were spread on Saubouraud's agar plates. Absorbance of each diluted sample was measured at 600 nm (Vectra XM, Hewlett Packard, Waldbronn, Germany). Regression analysis of the respective absorbance *vs*. cfu/mL generated a representative calibration curve for the particular organism.

DNA Extraction

DNA was extracted according to the protocol described in the previous study (9). Briefly, cells from 300 µL of the blood or tissue fluid samples were collected by centrifugation at 8,000x*g* for 3 min in a microcentrifuge (235C, Fisher Scientific Co., Pittsburgh, PA). For solid tissues, approximately 0.03 cm^3 (≈ 30 mg) of each sample was grated, using a sterile scalpel and aseptic techniques, and then suspended in

270 µL of 10 mM $MgSO_4$. Pellets were suspended in Erythrocyte Lysis Solution (QIAGEN Inc., Valencia, CA) followed by centrifugation, and then reconstituted in Cell Suspension Solution (Gentra Inc., Minneapolis, MN). To facilitate the lysis of the microbial cell wall, the cell suspension was incubated at 37°C for 70 min in the presence of 200 U lyticase (Sigma, St. Louis, MO) and 0.5% β-mercaptoethanol (Fisher Scientific Co., Pittsburgh, PA). DNA was isolated using the QIAmp® Tissue Kit, as described in the manufacturer's protocol. Along with each batch of samples, a negative blood control and a reagent blank were processed, as well as 3 positive control samples of *E. coli, P. vulgaris*, and *C. albicans*.

The positive control samples were prepared for each microorganism by inoculating 200 µL of negative human blood (Oklahoma Blood Institute, Oklahoma City, OK) with 100 µL of the microbial cell suspensions collected from overnight cultures. The positive control samples contained 10^5 cfu of the bacteria or 10^3 cfu of the yeast. Uninoculated human blood and 10 mM $MgSO_4$ were used for negative and reagent control in 300-µL aliquots, respectively.

DNA Amplification

To detect the target DNA in the extracts, PCR was performed according to the protocol developed in the earlier study (9). Previously designed oligonucleotide primers (9) were synthesized on a Beckman Oligo 1000M DNA Synthesizer (Beckman Instruments, Houston, TX). Approximately 20 ng of the extracted DNA (8 µL of the extract) from each sample was added to 3 separate reaction mixtures that contained the primer pair specific for either *C. albicans*, *P. vulgaris*, or other enteric bacteria. Those primers were FAA37, 11 for *C. albicans*; FAA46, 45 for *P. vulgaris*; and FAA30, 31 for *E. coli, P. vulgaris*, and other enteric bacteria (9). The PCR was performed in a Perkin-Elmer 9600 thermocycler (Perkin Elmer Corporation, Norwalk, CT) in 35 cycles, following a 5-min denaturation at 95°C. Each thermal cycle consisted of 3 steps: denaturation (94°C, 1 min), primer annealing (50°C, 2 min), and primer extension (72°C, 3 min). The last cycle was followed by the extension step at 72°C for 10 min and then cooling to 15°C for storage. To inactivate the polymerase, 5 µL of 200 mM EDTA was added to each of the amplified samples and stored at –20°C until used.

Semiquantitation of Microbial Cells by Duplex PCR

Optimization for the duplex amplification was conducted using *E. coli* as the representative organism and *C. albicans* as the internal control. The control template was prepared from a pool of the extracts obtained with a blood matrix containing $3.0x10^5$ cfu *C. albicans* and then diluted in 2 parts of sterile water. The diluted extract of the control template (8 µL; ≈20 ng DNA) was added to each reaction mixture. The *E. coli* template was prepared from a series of blank blood that had been supplemented with 10^5, 10^6, and 10^7 microbial cells. The PCR conditions were optimized with respect to the amounts of the *Taq*-polymerase (M186B, Promega, Madison, WI), the $MgCl_2$ buffer (M188J, Promega, Madison, WI), and the dNTPs (100 mM, Pharmacia Biotech, Piscataway, NJ). The thermal cycles and the primer concentration for each template were the same as previously described in the single target sequence amplification.

