


รายงานวิจัยฉบับสมบูรณ์

โครงการ การตรวจดีเอ็นเอจากซองกระสุน กระสุน และปลอกกระสุน ด้วยวิธีไดเร็คพีซีอาร์

Direct PCR DNA typing from touch DNA on cartridges, bullets, and casings (CBCs)

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ตุลาคม 2561

สัญญาเลขที่ MRG5580105

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา สำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยสงขลานครินทร์

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ MRG5580105

ชื่อโครงการ โครงการการตรวจดีเอ็นเอจากซองกระสุน กระสุน และปลอกกระสุนด้วยวิธีไดเร็คพีซีอาร์

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กระสุนปืนและปลอกกระสุนปืนถูกพบได้บ่อยในที่เกิดเหตุของคดีความที่มีการใช้ปืน ซึ่งอาจจะเป็นแหล่งของดีเอ็นเอที่เกิดจากการสัมผัสระหว่างการบรรจุกระสุนลงซองกระสุนหรือการใส่ลูกกระสุน อย่างไรก็ตาม การตรวจพิสูจน์ดีเอ็นเอจากหลักฐานดังกล่าวด้วยวิธีมาตรฐานนั้นให้รูปแบบลายพิมพ์ดีเอ็นเอ (STR profile) ที่ไม่สมบูรณ์หรือไม่สามารถนำไปใช้การในชั้นศาลได้ กระบวนการไดเร็คพีซีอาร์ (Direct PCR) หรือการจัดทำลายพิมพ์ดีเอ็นเอจากวัตถุพยานโดยตรงโดยไม่ผ่านการสกัดดีเอ็นเอได้ถูกแสดงให้เห็นว่าสามารถเพิ่มความสมบูรณ์ของรูปแบบลายพิมพ์ดีเอ็นเอจากวัตถุพยานที่มีดีเอ็นเอจากการสัมผัส โดยมีงานวิจัยเพียงแค่สองงานก่อนหน้าที่นำกระบวนการไดเร็คพีซีอาร์มาใช้เพื่อเพิ่มปริมาณดีเอ็นเอจากกระสุนปืนและปลอกกระสุนปืน และทั้งสองงานยังมีขอบเขตการวิจัยที่จำกัด ดังนั้น ในงานวิจัยนี้ทางผู้วิจัยจึงนำกระบวนการไดเร็คพีซีอาร์มาใช้ในการเพิ่มประสิทธิภาพและความสำเร็จในการจัดทำรูปแบบลายพิมพ์ดีเอ็นเอ ผลการทดลองแสดงให้เห็นว่า การสกัดดีเอ็นเอมีประสิทธิภาพต่ำและทำให้เกิดการสูญเสียดีเอ็นเอไปมากถึงร้อยละ 40 ของดีเอ็นเอตั้งต้น ในขณะที่กระบวนการยิงปืนทำให้สูญเสียดีเอ็นเอไปอีกร้อยละ 27 เมื่อทำการเปรียบเทียบการจัดทำลายพิมพ์ดีเอ็นเอแบบมาตรฐาน การจัดทำลายพิมพ์ดีเอ็นเอแบบไดเร็คพีซีอาร์ และการจัดทำลายพิมพ์ดีเอ็นเอแบบไดลูชั่นพีซีอาร์ จากปลอกกระสุนปืน 9 มม. ที่ผ่านการยิงแล้ว พบว่าการจัดทำลายพิมพ์ดีเอ็นเอแบบไดเร็คพีซีอาร์สามารถให้จำนวนอัลลีลที่สูงที่สุดเมื่อเทียบกับอีกสองวิธี โดยมีค่าเฉลี่ยและช่วงเชื่อถือ (Credible interval) อยู่ที่ 11.1 (7.9–13.9) สำหรับวิธีไดเร็คพีซีอาร์ 5.6 (3.0–7.7) สำหรับวิธีมาตรฐาน และ 2.3 (0.2–4.0) สำหรับวิธีไดลูชั่นพีซีอาร์ การจัดทำลายพิมพ์ดีเอ็นเอแบบไดเร็คพีซีอาร์จากปลอกกระสุนปืนที่ผ่านการยิงแล้วสามชนิด (9 มม. 7.62 มม. และ 5.5 มม.) แสดงให้เห็นว่าจำนวนอัลลีลที่ได้รับจากกระสุนและปืนทั้งสามชนิดไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ การทดลองการจำลอง

เหตุการณ์จริงที่มีการใช้ปืนร่วมกันแสดงให้เห็นว่ามีรูปแบบลายพิมพ์ดีเอ็นเอผสมถึงร้อยละ 40 ของตัวอย่าง ดังนั้นการแปลผลจากคดีความจริงจึงมีน่าจะความยุ่งยากและซับซ้อน ซึ่งน่าจะต้องมีการนำระบบการแปลผลที่ใช้โมเดลการคำนวณค่าความน่าจะเป็น (Expert interpretation system using continuous model) มาใช้ โดยสรุป การนำเทคนิคไดเร็คพีซีอาร์มาใช้เพื่อจัดทำลายพิมพ์ดีเอ็นเอจากปลอกกระสุนประสบความสำเร็จ มีประสิทธิภาพสูง รวดเร็ว และประหยัดค่าใช้จ่าย เหมาะกับการนำไปใช้กับตัวอย่างปลอกกระสุนจากคดีความจริงต่อไป

คำหลัก: ไดเร็คพีซีอาร์ ปลอกกระสุน กระสุนที่ผ่านการยิง ดีเอ็นเอจากการสัมผัส เครื่องกระสุน

Abstract

Project Code: MRG5580105

Project Title: Direct PCR DNA typing from touch DNA on cartridges, bullets, and casings (CBCs)

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Project Period: 2 July 2012 to 1 July 2014

Cartridges, bullets, and casings (CBCs) are commonly encountered in shooting incidences and could provide valuable DNA information from touch DNA that has been left during bullet handling and gun loading; however, conventional DNA analysis has yielded very poor results. Direct PCR, in which the DNA extraction and quantification steps are bypassed, has been shown to provide comparable and sometimes improved results from touch DNA and trace DNA samples. Here, we aimed to apply direct PCR with bullet casings (from three ammunition types and guns) and evaluate whether it should be recommended as a standard operating protocol for forensic DNA analysts. Three experiments were carried out to investigate the following: the effect of firing on DNA deposited on bullet casings; the effect of gun and ammunition types on STR profile quality; and the feasibility of using direct PCR with actual cases via typing of mock casework samples. DNA extraction resulted in a loss of about 40% of DNA originally deposited, and firing a bullet decreased the amount of DNA recovered by 27%. We recovered means (and 95% credible intervals) of 11.1 (7.9 to 13.9), 5.6 (3.0 to 7.7), 2.3 (0.2 to 4.0) alleles from touch DNA on fired bullet casings using the direct PCR protocol, conventional extraction protocol, and dilution protocol, respectively. No statistical difference in alleles recovered was observed between different fired ammunition types from three guns (9mm, 7.62 mm, and 5.56 mm from Glock Model 19, AK47, and Tavor T-21, respectively). As expected, mixed DNA profiles were observed in 40% of mock casework samples in which guns are shared between volunteers, which can complicate profile interpretation. This study showed that direct PCR from bullet casings improved STR profiles. As the direct PCR protocol is quicker, cheaper, and resulted in more alleles recovered, forensic DNA analysts may benefit from using direct PCR.

Keywords: Direct PCR; bullet casings; fired bullets; touch DNA; ammunition

Executive summary

Gun crimes are undoubtedly a significant problem in the Thai society. Crimes involving guns, ammunitions, and explosives are among the top five in terms of the number of cases that go to court. In the past three years, there were 60,000 cases that reach the provincial court and an additional 9,000 cases for the juvenile and family court. In other words, there were more than 70 cases per day being decided. These numbers grossly underestimate the actual number of incidences, as there are numerous cases that are not filed to the courts by the public prosecutors due to various reasons such as the lack of eyewitness testimony.

Since the start of the insurgencies in the southern provinces fourteen years ago, there have been more than four thousand incidences of shooting to date [1]. This accounts for approximately 43 percent of all terrorist-related incidences [1]. While over 290 billion baht has been spent, Thailand has not been able to put a stop to the problem [1]. As a result of these terrorist activities, more than four thousand people have died and ten thousand people have been injured. In the latest Global Terrorism Index published by Institute for Economics and Peace – a global risk assessor – Thailand was ranked 16 of 130 and was classified as extremely at risk [2].

However, these incidences have rarely led to a successful prosecution by the criminal justice system. No perpetrator was identified in about 70 percent of all national security-related cases that reached the court. Of the remaining 30 percent where the perpetrator(s) could be identified, 25 percent of these cases resulted in no arrest [3]. This resulted from a combination of the following reasons: lack of physical evidence linking suspects to the crimes; lack of experience of investigators; lack of eyewitness testimony; the inability to protect key witnesses; and the difficulty in obtaining permission to perform autopsies on Muslim victims [4].

A DNA typing technique called direct PCR has been gathering attention lately. The process has been applied to touch or trace DNA on many substrates and casework samples [5–10]. A modified direct PCR protocol called the dilution or pre-PCR solution protocol also have been applied to forensically-relevant samples with good success rates [11–13]. Direct PCR bypasses the DNA extraction and

quantification step. This could be beneficial for a low-level DNA sample, as the process of DNA extraction has very low efficiency [14, 15]. An additional benefit is that all available cellular materials in the sample are added to the PCR reaction as opposed to only a small portion of the DNA extract. Direct PCR also saves time and resources, as no extraction is needed. It also enables a researcher to tease out the effect of a variable being studied directly as the variation in extraction efficiency is eliminated. Only one limited study by our group investigated direct PCR for fired bullets [16], and another study applied direct PCR to unfired bullet casings [9]. These two previous results showed that direct PCR has the potential to increase the number of alleles obtained from bullet casings. A more detailed study with a larger sample size and conditions that reflect real-world scenarios would help determine if direct PCR could be beneficial for this evidence type. Direct PCR could also help forensic analysts understand and quantify the effects of firing, gun type, and ammunition type on touch DNA.

Therefore, the aims of the study were (1) to evaluate whether direct PCR would improve the STR profiles obtained from bullet casings, (2) to further understand the effects of ammunition type and firing on STR profiles and (3) to develop a baseline information for real casework analysis of bullet casings from different gun and ammunition types.

Introduction

Firearm–related crimes are undoubtedly a significant problem in many nations. In the United States, over 400,000 people were victims of a crime committed with a firearm each year. Firearms are used in almost 70% of murders and half of robbery offences. In a country with political unrest and terrorism–related activities such as Thailand, over six thousand terrorist attacks (54% of all attacks) in the past 10 years involved firearms. It is not uncommon for cartridges, bullets, and casings (CBCs) to be left at a crime scene involving shootings [17]. During loading of guns, the handler touches the bullets and hence these casings can be a valuable source of touch DNA [18]. Firearms are often shared in an organized crime setting, which adds further complications to the STR typing and interpretation process. A detailed previous study on development of STR profiles from CBCs resulted in a very low success rate, as most profiles obtained were either a partial or a no profile [19–21]. It is presumed that the heat and pressure exerted on the sample during the shooting process is detrimental to DNA – lowering the amount of DNA from an already low–template sample – and that the metal ions in the CBCs are potential inhibitors [22]. However, only two previous studies examined the different factors that could affect the STR typing process [20, 21].

A DNA typing technique called direct PCR has been gathering attention lately. The process has been applied to touch or trace DNA on many substrates and casework samples [5–10]. A modified direct PCR protocol called the dilution or pre–PCR solution protocol also have been applied to forensically–relevant samples with good success rates [11–13]. Direct PCR bypasses the DNA extraction and quantification step. This could be beneficial for a low–level DNA sample, as the process of DNA extraction has very low efficiency [14, 15]. An additional benefit is that all available cellular materials in the sample are added to the PCR reaction as opposed to only a small portion of the DNA extract. Direct PCR also saves time and resources, as no extraction is needed. It also enables a researcher to tease out the effect of a variable being studied directly as the variation in extraction efficiency is eliminated. Only one limited study by our group investigated direct PCR for fired bullets [16], and another study applied direct PCR to unfired bullet casings [9]. These two previous results showed

that direct PCR has the potential to increase the number of alleles obtained from bullet casings. A more detailed study with a larger sample size and conditions that reflect real-world scenarios would help determine if direct PCR could be beneficial for this evidence type. Direct PCR could also help forensic analysts understand and quantify the effects of firing, gun type, and ammunition type on touch DNA.

Therefore, the aims of the study were (1) to evaluate whether direct PCR would improve the STR profiles obtained from bullet casings, (2) to further understand the effects of ammunition type and firing on STR profiles and (3) to develop a baseline information for real casework analysis of bullet casings from different gun and ammunition types.

Methodology:

Experiments

Four experiments were conducted. Three guns and ammunition types were used in this study: (1) Glock Model 19 Gen4 with 9x19mm Luger (Bullet Master Co., Ltd., Bangkok, Thailand), (2) AK47 with 7.62x39mm (Ordnance Department, Royal Thai Army, Thailand), and (3) Tavor TAR-21 with 5.56x45mm NATO (Ordnance Department, Royal Thai Army, Thailand). The floor, guns, and ammunitions were cleaned with de-ionized water and 70% ethanol prior to testing. The bullets and the oils used for the guns were UV irradiated for 2 h to remove any extraneous DNA. All volunteers washed their hands one hour prior to handling the bullets. The guns were fired at angle by a gloved ballistics expert (except for the mock casework experiment) to direct the bullet casing to the floor. Clean forceps were used to collect the casings into an envelope. The research has been approved by the Ethical Committee, Faculty of Medicine, Prince of Songkla University (approval no. 57-027-19-2).

Fired vs. unfired

In this experiment, 60 stains of 5 uL of buffy coat (containing 21 ng of DNA) were deposited using a micropipette and smeared on 9mm bullet casings. Each bullet received one stain. The stains were

left to dry for 24 h. Thirty bullets were randomized to the unfired group and another thirty were randomized to the fired group.

Extraction vs. direct protocol vs. dilution protocol

Ten volunteers with unknown shedder status held nine 9mm bullets for 15 s in each hand. All bullets were fired by a gloved ballistics expert with the order of the volunteers randomized and the guns cleaned between each volunteer. Thirty bullet casings (three from each volunteer) were subjected to DNA extraction, quantification, and STR profiling. Another 30 casings were subjected to a direct PCR protocol described below. The last 30 casings were subjected to a pre-PCR dilution protocol [11].

Ammunition type

Ten volunteers each held three bullets from each ammunition type (5.56, 7.62, and 9 mm bullets) for 15 s in one hand, transferred the bullets to the other hand, and then held them for another 15 s. All bullets were fired. The guns were cleaned in-between using bullets from each volunteer. All bullets were subjected to direct PCR.

Mock casework

Ten volunteers held three bullets from each ammunition type (5.56, 7.62, and 9 mm bullets) as long as he/she needed to load the bullet into the magazine. The volunteers then fired the gun without wearing any glove. The guns were thoroughly cleaned with deionized water, wiped down with 70% ethanol, and oiled with fresh, UV-irradiated oil after the third and the seventh volunteer. After completing the experiment for one gun and ammunition type, all volunteers washed their hands thoroughly, waited for at least one hour, and continued with the next gun and ammunition type.

Sample collection

Single swab technique using EO cotton swab (Thai Gauze, Bangkok, Thailand) moistened with phosphate-buffered saline (PBS) (Sigma-Aldrich, MO, USA) was used to swab the bullet casings. Swabbing was carried out by swabbing lengthwise direction of the casing with only the distal end

of the swab head touching the casing. The same swab was then used to swab widthwise direction, again with only the distal end touching the casing. An unused, DNA-free bullet was swabbed as a negative control. The swabs were then air dried in a fume hood. Swabs were then frozen at -20C and only thawed for batch processing to minimize confounding factors.

DNA extraction

Samples to be processed with conventional PCR were first extracted using the DNA IQ™ System (Promega Corporation, WI, USA) with the small sample casework protocol per the manufacturer's recommendation. DNA was eluted in 30 uL elution buffer and stored at -20C until used. A negative extraction control was included with each batch.

DNA quantification

Extracted DNA were quantified using the Quantifiler® Human DNA Quantification Kit (Thermo Fisher Scientific, CA, USA) per the manufacturer's protocol. DNA standards and negative controls were included with each plate.

STR amplification

STR amplification of extracted DNA were performed in 25 uL reaction of the Identifiler® Plus Kit (Thermo Fisher Scientific), following the manufacturer's protocol. One ng of DNA was the optimal DNA input amount. Samples with DNA concentrations higher than the target were diluted to 0.1 ng/uL, and 10 uL of DNA extract was added. Similarly, maximum input volume of 10 uL was used for samples with DNA concentration of less than 0.1 ng/uL.

For the direct PCR protocol, ten 1-mm^2 pieces were cut from the distal end of the swab and placed into a PCR tube and amplification-grade water was used to make up the final volume.

For the pre-PCR solution protocol, the distal end the swab was cut into 20 pieces of 1-mm^2. They were then added to 20 uL PBS in a 1.5 mL microcentrifuge tube. The tube was vortexed for 30 s and then heated at 98C for 2 min. Ten uL was used for STR amplification.

All PCR reactions were amplified using a Bio–Rad T100™ thermal cycler (Bio–Rad, CA, USA). PCR conditions were as follows: initial denaturation at 95C for 11 min, 29 cycles of 94C for 20 s and 59C for 3 min, and a final extension at 60C for 10min.

Detection and analysis

PCR products were detected using an ABI 3130*xl* Genetic Analyzer using the manufacturer's protocol. Analysis of raw data were carried out using GeneMapper® ID v 3.2.1. Relevant data were exported to the open–source statistical program R [23]. An analytical threshold of 50 RFU and a stochastic threshold of 200 RFUs were used.

Three factors were investigated: (1) effect of DNA extraction on the amount of DNA recovered, (2) the effect of firing on DNA loss, (3), the effect of gun type and ammunition type, and (4) the number of donor's alleles and non–donor's alleles observed. All statistical comparisons were carried out using Bayesian credible intervals. Bayesian 95% credible intervals of the means and 95% credible intervals for effect sizes were calculated using "Bayesian Estimation Supersedes the T–test (BEST)" [24]. Burn–in length and sampling length of 40,000 were used. Bayesian credible intervals provide a direct, probabilistic statements through the use of posterior distributions and can accommodate different prior beliefs. Non–overlapping credible intervals indicate a high probability of having a, in frequentist terms, statistically significant difference ($p < 0.001$) [24, 25]. Additionally, these calculations are robust to outliers and do not depend on normal distribution of the data at hand. For meaningful interpretation of effect sizes, we referred to an interactive website (http://rpsychologist.com/d3/cohend/).

Results and discussion

Fired vs unfired

In order to quantify the effect of DNA extraction and firing on DNA on bullets, 5 uL of buffy coat (containing 21 ng of DNA in total) were deposited onto sixty 9mm bullets. Figure 1 shows the percent of DNA recovered from the 30 bullets that were fired and 30 bullets that were not fired. On average,

we were able to recovered 45% of DNA originally deposited (95% credible interval: 36% to 51%). Percent recovery were clustered around the arithmetic means with longer tail toward the higher percentages. Firing the bullets reduced the percent recovery to 17% (95% credible interval: 7% to 24%). As such, firing the bullets was responsible for a further 27% decrease in percent DNA recovery (effect size of 3.68 with 95% HDI of 2.06 to 5.40). Most of the data points for fired bullets were clustered around 0% to 10% with only two samples exhibiting more 50% percent DNA recovery.

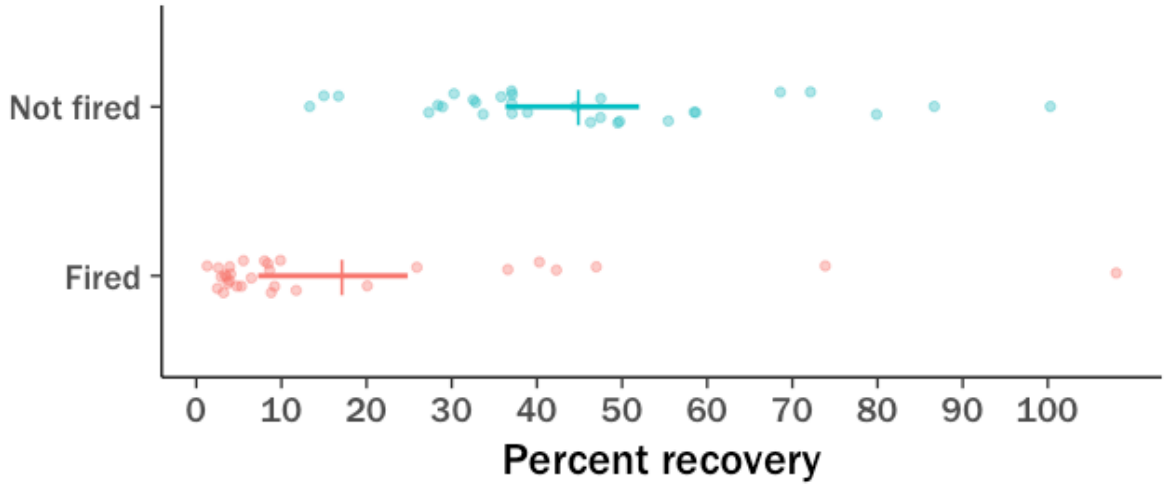

Figure 1. RDI plot for percent DNA recovery from fired and not fired 9mm bullets (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

The loss in DNA was attributed to the deposition, collection, and DNA extraction process. The amount of DNA lost through collection and DNA extraction process were in line with previous studies [14, 15, 26]. DNA extraction efficiency has been shown to range from 30% to 70%, depending on the method used and the sample type. The percent of DNA lost through firing (27%) in our study agrees with Montpetit *et al.* [21], who showed firing had detrimental effects to STR typing. However, Horsman–Hall *et al.* [20] did not see any significant decrease in the amount of DNA between fired and unfired bullet casings. The difference between these two studies was the small sample size used in Horsman–Hall *et al.* (n = 5 vs 335) and the different extraction and quantification kits, which could affect the amount of DNA recovered, amount of inhibitor co–extracted, and also robustness to inhibitor.

We chose buffy coat for this experiment instead of touch DNA because we wanted to minimize confounding effect from variations in shedder status. Buffy coat has been used as a substitute for touch DNA in previous studies [27–29]. Although one could control the amount of time a volunteer used to touch certain object, the amount of touch DNA actually deposited could vary up to two orders of magnitude, due to many factors such as shedder status and personal behavior prior to touch DNA deposition [30–33]. As such, using touch DNA would obfuscate direct comparison between the fired and unfired bullets.