Estimation of the microbial concentration in the forensic samples was accomplished by performing the duplex PCR for selected samples under optimized conditions. The *E. coli* calibration curve was examined in the range from 10^3 to $5.5x10^7$ cfu/300 µL. The assay precision was investigated in 5 replicates, using the same extract from 6 *E. coli* standards, ranging from 10^3 to $5.5x10^7$ cfu/300 µL, which were analyzed on separate occasions. Precision was evaluated by means of the normalized ratios obtained from the extrapolated cfu values for *E. coli* and *C. albicans*.

PCR Product Gel

Gel electrophoresis was performed to identify the PCR products using a Horizontal Mini-Gel Electrophoresis Unit (Sigma-Aldrich, St. Louis, MO). The 10x7.5x0.4-cm gels were cast from a solution of 2% agarose in 0.5X TBE buffer (45 mM Tris, 45 mM borate, and 1 mM EDTA) and 0.5 µg/µL ethidium bromide (Sigma, St. Louis, MO). Each 8 µL of the amplified sample was mixed with 2 µL of the gel loading buffer (20 mM Tris, 2.5 mM EDTA, 50% glycerol, and 0.2% bromophenol blue). This mixture was electrophoresed in parallel with a 123 Base Pair DNA ladder (Life Technologies Inc., Gaithersburg, MD). Electrophoresis was carried out at 105 volts (DPS 1000, Buchler Instrument Inc., Fort Lee, NJ)

for approximately 1.5 hours, at which time the bromophenol blue dye front had traveled at least 7.5 cm from the wells. Subsequently, the gels were photographed under UV-illumination (U.V.P. Inc., San Gabriel, CA), using a red 23A filter (Tiffen Manufacturing Corp., Hauppage, NY). The intensity of the DNA bands represents the relative amount of specific amplification product, when compared with standard samples of known concentration.

RESULTS

A typical calibration curve for determining the concentration of the microbial cell suspensions in the $MgSO_4$ solution is presented in Fig. 1. The calibration curves were linear over the range examined with $r^2 = 0.986$ for *E. coli*, 0.997 for *P. vulgaris*, and 0.960 for *C. albicans*. The colony count approximated the number of viable bacteria or yeast cells, while the absorbance represented the amount of viable and unviable microbial cells.

The PCR-products obtained from the positive control samples of the 3 microorganisms are shown in triplicate (Fig. 2). *C. albicans* yielded a 704 bp fragment at 10^3 cfu. The 384 bp and 1226 bp fragments were produced with *E. coli* and *P. vulgaris* at 10^5 cfu, respectively. The detectable levels for each species in a mixed microbial blood matrix were determined to be 10^3 cfu/300 µL (not shown for *E. coli* and *P. vulgaris*). These levels were detected in 70% of the tested control samples.

The forensic samples from all 3 batches were screened using the 3 DNA primer pairs in separate amplification reactions. Preliminary screening results indicated that amplicons produced by the primer pair FAA30, 31 were detectable with high intensity in samples of Batch 1 and Batch 3, while only 13% of samples in Batch 2 (ethanol due to ingestion) were positive (Table 1). *C. albicans* was detected in 9 to 33% of the samples from all the batches. *P. vulgaris* was found only in Batch 3 samples, at a frequency of less than 10%.

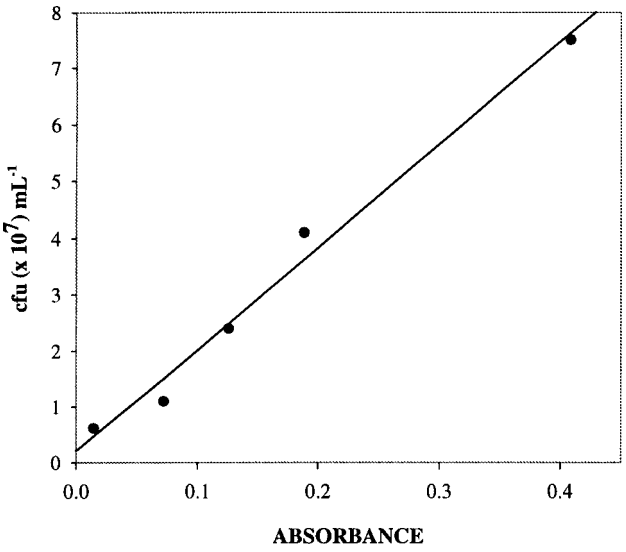

Fig. 1. The calibration curve for *E. coli* suspended in 10 mM $MgSO_4$. A typical linear curve ($r^2 = 0.986$) used for determining the concentration of viable cells/mL of the microorganism is shown. Absorbance was measured at 600 nm. cfu: colony-forming units.