Extraction vs direct vs dilution protocols

Using three STR typing methods, the direct protocol recovered the highest number of alleles from fired bullet casings, followed conventional extraction and then by dilution protocol (Figure 2). No full profile was obtained from any of the 90 casings typed. No alleles were seen in three samples (10%) for direct protocol, 10 samples (33%) for conventional extraction protocol, and 24 samples (80%) for the dilution protocol. The means (and 95% credible intervals) were 11.1 (7.9 to 13.9), 5.6 (3.0 to 7.7), 2.3 (0.2 to 4.0) alleles for direct protocol, conventional extraction protocol, and dilution protocol, respectively. The mean effect sizes of the difference were 0.71 (0.21 to 1.32), 1.37 (0.31 to 2.02), and 2.09 (1.14 to 3.53) for direct protocol vs. extraction protocol, extraction protocol vs. dilution protocol, and direct protocol vs. dilution protocol, respectively. In other words, the probability of superiority (the chance that a sample picked at random from one group will have more alleles than the other group) was 69% for direct protocol vs. extraction protocol.

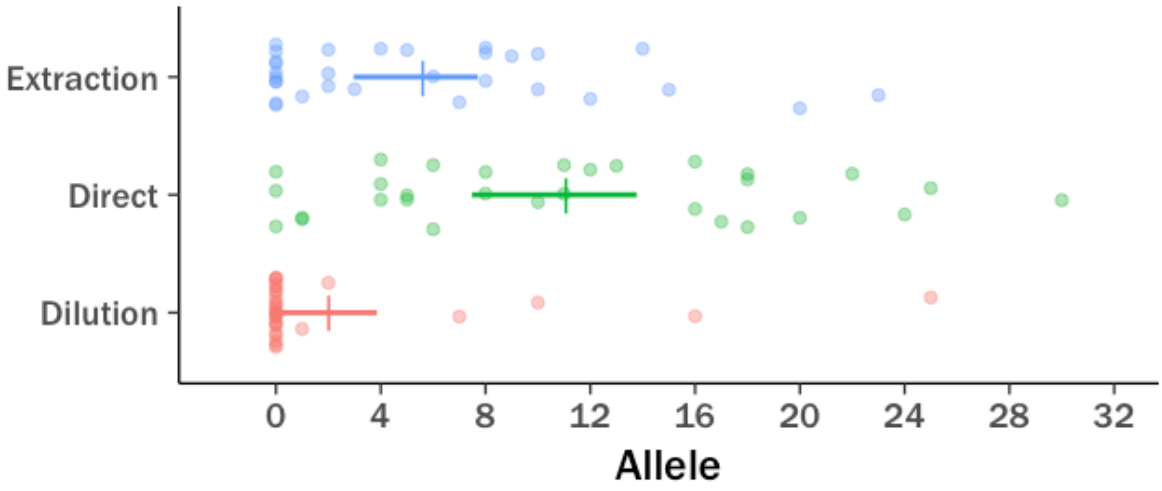

Figure 2. RDI plot for the number of alleles recovered using three different pre-PCR methods: conventional extraction, direct STR typing with direct protocol, and direct STR typing with the dilution protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference ($p < 0.001$).

DNA extractions from bullet casings resulted in very low DNA yields (Figure 3). Twenty–two of 30 samples (73%) had DNA concentrations of less than 10 pg/uL. Only two samples had DNA concentration of over 50 pg/uL. Even with 10 uL input, the total amounts of DNA added to the Identifiler Plus reaction mix were still lower than the optimal amount of DNA input required (~1000 ng) for a full STR profile [34]. As such, it is not surprising that we were not able to obtain even one full STR profile from these samples.

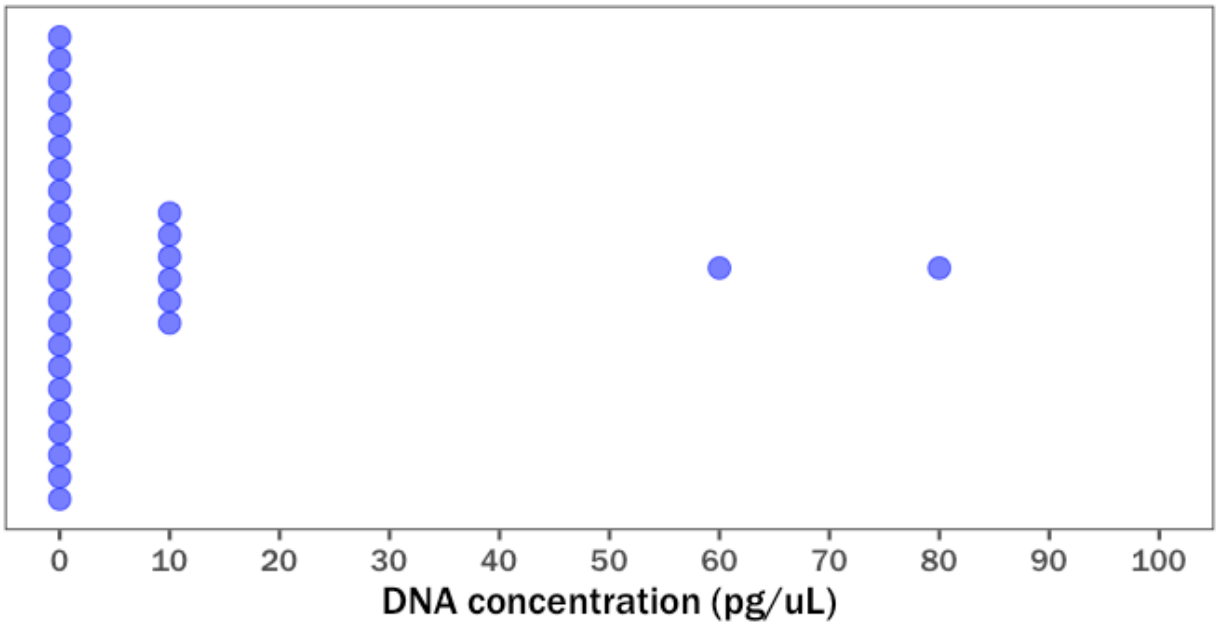

Figure 3. Dotplot showing the distribution of the concentration of extracted DNA (in pg/uL) from touch DNA on bullet casings. Each dot represents one sample. One column of dot represents an increment of 50 pg. For example, there are 22 samples with DNA concentration between 0 and 10 pg/uL and six samples with DNA concentration between 10 to 20 pg/uL.

Due to the inefficiency of the DNA extraction process, even a whole swab head did not yield more alleles when compared to the direct protocol wherein only ten pieces of the swab was added directly to the PCR reaction. Direct PCR protocol has been shown to improve STR profiles from touch DNA [7, 9]. The differences between the extraction protocol and direct PCR protocol in this study was not as significant compared with those reported by Martin *et al.* [9]. This could be due to the difference between the use of unfired casings in their study compared to fired casings here. Fired casings are covered in gunshot residues, which could inhibit PCR reactions. DNA extraction process could help to eliminate these inhibitors, while they are present during a direct PCR protocol. As such, there may be a trade-off between the inefficiency of DNA extraction and the removal of inhibitors from collected samples. We tried to tackled this trade-off with direct PCR via a dilution protocol. Unlike the successes of the dilution protocol for other evidence types in previous reports [11, 13], our results indicated that the dilution protocol is not suitable for touch DNA on bullet casings. The reason the dilution protocol yielded fewer alleles than the two other protocols could be due to the dilution of the already low template DNA present on the bullet casings while not being able to sufficiently remove PCR inhibitors.

We did not report a profile as interpretable, as this depends on the laboratory performing the analyses and the criteria used could be different between different laboratories and countries. Instead, we opted to report the number of alleles detected directly. For direct PCR, the difference in this study and the previous study [16] reported by us was the reaction volume and the number of PCR cycle. The full reaction volume (25 uL) and the 29 cycles used here could have mitigated the negative effect of PCR inhibitors present in the pieces of swab. Our extraction protocol result agrees with previous studies that performed STR typing from extracted DNA on fired bullet casings [20].

Ammunition type

We investigated the effect of ammunition and gun types on the probability of obtaining an STR profile from bullet casings. Three guns and ammunition types were used. Figure 3 shows the RDI plot for the number of alleles recovered from fired touched bullets. We recovered, on average (and 95% credible intervals), 10.9 (7.1 to 13.4), 10.5 (6.8 to 13.3), and 11.2 (7.9 to 13.8) alleles from 9mm, AK47, and Tavor bullet casings, respectively. The overlapping 95% Bayesian credible intervals between the three ammunition types indicate no credible difference between the three. In other words, despite a four times difference in power factor between the two assault rifles and the handgun, one could expect similar STR profiles from the bullet casings. We did not calculate the effect size for these comparisons.

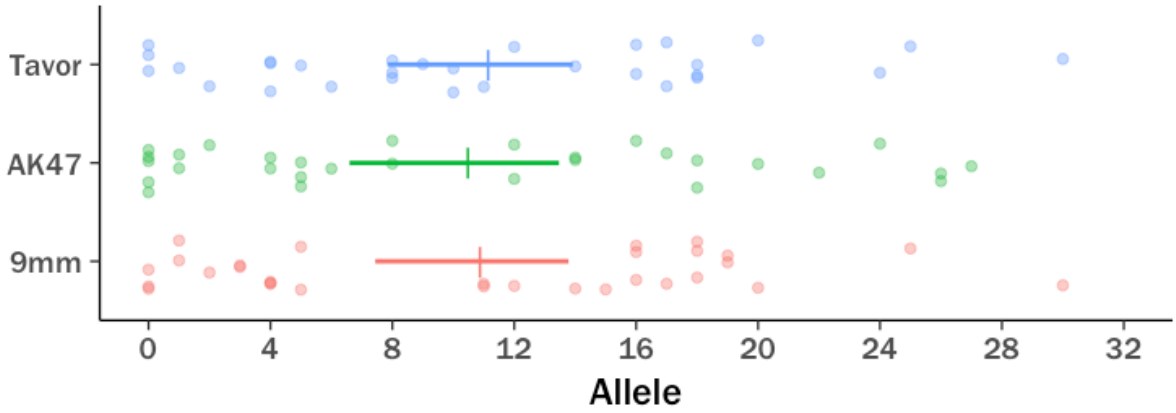

Figure 4. RDI plot for the number of alleles recovered using three different ammunition and gun types: 9mm, AK47, and Tavor 21 using the direct PCR protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the

arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate highly credible difference (equivalent to frequentist p < 0.001).

This was surprising, as we expected the automatic rifles to exert a more detrimental effect on the touch DNA found on bullet casings. Perhaps this was counteracted by the increased surface area of the bullet. The Tavor bullets (5.56 x 45 mm) and the AK47 bullets (7.62 x 39 mm) are much longer than the 9mm bullets (9 x 19 mm), resulting in about 60% increase in surface area. A previous study also found no statistical difference in the number of alleles observed from five different ammunition types [20].