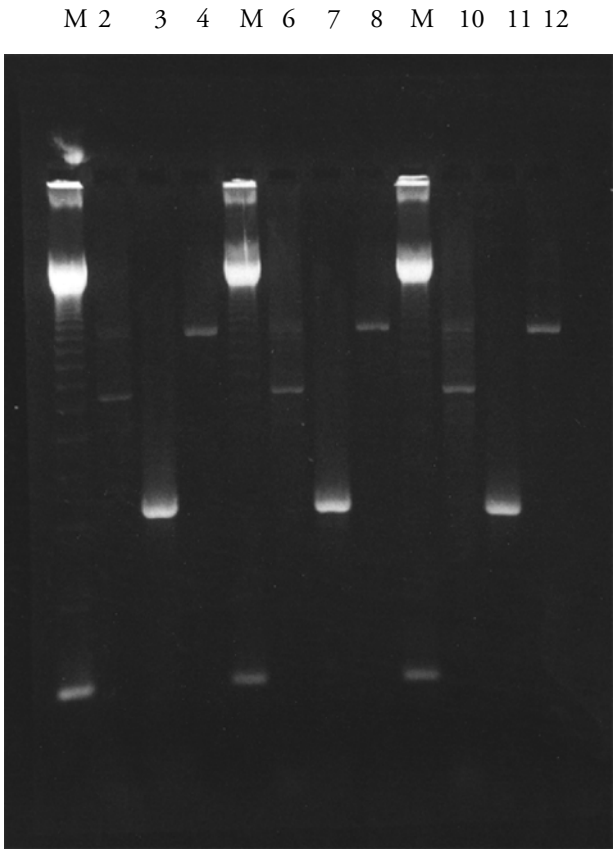

Fig. 2. The PCR products obtained from representative microorganisms using specific primer pairs. Lanes 2, 6, 10 *C. albicans* (704 bp); lanes 3, 7, 11 *E. coli* (384 bp); lanes 4, 8, 12 *P. vulgaris* (1226 bp). Marker lanes (M).

Table 1. Percentage of samples in a particular batch where the targeted microbial species were detected using the specific DNA primer pairs.

Sample	Percentage of samples*		
	FAA37, 11 ***(for C. albicans)***	**FAA46, 45** ***(for C. albicans)***	**FAA46, 45** **(for *E. coli* and other microbes)**
Batch 1 samples (No ethanol detected)	26%	Below detectable level	97%
Batch 2 samples (Antemortem ethanol ingestion)	33%	Below detectable level	13%
Batch 3 samples (Postmortem ethanol production)	9%	9%	95%

*The results are expressed in percentage of samples in which microbes under a particular category were detected. The detectable levels for representative microbial species were 10^3 cfu.

Since a significant number of samples with (Batch 3) and without (Batch 1) ethanol showed positive results with the primer pair FAA30, 31 (Table 1), it was suggested that the relative amounts of the microorganisms were responsible for the observed differences in the ethanol content between the 2 batches. A semi-quantitative assessment of the microbial DNA content among the samples was achieved by the duplex PCR procedure. The goal was to determine the relative numbers of the microorganisms present in the forensic samples, both viable and unviable, without reference to the exact number of their target DNA templates. *C. albicans* template equivalent to 10^5 cells was employed to normalize the efficiency of the amplification process. In optimizing the duplex PCR, the variables that consistently produced the largest yield of the duplex targets, while remaining within the linear amplification range, were selected for use. Since the melting temperatures for primer pairs FAA30, 31 and FAA37, 11 are similar ($T_m \approx 60$-64°C), their optimal annealing temperatures are also equivalent (10). Hence, the conditions of the thermal cycles and the concentration of the primers used for the previous reaction (single template amplification) were adopted for duplex priming. The concentrations of the polymerase, the dNTPs, and the $MgCl_2$ buffer were the variables reexamined for assay optimization. The results confirmed the robustness of the existing PCR condition. Accordingly, the polymerase at 5 units, the dNTPs at 0.2 µmole, and $MgCl_2$ at 1.5 µmoles in a 100-µL final volume of the reaction mixture were optimal for the duplex amplification. A standard curve representing the range 10^3-$5.5x10^7$ cfu/300 µL of *E. coli* was prepared by plotting the ratio of the equivalent cfu for *E. coli:C. albicans vs. E. coli* cfu (Fig. 3). The estimation of *C. albicans* cfu was based on calibration samples prepared by amplifying, in the absence of the *E. coli* template, the *C. albicans* standards at the concentrations from 10^3 to 10^5 cfu. In examining Table 2 and Figs. 3 and 4, it is observable that the *C. albicans* and *E. coli* products accumulated with similar efficiencies, and the product ratios in Table 2 and Fig. 3 demonstrate the linear quantitative range for the *E. coli* target ($r^2 = 0.998$). The precision of the assay was assessed based on a relative standard deviation (RSD) ≤ 20%, which indicated the tolerable reproducibility of the quantitative estimation. A relative change in the PCR products could be detected with a minimum of a 5-fold change of the starting *E. coli* DNA template concentration.

A relative estimate of the microbial content in the forensic samples was examined by using the primer pair FAA30, 31. The bacterial concentrations in samples from Batch 1 and Batch 3 were extrapolated from the respective standard curve for *E. coli* obtained by the duplex PCR procedure (Fig. 3); the results are given in Tables 3 and 4. Since the internal control template in the duplex procedure was *C.*

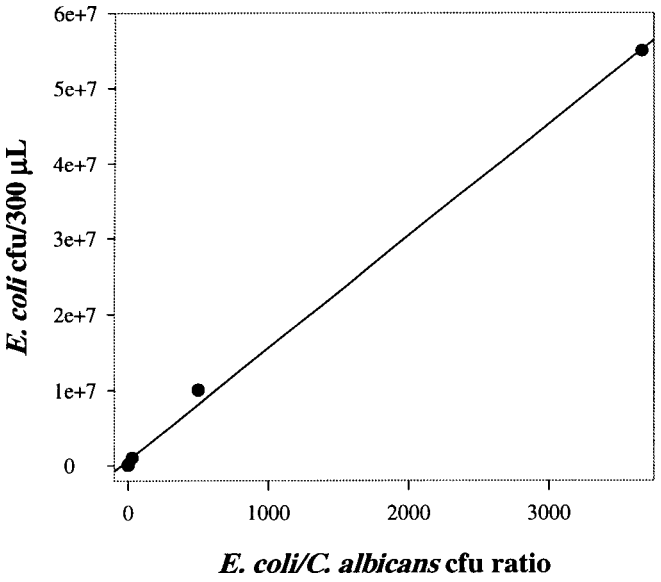

Fig. 3. Calibration curve for the estimation of microbial content in selected samples from Batch 1 and Batch 3 by a duplex PCR procedure (r^2 = 0.998). This curve was prepared using *E. coli* standards as the representative organism in the range from 10^3 to 5.5x10^7 cfu/300 µL; the internal control template was prepared from *C. albicans* at a constant concentration of 10^5 cfu. The cfu for *C. albicans* was obtained by comparing the relative intensity of the *C. albicans* product bands with that of the *C. albicans* standard samples amplified in the absence of *E. coli*. Each cfu ratio was calculated based on the *E. coli* concentration in the standard sample and the respective estimated cfu for *C. albicans* product (cfu: colony-forming units).