Mock casework

In order to mimic real casework, volunteers were asked to load bullets into a magazine, fired them, and handed the guns to the next volunteers. Cleaning was performed before the 4th volunteer and the 8th volunteer to mimic gun sharing. Alleles belonging to the volunteer (self) and others (non–self) were noted in the STR profiles. Similar to the previous experiments, the means (and 95% credible interval) of alleles recovered were 10.5 (7.6 to 13.3), 10.5 (7.3 to 13.2), and 12.0 (8.2 to 15.1) for 9mm, AK47, and Tavor, respectively. The overlapping 95% credible intervals suggest no credible difference between the number of alleles recovered for the three gun and ammunition types. Also, the numbers of self–alleles and non–self–alleles did not credibly differ between the three ammunition types (95% credible intervals overlapping 0s for all pairwise comparisons; data not shown).

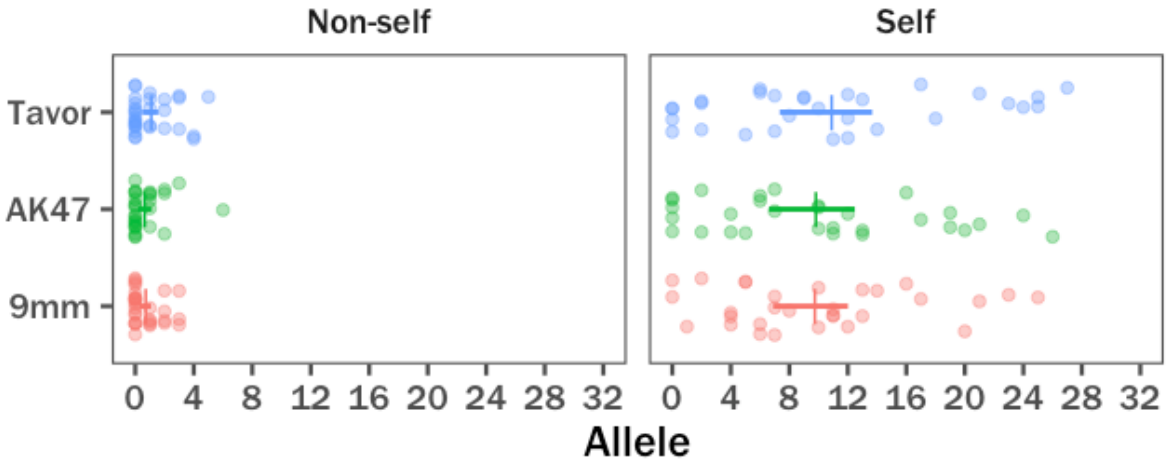

Figure 5. RDI plot for the number of self and non-self alleles recovered using three different ammunition and gun types: 9mm, AK47, and Tavor 21 using the direct PCR protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

Combining data from the three ammunition types, 41% of the profiles contained one or more allele that do not belong to the loader's known genotype, 44% were consistent with single–source contribution (or masking due to overlapping allele from minor contributors), and 10% failed to produce any allele at all (Table 1). Bayesian proportion test showed no credible differences between the gun and ammunition types in the proportions of single–source, mixture, and no profile samples (all 95% pairwise credible intervals overlap zero).

Table 1. Number of mock casework samples categorized by profile type (single-source, mixture, or no profile) for each gun and ammunition type. ND denotes "no credible difference" (95% credible interval overlapping 0) in all pairwise comparisons (9mm vs AK47 vs Tavor).

	9mm	AK47	Tavor	Total	Pairwise difference
Single source	16 (53%)	15 (50%)	13 (43%)	44 (49%)	ND
Mixture	12 (40%)	11 (37%)	14 (47%)	37 (41%)	ND
No profile	2 (7%)	4 (13%)	3 (10%)	9 (10%)	ND
Total	30	30	30	90	–

In order for an STR profile to be uploadable to the Royal Thai Police DNA database, it must contain at least 16 alleles from any STR locus (including the amelogenin locus). Using this criterion, the percentages of mock casework profiles that could be uploaded to the database for each gun/ammunition type were calculated (Figure 6). Overall, less than 30% of profiles were considered uploadable. These numbers suggest that Thai forensic DNA analysts must weigh the cost of generating an STR profile against the low probability of obtaining an interpretable profile. No public information regarding STR typing from bullet casings was available from the Royal Thai Police, but bullet casings are not generally processed due to their low probability of obtaining an interpretable profile (personal comm.). Other touch DNA evidence processed at the Police Forensic Science Center 10, Office of Forensic Science, Royal Thai Police, generated about 10% interpretable profiles, using the conventional PCR technique [27].

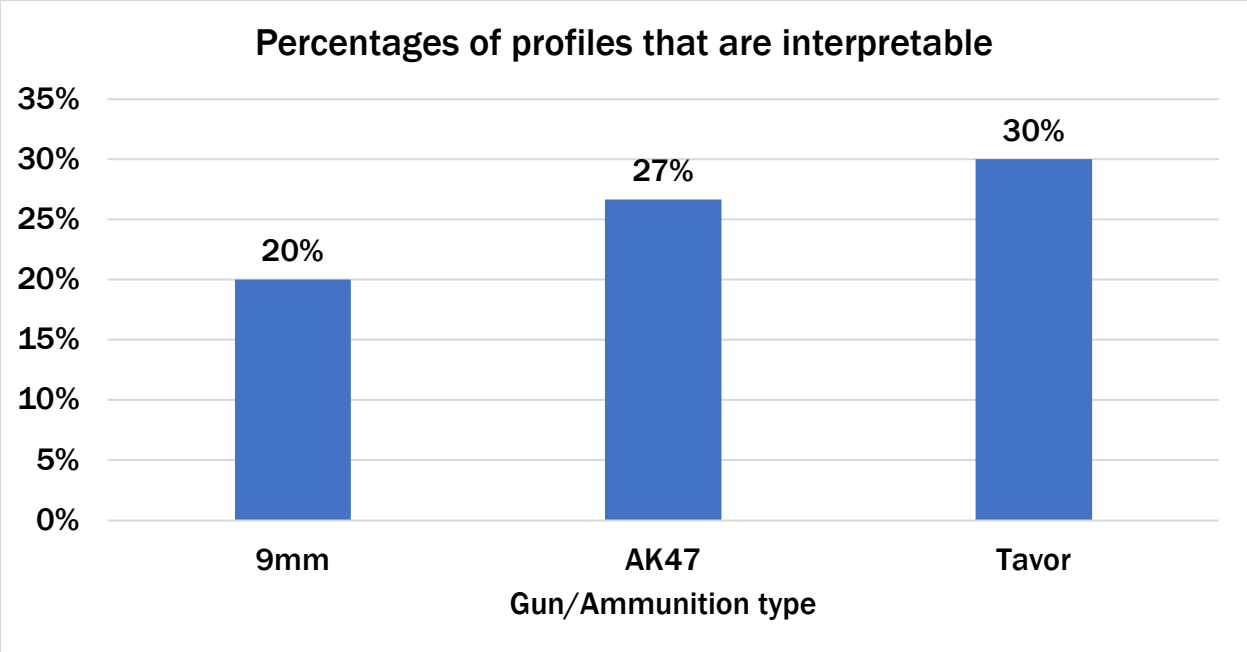

Figure 6. Percentages of profiles that are interpretable (more than 16 alleles detected) under the Royal Thai Police DNA Database for each gun/ammunition type is shown.

Our finding agrees with Horsman–Hall *et al.* [20], Martin *et al.* [9], and Montpetit *et al.* [21], all of which showed that mixtures are common from gun surfaces, bullets, and cartridges in both controlled experimental conditions and from real casework evidence. Mixtures could have arisen from at least two scenarios: (1) other people's DNA already presented on the volunteers' hands prior to touching

the bullets and loading the guns, and (2) the transfer of DNA from casings to the guns and from the guns to the casings. As volunteers carried out their daily activities during the one–hour period post–handwashing, it was highly likely that they picked up DNA from interacting with other individuals and from daily objects [35, 36]. Additionally, it was also possible for touch DNA on the bullet casings to be transferred to the inner gun surfaces and then back to another bullet casing. Previous studies have shown that objects can act as an intermediary for DNA transfer (i.e. secondary and tertiary transfers) [37–39].

All shedder statuses (light, intermediate, and heavy) were observed (Figure 7). Volunteer 7 seemed to a be a heavy shedder, as not even a single bullet casing resulted in a no profile. On the other hand, volunteer 6 and 8 seemed to be a light shedder, as only three and one STR profiles had over 12 alleles. The other shedders fell into the moderate shedder status category. A recent study that examined shedder status directly using fluorescent staining of cellular materials from fingermarks also established that there may be a normal distribution of shedder status, with fewer people being extremely light or extremely heavy shedders [33], a finding that was also supported by other previous studies [37, 40]. Large variations in the number of alleles were observed for most volunteers. This finding agrees with Kanokwongnuwut *et al.* [33], who found that although shedder status is reproducible when the number of cells deposited was used, intra–individual variations in STR profile quality could be seen. We attributed these variations to other confounding factors associated with this study, such as variations in the amount of DNA degradation resulting from the firing process, variations in the amount of touch DNA transferred to other materials (e.g. the floor lining) prior to the collection and PCR process, and variations in PCR efficiency due to the amount of PCR inhibitors collected by the swabs.

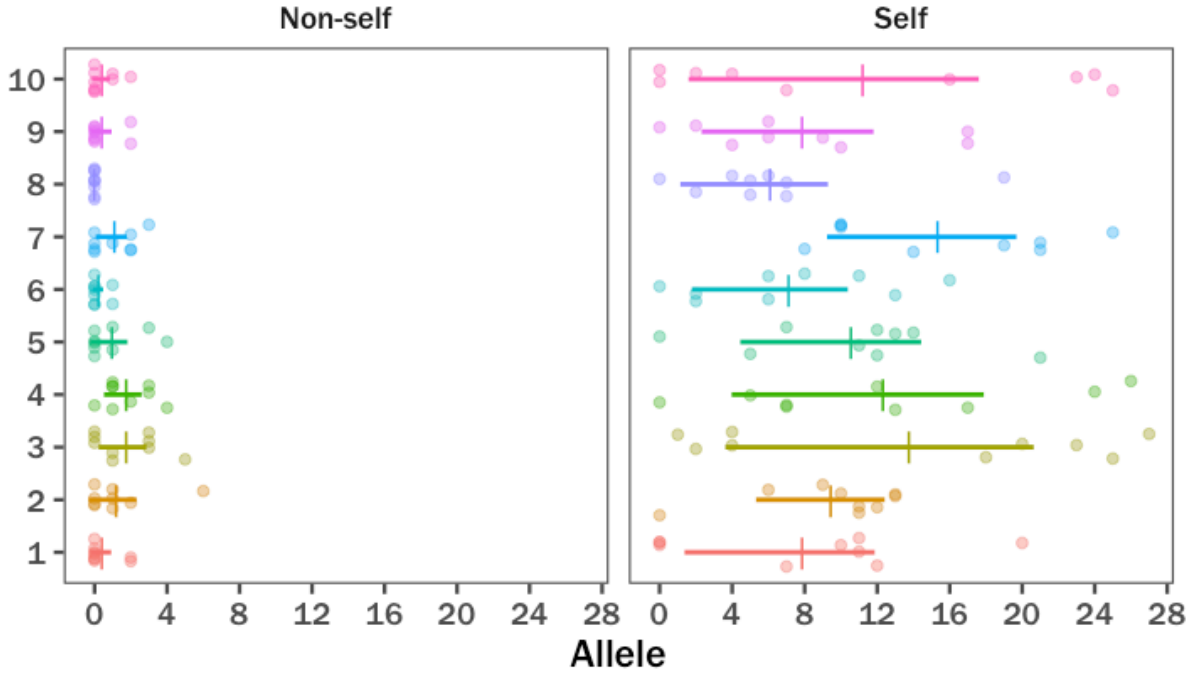

Figure 7. RDI plot for the number of self and non-self alleles recovered for each volunteer (N = 9 for each volunteer). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

Due to the high incidences of mixtures obtained in mock casework experiments, we encourage careful interpretation of STR profiles generated from touch DNA on bullet casings. Expert interpretation systems, both commercial and free, that utilize continuous models have been validated in recent years [41, 42] and should be useful for interpreting mixed profiles, especially from casework bullet casings in which the number of contributors are not known . Gun sharing is not uncommon in organized crimes, and the presence of multiple contributors could complicate profile interpretation, particularly when dealing with touch DNA and low-template DNA amplifications. With ever increasing analytical sensitivity, forensic DNA analysts must strive to be vigilant in their practices and cautious in their interpretations.

Conclusion

The use of direct PCR for touch DNA on bullet casings showed an increase in the number of alleles obtained. However, the difference between extraction and direct PCR is not as stark compared with earlier studies, probably due to the inhibitors and the low level of DNA present on the casings.

Different gun types and ammunition types had no effect on the STR profiles, despite our initial hypothesis. Mock casework experiment, in which the guns were not cleaned between used to mimic real-world gun sharing, showed that direct PCR mainly picked up the alleles of the person who loaded the bullets. Continuum in shedder statuses among the volunteers were observed. The high incidence of mixed STR profiles indicated that expert interpretation systems should be incorporated in laboratories that performed touch DNA typing. In conclusion, direct PCR should be considered for processing bullet casings, and bullet casings can provide a valuable source of DNA.

Acknowledgements

This work was supported by the Thailand Research Fund [grant numbers MRG5580105]. We acknowledge the help of Ms. Dalad Mattayat, Pol. Maj. Sukanya Phetpeng, and the DNA Analysis Center, Police Forensic Science Center 10, Royal Thai Police.

Conflict of interest

None

Reference

1. ศูนย์ข่าวภาคใต้. *14* ปีไฟใต้ละลายงบ *3* แสนล้าน กองทัพเชื่อมั่นปีหน้าปิดเกม! 2018 [cited 2018 11 October]; Available from: https://www.isranews.org/content-page/67-south-slide/62488-success.html.
2. *Global Terrorism Index 2017*. 2017, Institute for Economics and Peace: Sydney, Australia.
3. ป. ย. วรศิริ, อ. แต้ชูตระกูล and ก. ศุภวรรธนะกูล, ยุติธรรมไม่มา ปัญหาไฟใต้ไม่จบ, in สุขภาพคนไทย *2554 :* เอชไอเอ กลไกพัฒนานโยบายสาธารณะเพื่อชีวิตและสุขภาพ, ช. น. ย. กาญจนะจิตรา, Editor. 2011, อมรินทร์พริ้นติ้งแอนด์พับลิชชิ่ง จำกัด: นครปฐม. p. 55-58.
4. ทีมข่าวอิศรา, ข้อมูลตำรวจ...ไฟใต้ 7 ปี 7 เดือนตายทะลุ 4.7 พันราย ประทุษร้ายพระ 33 คดี 194 หมู่บ้านป่วน, Isranews Agency, 2011.
5. R. Blackie, D. Taylor and A. Linacre, DNA profiles from clothing fibers using direct PCR. Forensic Sci. Med. Pathol. 12 (2016) 331-5.
6. J. E. Templeton, D. Taylor, O. Handt, P. Skuza and A. Linacre, Direct PCR Improves the Recovery of DNA from Various Substrates. J. Forensic Sci. 60 (2015) 1558-62.
7. J. E. Templeton and A. Linacre, DNA profiles from fingermarks. BioTechniques 57 (2014) 259-66.

8. A. Linacre, V. Pekarek, Y. C. Swaran and S. S. Tobe, Generation of DNA profiles from fabrics without DNA extraction. Forensic Sci Int Genet 4 (2010) 137–41.
9. B. Martin, R. Blackie, D. Taylor and A. Linacre, DNA profiles generated from a range of touched sample types. Forensic Sci Int Genet 36 (2018) 13–19.
10. S. Verheij, J. Harteveld and T. Sijen, A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples. Forensic Sci Int Genet 6 (2012) 167–75.
11. T. Kitpipit, W. Chotigeat, A. Linacre and P. Thanakiatkrai, Forensic animal DNA analysis using economical two-step direct PCR. Forensic Sci. Med. Pathol. 10 (2014) 29–38.
12. T. Kitpipit, K. Sittichan and P. Thanakiatkrai, Direct-multiplex PCR assay for meat species identification in food products. Food Chem. 163 (2014) 77–82.
13. P. Thanakiatkrai, K. Raham, J. Pradutkanchana, S. Sotthibandhu and T. Kitpipit, Direct-STR typing from presumptively-tested and untreated body fluids. Forensic Sci Int Genet 30 (2017) 1–9.
14. R. Kishore, W. Reef Hardy, V. J. Anderson, N. A. Sanchez and M. R. Buoncristiani, Optimization of DNA extraction from low-yield and degraded samples using the BioRobot EZ1 and BioRobot M48. J. Forensic Sci. 51 (2006) 1055–61.
15. A. Colussi, M. Viegas, J. Beltramo and M. Lojo, Efficiency of DNA IQ System® in recovering semen DNA from cotton swabs. Forensic Sci. Int. Genet. Suppl. Ser. 2 (2009) 87–88.
16. P. Thanakiatkrai and B. Rerkamnuaychoke, Direct STR typing from bullet casings. Forensic Science International Genetics Supplement Series 6 (2017) E164–E166.
17. B. a. J. Fisher, Techniques of Crime Scene Investigation. (2004).
18. R. A. Van Oorschot, K. N. Ballantyne and R. J. Mitchell, Forensic trace DNA: a review. Investig Genet 1 (2010) 14.
19. P. Dieltjes, R. Mieremet, S. Zuniga, T. Kraaijenbrink, J. Pijpe and P. De Knijff, A sensitive method to extract DNA from biological traces present on ammunition for the purpose of genetic profiling. Int. J. Legal Med. 125 (2011) 597–602.
20. K. M. Horsman-Hall, Y. Orihuela, S. L. Karczynski, A. L. Davis, J. D. Ban and S. Greenspoon, Development of STR profiles from firearms and fired cartridge cases. Forensic science international. Genetics 3 (2009) 242–50.
21. S. Montpetit and P. O'donnell, An optimized procedure for obtaining DNA from fired and unfired ammunition. Forensic Sci Int Genet 17 (2015) 70–74.
22. L. G. Combs, J. E. Warren, V. Huynh, J. Castaneda, T. D. Golden and R. K. Roby, The effects of metal ion PCR inhibitors on results obtained with the Quantifiler ® Human DNA Quantification Kit. Forensic Sci. Int. Genet. 19 (2015) 180–189.
23. R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2018.
24. J. K. Kruschke, Bayesian estimation supersedes the t test. J. Exp. Psychol. Gen. 142 (2013) 573–603.
25. J. K. Kruschke, Bayesian data analysis. Wiley Interdiscip. Rev. Cogn. Sci. 1 (2010) 658–76.

26. R. a. H. Van Oorschot, D. G. Phelan, S. Furlong, G. M. Scarfo, N. L. Holding and M. J. Cummins, Are you collecting all the available DNA from touched objects? International Congress Series 1239 (2003) 803–807.
27. S. Phetpeng, T. Kitpipit and P. Thanakiatkrai, Systematic study for DNA recovery and profiling from common IED substrates: From laboratory to casework. Forensic Sci Int Genet 17 (2015) 53–60.
28. J. J. Raymond, R. A. Van Oorschot, P. R. Gunn, S. J. Walsh and C. Roux, Trace evidence characteristics of DNA: A preliminary investigation of the persistence of DNA at crime scenes. Forensic Sci Int Genet 4 (2009) 26–33.
29. J. J. Raymond, S. J. Walsh, R. a. H. Van Oorschot, P. R. Gunn, L. Evans and C. Roux, Assessing trace DNA evidence from a residential burglary: Abundance, transfer and persistence. Forensic Sci. Int. Genet. Suppl. Ser. 1 (2008) 442–443.
30. D. J. Daly, C. Murphy and S. D. Mcdermott, The transfer of touch DNA from hands to glass, fabric and wood. Forensic Sci Int Genet 6 (2012) 41–6.
31. R. A. Wickenheiser, Trace DNA: A review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. J. Forensic Sci. 47 (2002) 442–450.
32. R. W. Allen, J. Pogemiller, J. Joslin, M. Gulick and J. Pritchard, Identification Through Typing of DNA Recovered From Touch Transfer Evidence: Parameters Affecting Yield of Recovered Human DNA. Journal of Forensic Identification 58 (2008) 33–41.
33. P. Kanokwongnuwut, B. Martin, K. P. Kirkbride and A. Linacre, Shedding light on shedders. Forensic Sci Int Genet 36 (2018) 20–25.
34. D. Y. Wang, C. W. Chang, R. E. Lagace, L. M. Calandro and L. K. Hennessy, Developmental validation of the AmpFlSTR(R) Identifiler(R) Plus PCR Amplification Kit: an established multiplex assay with improved performance. J. Forensic Sci. 57 (2012) 453–65.
35. B. Szkuta, K. N. Ballantyne, B. Kokshoorn and R. a. H. Van Oorschot, Transfer and persistence of non–self DNA on hands over time: Using empirical data to evaluate DNA evidence given activity level propositions. Forensic Sci Int Genet 33 (2018) 84–97.
36. B. Szkuta, K. N. Ballantyne and R. a. H. Van Oorschot, Transfer and persistence of DNA on the hands and the influence of activities performed. Forensic Sci Int Genet 28 (2017) 10–20.
37. A. E. Fonnelop, M. Ramse, T. Egeland and P. Gill, The implications of shedder status and background DNA on direct and secondary transfer in an attack scenario. Forensic Sci Int Genet 29 (2017) 48–60.
38. D. Taylor, A. Biedermann, L. Samie, K. M. Pun, T. Hicks and C. Champod, Helping to distinguish primary from secondary transfer events for trace DNA. Forensic Sci Int Genet 28 (2017) 155–177.
39. A. E. Fonneløp, H. Johannessen and P. Gill, Persistence and secondary transfer of DNA from previous users of equipment. Forensic Sci. Int. Genet. Suppl. Ser. 5 (2015) e191–e192.
40. G. E. Meakin, E. V. Butcher, R. a. H. Van Oorschot and R. M. Morgan, Trace DNA evidence dynamics: An investigation into the deposition and persistence of directly– and indirectly–transferred DNA on regularly–used knives. Forensic Sci Int Genet 29 (2017) 38–47.

41. J. S. Buckleton, J. A. Bright, S. Gittelson, T. R. Moretti, A. J. Onorato, F. R. Bieber, B. Budowle and D. A. Taylor, The Probabilistic Genotyping Software STRmix: Utility and Evidence for its Validity. J. Forensic Sci. (2018).

42. S. Manabe, C. Morimoto, Y. Hamano, S. Fujimoto and K. Tamaki, Development and validation of open–source software for DNA mixture interpretation based on a quantitative continuous model. PLoS One 12 (2017) e0188183.