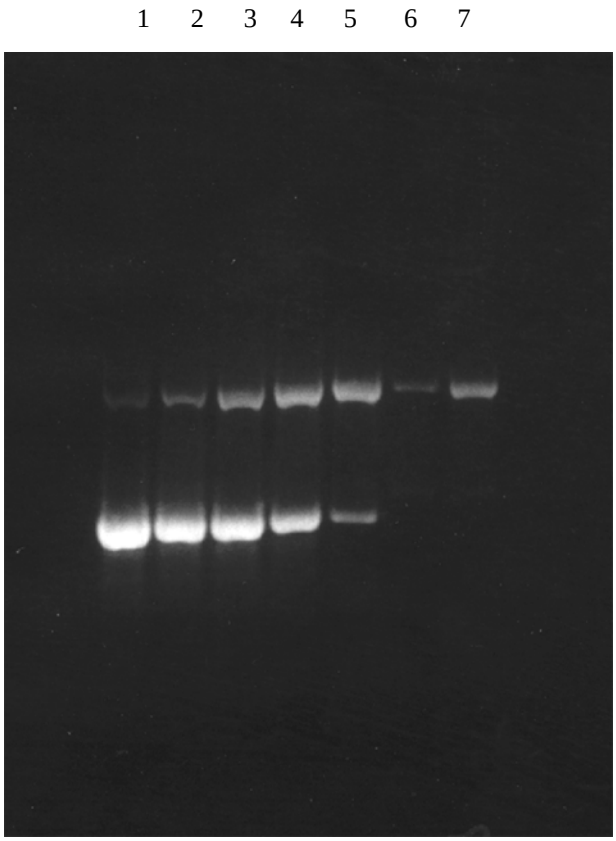

Fig. 4. The amplicons obtained by duplex PCR procedure from the standard samples (also refer to Fig. 3). The top amplicons are *C. albicans*, while the lower amplicons are *E. coli*. *C. albicans* template equivalent to 10^5 cfu was added to the *E. coli* standards as the control template. *E. coli* amplicons in lanes 1-7 represent 5.5x10^7, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cfu/300 µL, respectively.

Table 2. Assessment of the precision for the estimation of *E. coli* cells present in 300 µL blood matrix by duplex PCR.

***E. coli* Standard sample (cfu/300 µL)**	**Mean ratio (SD)***	**RSD (%)****
5.5×10^7	4861.1 (666.1)	13.7
10^7	553.3 (73.0)	13.2
10^6	22.4 (3.2)	14.1
10^5	2.4 (0.4)	15.7
10^4	0.13 (0.025)	18.7
10^3	0.035 (0.007)	20.2

*Ratio of the equivalent colony-forming unit for *E. coli*:*C. albicans.* The equivalent cfu was derived from the evaluation of the relative intensity of the PCR product bands. The *E. coli* product bands were compared with those of the samples that made up the calibration curve (see Figs. 3 and 4). The *C. albicans* product bands were compared with those of the *C. albicans* standard samples amplified in the absence of *E. coli*. This same procedure was used in evaluating the forensic samples. The mean values were obtained from 5 replicate analyses of the standard samples. The primer pairs used were FAA30, 31 and FAA37, 11. The *C. albicans* template equivalent to 10^5 cfu/mL was added to each reaction as the internal control.

**RSD: relative standard deviation

albicans, any forensic sample contaminated with *C. albicans* was excluded from the assay. The target DNA detectable by the FAA30, 31 primer set was found at concentration levels correlating to 10^6-10^8 cfu/mL in the majority of samples from both Batch 1 and Batch 3. The DNA concentration was 3 orders of magnitude lower ($\leq 10^3$ cfu) in about 10% of the tested samples. The absence of both amplicons (target DNA and control DNA) in 5-17% of samples suggests the reaction was inhibited, which might be due to the putrefactive condition of those samples. Thus, samples from Batch 1 and Batch 3 yielded similar results when tested with the primer pair FAA30, 31. These results indicate that ethanol production in Batch 3 was not related to any significant difference in the amount of the microbial contamination (Mann-Whitney, $p = 0.9399$).

DISCUSSION

The specificity of the primers employed in this study allowed the detection of 3 commonly encountered microbial species—*C. albicans*, *P. vulgaris*, and *E. coli*. As discussed previously (9), the FAA30, 31 primer pair was designed from a conserved region of the 16S rDNA in the *Enterobacteriaceae*. Therefore, the PCR products obtained by this primer pair could provide an indication for the presence of any enteric species, including *Klebsiella, Yersinia, Samonella, Proteus,* and *Escherichia*. Many of these strains are established ethanol producers (11).

The present results agree with the observation made by Cory (11) that the greatest increases in ethanol levels were usually associated not with yeast contamination, but with enterococci or enteric bacilli.