Outputs

1. Manuscript entitled “Direct STR typing from fired and unfired bullet casings” ready to be submitted to an international forensic journal indexed in the Web of Science (WOS/ISI) database.
2. Poster presentation at งานประชุม “นักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส” ครั้งที่ 14 at Ambassador City Jomtien Hotel, Chonburi. 23 October 2014.

DIRECT STR TYPING FROM FIRED AND UNFIRED BULLET CASINGS

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Abstract

Cartridges, bullets, and casings (CBCs) are commonly encountered in shooting incidences and could provide valuable DNA information from touch DNA that has been left during bullet handling and gun loading; however, conventional DNA analysis has yielded very poor results. Direct PCR, in which the DNA extraction and quantification steps are bypassed, has been shown to provide comparable and sometimes improved results from touch DNA and trace DNA samples. Here, we aimed to apply direct PCR with bullet casings (from three ammunition types and guns) and evaluate whether it should be recommended as a standard operating protocol for forensic DNA analysts. Three experiments were carried out to investigate the following: the effect of firing on DNA deposited on bullet casings; the effect of gun and ammunition types on STR profile quality; and the feasibility of using direct PCR with actual cases via typing of mock casework samples. DNA extraction resulted in a loss of about 40% of DNA originally deposited, and firing a bullet decreased the amount of DNA recovered by 27%. We recovered means (and 95% credible intervals) of 11.1 (7.9 to 13.9), 5.6 (3.0 to 7.7), 2.3 (0.2 to 4.0) alleles from touch DNA on fired bullet casings using the direct PCR protocol, conventional extraction protocol, and dilution protocol, respectively. No statistical difference in alleles recovered was observed between different fired ammunition types from three guns (9mm, 7.62 mm, and 5.56 mm from Glock Model 19, AK47, and Tavor T-21, respectively). As expected, mixed DNA profiles were observed in 40% of mock casework samples in which guns are shared between volunteers, which can complicate profile interpretation. This study showed that direct PCR from bullet casings improved STR profiles. As the direct PCR protocol is quicker, cheaper, and resulted in more alleles recovered, forensic DNA analysts may benefit from using direct PCR.

Keywords: Direct PCR; bullet casings; fired bullets; touch DNA; ammunition

Introduction

Firearm-related crimes are undoubtedly a significant problem in many nations. In the United States, over 400,000 people were victims of a crime committed with a firearm each year. Firearms are used in almost 70% of murders and half of robbery offences. In a country with political unrest and terrorism-related activities such as Thailand, over six thousand terrorist attacks (54% of all attacks) in the past 10 years involved firearms. It is not uncommon for cartridges, bullets, and casings (CBCs) to be left at a crime scene involving shootings (Fisher, 2004). During loading of guns, the handler touches the bullets and hence these casings can be a valuable source of touch DNA (R. A. van Oorschot, Ballantyne, & Mitchell, 2010). Firearms are often shared in an organized crime setting, which adds further complications to the STR typing and interpretation process. A detailed previous study on development of STR profiles from CBCs resulted in a very low success rate, as most profiles obtained were either a partial or a no profile (Dieltjes et al., 2011; Horsman-Hall et al., 2009; Montpetit & O'Donnell, 2015). It is presumed that the heat and pressure exerted on the sample during the shooting process is detrimental to DNA – lowering the amount of DNA from an already low-template sample – and that the metal ions in the CBCs are potential inhibitors (Combs et al., 2015). However, only two previous studies examined the different factors that could affect the STR typing process (Horsman-Hall et al., 2009; Montpetit & O'Donnell, 2015).

A DNA typing technique called direct PCR has been gathering attention lately. The process has been applied to touch or trace DNA on many substrates and casework samples (Blackie, Taylor, & Linacre, 2016; Linacre, Pekarek, Swaran, & Tobe, 2010; Martin, Blackie, Taylor, & Linacre, 2018; Templeton & Linacre, 2014; Templeton, Taylor, Handt, Skuza, & Linacre, 2015; Verheij, Harteveld, & Sijen, 2012). A modified direct PCR protocol called the dilution or pre-PCR solution protocol also have been applied to forensically-relevant samples with good success rates (Kitpipit, Chotigeat, Linacre, & Thanakiatkrai, 2014; Kitpipit, Sittichan, & Thanakiatkrai, 2014; Thanakiatkrai, Raham, Pradutkanchana, Sotthibandhu, & Kitpipit, 2017). Direct PCR bypasses the DNA extraction and quantification step. This could be beneficial for a low-level DNA sample, as the process of DNA extraction has very low efficiency (Colussi, Viegas, Beltramo, & Lojo, 2009; Kishore, Reef Hardy, Anderson, Sanchez, & Buoncristiani, 2006). An additional benefit is that all available cellular materials in the sample are added to the PCR reaction as opposed to only a small portion of the DNA extract. Direct PCR also saves time and resources, as no extraction is needed. It also enables a researcher to tease out the effect of a variable being studied directly as the variation in extraction efficiency is eliminated. Only one limited study by our group investigated direct PCR for fired bullets (Thanakiatkrai & Rerkamnuaychoke, 2017), and another study applied direct PCR to unfired bullet casings (Martin et al., 2018). These two previous results showed that direct PCR has the

potential to increase the number of alleles obtained from bullet casings. A more detailed study with a larger sample size and conditions that reflect real-world scenarios would help determine if direct PCR could be beneficial for this evidence type. Direct PCR could also help forensic analysts understand and quantify the effects of firing, gun type, and ammunition type on touch DNA.

Therefore, the aims of the study were (1) to evaluate whether direct PCR would improve the STR profiles obtained from bullet casings, (2) to further understand the effects of ammunition type and firing on STR profiles and (3) to develop a baseline information for real casework analysis of bullet casings from different gun and ammunition types.

Methodology:

Experiments

Four experiments were conducted. Three guns and ammunition types were used in this study: (1) Glock Model 19 Gen4 with 9x19mm Luger (Bullet Master Co., Ltd., Bangkok, Thailand), (2) AK47 with 7.62x39mm (Ordnance Department, Royal Thai Army, Thailand), and (3) Tavor TAR-21 with 5.56x45mm NATO (Ordnance Department, Royal Thai Army, Thailand). The floor, guns, and ammunitions were cleaned with de-ionized water and 70% ethanol prior to testing. The bullets and the oils used for the guns were UV irradiated for 2 h to remove any extraneous DNA. All volunteers washed their hands one hour prior to handling the bullets. The guns were fired at angle by a gloved ballistics expert (except for the mock casework experiment) to direct the bullet casing to the floor. Clean forceps were used to collect the casings into an envelope. The research has been approved by the Ethical Committee, Faculty of Medicine, Prince of Songkla University (approval no. 57-027-19-2).

Fired vs. unfired

In this experiment, 60 stains of 5 uL of buffy coat (containing 21 ng of DNA) were deposited using a micropipette and smeared on 9mm bullet casings. Each bullet received one stain. The stains were left to dry for 24 h. Thirty bullets were randomized to the unfired group and another thirty were randomized to the fired group.

Extraction vs. direct protocol vs. dilution protocol

Ten volunteers with unknown shedder status held nine 9mm bullets for 15 s in each hand. All bullets were fired by a gloved ballistics expert with the order of the volunteers randomized and the guns cleaned between each volunteer. Thirty bullet casings (three from each volunteer) were subjected to DNA extraction, quantification, and STR profiling. Another 30 casings were subjected to a direct PCR protocol described below. The last 30 casings were subjected to a pre-PCR dilution protocol (Kitpipit, Chotigeat, et al., 2014).

Ammunition type

Ten volunteers each held three bullets from each ammunition type (5.56, 7.62, and 9 mm bullets) for 15 s in one hand, transferred the bullets to the other hand, and then held them for another 15 s. All bullets were fired. The guns were cleaned in-between using bullets from each volunteer. All bullets were subjected to direct PCR.

Mock casework

Ten volunteers held three bullets from each ammunition type (5.56, 7.62, and 9 mm bullets) as long as he/she needed to load the bullet into the magazine. The volunteers then fired the gun without wearing any glove. The guns were thoroughly cleaned with deionized water, wiped down with 70% ethanol, and oiled with fresh, UV-irradiated oil after the third and the seventh volunteer. After completing the experiment for one gun and ammunition type, all volunteers washed their hands thoroughly, waited for at least one hour, and continued with the next gun and ammunition type.

Sample collection

Single swab technique using EO cotton swab (Thai Gauze, Bangkok, Thailand) moistened with phosphate-buffered saline (PBS) (Sigma–Aldrich, MO, USA) was used to swab the bullet casings. Swabbing was carried out by swabbing lengthwise direction of the casing with only the distal end of the swab head touching the casing. The same swab was then used to swab widthwise direction, again with only the distal end touching the casing. An unused, DNA-free bullet was swabbed as a negative control. The swabs were then air dried in a fume hood. Swabs were then frozen at -20C and only thawed for batch processing to minimize confounding factors.

DNA extraction

Samples to be processed with conventional PCR were first extracted using the DNA IQ™ System (Promega Corporation, WI, USA) with the small sample casework protocol per the manufacturer's recommendation. DNA was eluted in 30 uL elution buffer and stored at -20C until used. A negative extraction control was included with each batch.

DNA quantification

Extracted DNA were quantified using the Quantifiler® Human DNA Quantification Kit (Thermo Fisher Scientific, CA, USA) per the manufacturer's protocol. DNA standards and negative controls were included with each plate.

STR amplification

STR amplification of extracted DNA were performed in 25 uL reaction of the Identifiler® Plus Kit (Thermo Fisher Scientific), following the manufacturer's protocol. One ng of DNA was the

optimal DNA input amount. Samples with DNA concentrations higher than the target were diluted to 0.1 ng/uL, and 10 uL of DNA extract was added. Similarly, maximum input volume of 10 uL was used for samples with DNA concentration of less than 0.1 ng/uL.

For the direct PCR protocol, ten 1-mm² pieces were cut from the distal end of the swab and placed into a PCR tube and amplification-grade water was used to make up the final volume.

For the pre-PCR solution protocol, the distal end the swab was cut into 20 pieces of 1-mm². They were then added to 20 uL PBS in a 1.5 mL microcentrifuge tube. The tube was vortexed for 30 s and then heated at 98C for 2 min. Ten uL was used for STR amplification.

All PCR reactions were amplified using a Bio-Rad T100™ thermal cycler (Bio-Rad, CA, USA). PCR conditions were as follows: initial denaturation at 95C for 11 min, 29 cycles of 94C for 20 s and 59C for 3 min, and a final extension at 60C for 10min.

Detection and analysis

PCR products were detected using an ABI 3130*xl* Genetic Analyzer using the manufacturer's protocol. Analysis of raw data were carried out using GeneMapper® ID v 3.2.1. Relevant data were exported to the open-source statistical program R (R Core Team, 2018). An analytical threshold of 50 RFU and a stochastic threshold of 200 RFUs were used.

Three factors were investigated: (1) effect of DNA extraction on the amount of DNA recovered, (2) the effect of firing on DNA loss, (3), the effect of gun type and ammunition type, and (4) the number of donor's alleles and non-donor's alleles observed. All statistical comparisons were carried out using Bayesian credible intervals. Bayesian 95% credible intervals of the means and 95% credible intervals for effect sizes were calculated using "Bayesian Estimation Supersedes the T-test (BEST)" (Kruschke, 2013). Burn-in length and sampling length of 40,000 were used. Bayesian credible intervals provide a direct, probabilistic statements through the use of posterior distributions and can accommodate different prior beliefs. Non-overlapping credible intervals indicate a high probability of having a, in frequentist terms, statistically significant difference ($p < 0.001$) (Kruschke, 2010, 2013). Additionally, these calculations are robust to outliers and do not depend on normal distribution of the data at hand. For meaningful interpretation of effect sizes, we referred to an interactive website (http://rpsychologist.com/d3/cohend/).

Results and discussion

Fired vs unfired

In order to quantify the effect of DNA extraction and firing on DNA on bullets, 5 uL of buffy coat (containing 21 ng of DNA in total) were deposited onto sixty 9mm bullets. Figure 1 shows

the percent of DNA recovered from the 30 bullets that were fired and 30 bullets that were not fired. On average, we were able to recovered 45% of DNA originally deposited (95% credible interval: 36% to 51%). Percent recovery were clustered around the arithmetic means with longer tail toward the higher percentages. Firing the bullets reduced the percent recovery to 17% (95% credible interval: 7% to 24%). As such, firing the bullets was responsible for a further 27% decrease in percent DNA recovery (effect size of 3.68 with 95% HDI of 2.06 to 5.40). Most of the data points for fired bullets were clustered around 0% to 10% with only two samples exhibiting more 50% percent DNA recovery.

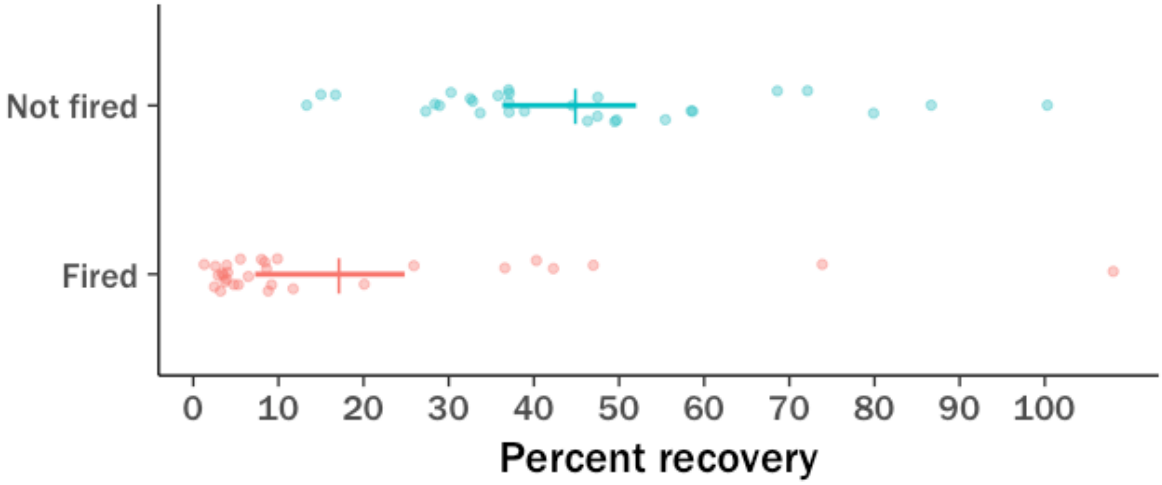

Figure 1. RDI plot for percent DNA recovery from fired and not fired 9mm bullets (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

The loss in DNA was attributed to the deposition, collection, and DNA extraction process. The amount of DNA lost through collection and DNA extraction process were in line with previous studies (Colussi et al., 2009; Kishore et al., 2006; R. A. H. van Oorschot et al., 2003). DNA extraction efficiency has been shown to range from 30% to 70%, depending on the method used and the sample type. The percent of DNA lost through firing (27%) in our study agrees with Montpetit *et al.* (Montpetit & O'Donnell, 2015), who showed firing had detrimental effects to STR typing. However, Horsman-Hall *et al.* (Horsman-Hall et al., 2009) did not see any significant decrease in the amount of DNA between fired and unfired bullet casings. The difference between these two studies was the small sample size used in Horsman-Hall *et al.* (*n* = 5 vs 335) and the different extraction and quantification kits, which could affect the amount of DNA recovered, amount of inhibitor co-extracted, and also robustness to inhibitor.

We chose buffy coat for this experiment instead of touch DNA because we wanted to minimize confounding effect from variations in shedder status. Buffy coat has been used as a substitute for touch DNA in previous studies (Phetpeng, Kitpipit, & Thanakiatkrai, 2015; J. J. Raymond,

van Oorschot, Gunn, Walsh, & Roux, 2009; Jennifer J. Raymond et al., 2008). Although one could control the amount of time a volunteer used to touch certain object, the amount of touch DNA actually deposited could vary up to two orders of magnitude, due to many factors such as shedder status and personal behavior prior to touch DNA deposition (Allen, Pogemiller, Joslin, Gulick, & Pritchard, 2008; Daly, Murphy, & McDermott, 2012; Kanokwongnuwut, Martin, Kirkbride, & Linacre, 2018; Wickenheiser, 2002). As such, using touch DNA would obfuscate direct comparison between the fired and unfired bullets.

Extraction vs direct vs dilution protocols

Using three STR typing methods, the direct protocol recovered the highest number of alleles from fired bullet casings, followed conventional extraction and then by dilution protocol (Figure 2). No full profile was obtained from any of the 90 casings typed. No alleles were seen in three samples (10%) for direct protocol, 10 samples (33%) for conventional extraction protocol, and 24 samples (80%) for the dilution protocol. The means (and 95% credible intervals) were 11.1 (7.9 to 13.9), 5.6 (3.0 to 7.7), 2.3 (0.2 to 4.0) alleles for direct protocol, conventional extraction protocol, and dilution protocol, respectively. The mean effect sizes of the difference were 0.71 (0.21 to 1.32), 1.37 (0.31 to 2.02), and 2.09 (1.14 to 3.53) for direct protocol vs. extraction protocol, extraction protocol vs. dilution protocol, and direct protocol vs. dilution protocol, respectively. In other words, the probability of superiority (the chance that a sample picked at random from one group will have more alleles than the other group) was 69% for direct protocol vs. extraction protocol.

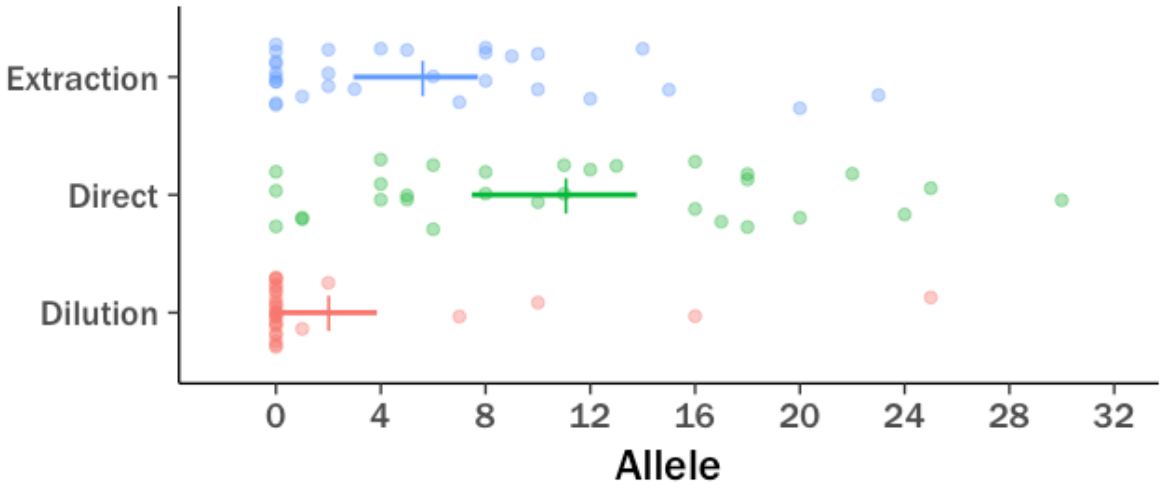

Figure 2. RDI plot for the number of alleles recovered using three different pre-PCR methods: conventional extraction, direct STR typing with direct protocol, and direct STR typing with the dilution protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference ($p < 0.001$).

Due to the inefficiency of the DNA extraction process, even a whole swab head did not yield more alleles when compared to the direct protocol wherein only ten pieces of the swab was added directly to the PCR reaction. Direct PCR protocol has been shown to improve STR profiles from touch DNA (Martin et al., 2018; Templeton & Linacre, 2014). The differences between the extraction protocol and direct PCR protocol in this study was not as significant compared with those reported by Martin *et al.* (Martin et al., 2018). This could be due to the difference between the use of unfired casings in their study compared to fired casings here. Fired casings are covered in gunshot residues, which could inhibit PCR reactions. DNA extraction process could help to eliminate these inhibitors, while they are present during a direct PCR protocol. As such, there may be a trade-off between the inefficiency of DNA extraction and the removal of inhibitors from collected samples. We tried to tackled this trade-off with direct PCR via a dilution protocol. Unlike the successes of the dilution protocol for other evidence types in previous reports (Kitpipit, Chotigeat, et al., 2014; Thanakiatkrai et al., 2017), our results indicated that the dilution protocol is not suitable for touch DNA on bullet casings. The reason the dilution protocol yielded fewer alleles than the two other protocols could be due to the dilution of the already low template DNA present on the bullet casings while not being able to sufficiently remove PCR inhibitors.

We did not report a profile as interpretable, as this depends on the laboratory performing the analyses and the criteria used could be different between different laboratories and countries. Instead, we opted to report the number of alleles detected directly. For direct PCR, the difference in this study and the previous study (Thanakiatkrai & Rerkamnuaychoke, 2017) reported by us was the reaction volume and the number of PCR cycle. The full reaction volume (25 uL) and the 29 cycles used here could have mitigated the negative effect of PCR inhibitors present in the pieces of swab. Our extraction protocol result agrees with previous studies that performed STR typing from extracted DNA on fired bullet casings (Horsman-Hall et al., 2009).

Ammunition type

We investigated the effect of ammunition and gun types on the probability of obtaining an STR profile from bullet casings. Three guns and ammunition types were used. Figure 3 shows the RDI plot for the number of alleles recovered from fired touched bullets. We recovered, on average (and 95% credible intervals), 10.9 (7.1 to 13.4), 10.5 (6.8 to 13.3), and 11.2 (7.9 to 13.8) alleles from 9mm, AK47, and Tavor bullet casings, respectively. The overlapping 95% Bayesian credible intervals between the three ammunition types indicate no credible difference between the three. In other words, despite a four times difference in power factor between the two assault rifles and the handgun, one could expect similar STR profiles from the bullet casings. We did not calculate the effect size for these comparisons.