Table 3. Concentrations of the detectable microbes using the primer pair FAA30, 31 in selected samples of Batch 1 (no detectable ethanol).

Sample no.	Sample types	Cfu/mL (x 10^6)*
1	Blood	2.49
2	Blood	27.3
3	Blood	2.55
4	Blood	3.90
5	Blood	2.51
6	Blood	Inhibition
7	Muscle	2.49
8	Heart	2.49
9	Kidney	2.50
10	Muscle	27.3
11	Kidney	Inhibition
12	Muscle	Inhibition
13	Kidney	27.3
14	Kidney	2.61
15	Muscle	3.23
16	Kidney	0.003
17	Muscle	0.003

*The values represent the approximation of the number of bacteria present in 1 mL of sample expressed as cfu/mL.

Further, *P. vulgaris* was neither a major microbial contaminant nor a significant source of postmortem ethanol, based on the cases included in the present study. The results also reveal that the primers FAA30, 31 have broad specificity, suggesting that these primers are not specific for the ethanol-producing enterobacteria. This observation is based on culturing information obtained for selected samples of Batch 1, which consisted of cases with no detectable ethanol. While not all the microorganisms were identified, the predominant species isolated were *Streptococcus iniae, Vibrio splendidus, Enterobacter aerogenes*, and *Brochothrix thermosphacta*. All of these species are facultative anaerobes. Their major fermentative products are mixed acids, with ethanol as a minor component under appropriate condition (12,13). Thus, it is likely that the DNA sequence flanked by the FAA30, 31 primer pair is conserved in one or more of the above species and contributed the observed PCR product bands signifying their presence. Perhaps the tissue conditions were unfavorable for ethanol production. Therefore, ethanol was not detected in these samples.

Whereas the broad specificity of FAA30, 31 allowed the detection of *E. coli* and many related bacteria, the concentration of these species in most samples from Batch 2 (ethanol levels due to antemortem consumption) was at or below the detectable level of 10^3 cfu/mL. The existence of putrefaction in Batch 2 samples prompted the conclusion that species other than those characterized by the 3 primer pairs were the predominant microorganisms, possibly including *Staphylococci, Streptococci, Micrococcaceae, Pseudomonas, Clostridium, Bacteroids, Peptococcus*, other yeasts, molds, etc. (11). The genomic DNA of

Table 4. Concentrations of the detectable microbes using the primer pair FAA30, 31 in selected samples of Batch 3 (presence of ethanol attributable to postmortem production).

Sample no.	Sample types	Cfu/mL (x 10^6)*
1	Blood	2.62
2	Blood	2.62
3	Blood	2.52
4	Blood	2.49
5	Muscle	2.49
6	Muscle	2.49
7	Muscle	4.14
8	Muscle	57.5
9	Lung	0.003
10	Kidney	2.49
11	Muscle	2.49
12	Muscle	25.0
13	Liver	2.49
14	Lung	144.0
15	Muscle	2.91
16	Heart	Inhibition
17	Liver	0.001
18	Muscle	2.62
19	Liver	4.14
20	Kidney	2.88

* The values represent the approximation of the number of bacteria present in 1 mL of sample expressed as cfu/mL.

any of these predominant species would be extracted by the protocol used but was not amplified during the PCR. Therefore, designing additional PCR primers specific for these other recognized ethanol producers would expand the microbial detection range of this technique.

SUMMARY

The 3 primers examined in this study could be employed to identify microbial contaminants in putrid forensic samples. A positive result conferred by the primer sets could lend credence to the microbial origin of ethanol in postmortem alcohol production cases. However, the use of microbial primers does not distinguish other non-microbial sources of ethanol. Additionally, the absence of PCR products resulting from these primers does not exclude the presence of other ethanol-producing microorganisms. The application of this DNA-based technique in resolving the question of postmortem ethanol is pending the development of additional primer sets specific for other ethanol-producing bacteria and yeast.

Clearly, now that this approach has been shown to be feasible, additional primers could be developed for all implicated microbial species using unique ribosomal RNA gene regions as a source of primer sequences. Further studies should aim at expanding the range of this DNA-based test for detecting the presence of ethanol-producing microorganisms.

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