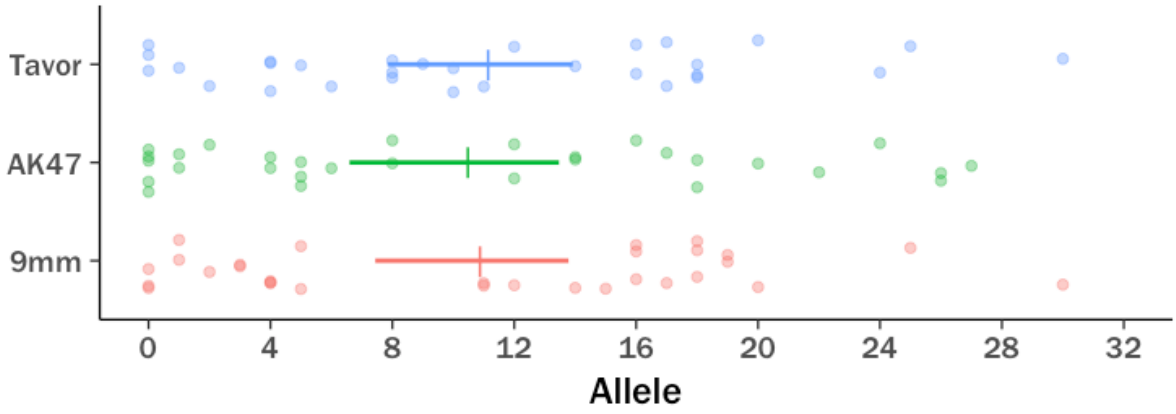

Figure 3. RDI plot for the number of alleles recovered using three different ammunition and gun types: 9mm, AK47, and Tavor 21 using the direct PCR protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate highly credible difference (equivalent to frequentist p < 0.001).

This was surprising, as we expected the automatic rifles to exert a more detrimental effect on the touch DNA found on bullet casings. Perhaps this was counteracted by the increased surface area of the bullet. The Tavor bullets (5.56 x 45 mm) and the AK47 bullets (7.62 x 39 mm) are much longer than the 9mm bullets (9 x 19 mm), resulting in about 60% increase in surface area. A previous study also found no statistical difference in the number of alleles observed from five different ammunition types (Horsman-Hall et al., 2009).

Mock casework

In order to mimic real casework, volunteers were asked to load bullets into a magazine, fired them, and handed the guns to the next volunteers. Cleaning was performed before the 4th volunteer and the 8th volunteer to mimic gun sharing. Alleles belonging to the volunteer (self) and others (non-self) were noted in the STR profiles. Similar to the previous experiments, the means (and 95% credible interval) of alleles recovered were 10.5 (7.6 to 13.3), 10.5 (7.3 to 13.2), and 12.0 (8.2 to 15.1) for 9mm, AK47, and Tavor, respectively. The overlapping 95% credible intervals suggest no credible difference between the number of alleles recovered for the three gun and ammunition types. Also, the numbers of self-alleles and non-self-alleles did not credibly differ between the three ammunition types (95% credible intervals overlapping 0s for all pairwise comparisons; data not shown).

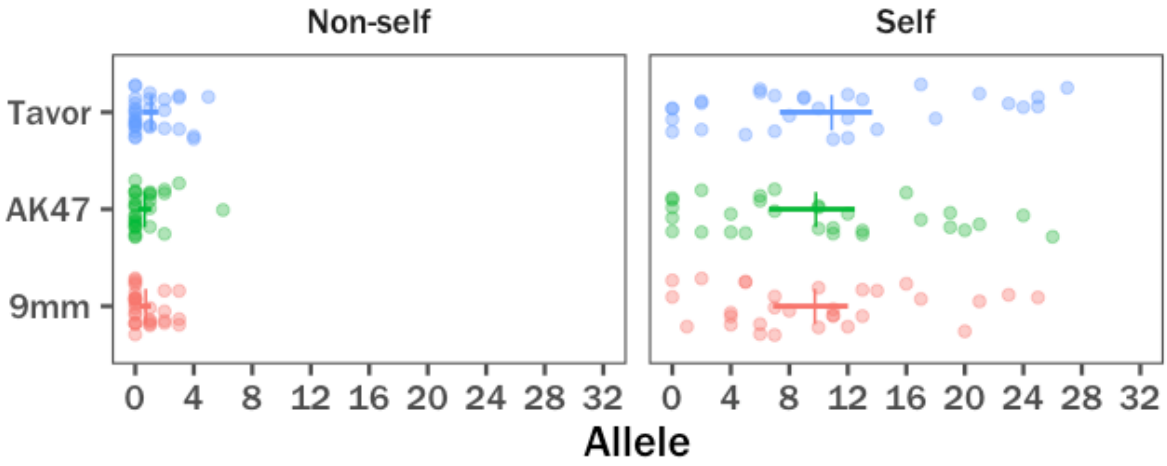

Figure 4. RDI plot for the number of self and non-self alleles recovered using three different ammunition and gun types: 9mm, AK47, and Tavor 21 using the direct PCR protocol (N = 30 for each treatment). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

Combining data from the three ammunition types, 41% of the profiles contained one or more allele that do not belong to the loader's known genotype, 44% were consistent with single-source contribution (or masking due to overlapping allele from minor contributors), and 10% failed to produce any allele at all (Table 1). Bayesian proportion test showed no credible differences between the gun and ammunition types in the proportions of single-source, mixture, and no profile samples (all 95% pairwise credible intervals overlap zero).

Table 1. Number of mock casework samples categorized by profile type (single-source, mixture, or no profile) for each gun and ammunition type. ND denotes "no credible difference" (95% credible interval overlapping 0) in all pairwise comparisons (9mm vs AK47 vs Tavor).

	9mm	AK47	Tavor	Total	Pairwise difference
Single source	16 (53%)	15 (50%)	13 (43%)	44 (49%)	ND
Mixture	12 (40%)	11 (37%)	14 (47%)	37 (41%)	ND
No profile	2 (7%)	4 (13%)	3 (10%)	9 (10%)	ND
Total	30	30	30	90	-

Our finding agrees with Horsman-Hall *et al.* (Horsman-Hall et al., 2009), Martin *et al.* (Martin et al., 2018), and Montpetit *et al.* (Montpetit & O'Donnell, 2015), all of which showed that mixtures are common from gun surfaces, bullets, and cartridges in both controlled experimental conditions and from real casework evidence. Mixtures could have arisen from at least two scenarios: (1) other people's DNA already presented on the volunteers' hands prior to touching the bullets and loading the guns, and (2) the transfer of DNA from casings to the

guns and from the guns to the casings. As volunteers carried out their daily activities during the one-hour period post-handwashing, it was highly likely that they picked up DNA from interacting with other individuals and from daily objects (Szkuta, Ballantyne, Kokshoorn, & van Oorschot, 2018; Szkuta, Ballantyne, & van Oorschot, 2017). Additionally, it was also possible for touch DNA on the bullet casings to be transferred to the inner gun surfaces and then back to another bullet casing. Previous studies have shown that objects can act as an intermediary for DNA transfer (i.e. secondary and tertiary transfers) (Fonneløp, Johannessen, & Gill, 2015; Fonnelop, Ramse, Egeland, & Gill, 2017; Taylor et al., 2017).

All shedder statuses (light, intermediate, and heavy) were observed (Figure 5). Volunteer 7 seemed to a be a heavy shedder, as not even a single bullet casing resulted in a no profile. On the other hand, volunteer 6 and 8 seemed to be a light shedder, as only three and one STR profiles had over 12 alleles. The other shedders fell into the moderate shedder status category. A recent study that examined shedder status directly using fluorescent staining of cellular materials from fingermarks also established that there may be a normal distribution of shedder status, with fewer people being extremely light or extremely heavy shedders (Kanokwongnuwut et al., 2018), a finding that was also supported by other previous studies (Fonnelop et al., 2017; Meakin, Butcher, van Oorschot, & Morgan, 2017). Large variations in the number of alleles were observed for most volunteers. This finding agrees with Kanokwongnuwut *et al.* (Kanokwongnuwut et al., 2018), who found that although shedder status is reproducible when the number of cells deposited was used, intra-individual variations in STR profile quality could be seen. We attributed these variations to other confounding factors associated with this study, such as variations in the amount of DNA degradation resulting from the firing process, variations in the amount of touch DNA transferred to other materials (e.g. the floor lining) prior to the collection and PCR process, and variations in PCR efficiency due to the amount of PCR inhibitors collected by the swabs.

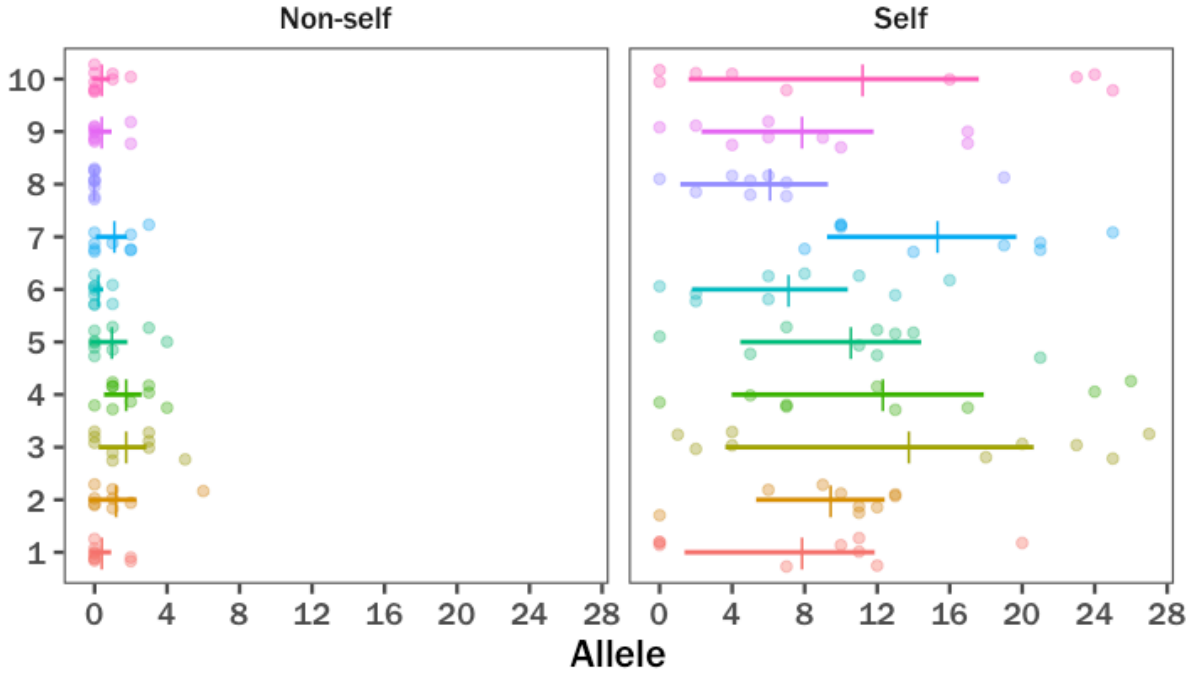

Figure 5. RDI plot for the number of self and non-self alleles recovered for each volunteer (N = 9 for each volunteer). The circles show the raw data, the vertical bars show the arithmetic means, and the horizontal lines show the 95% Bayesian credible intervals with 40000 iterations. Non-overlapping intervals indicate significant difference.

Due to the high incidences of mixtures obtained in mock casework experiments, we encourage careful interpretation of STR profiles generated from touch DNA on bullet casings. Expert interpretation systems, both commercial and free, that utilize continuous models have been validated in recent years (Buckleton et al., 2018; Manabe, Morimoto, Hamano, Fujimoto, & Tamaki, 2017) and should be useful for interpreting mixed profiles, especially from casework bullet casings in which the number of contributors are not known . Gun sharing is not uncommon in organized crimes, and the presence of multiple contributors could complicate profile interpretation, particularly when dealing with touch DNA and low-template DNA amplifications. With ever increasing analytical sensitivity, forensic DNA analysts must strive to be vigilant in their practices and cautious in their interpretations.

Conclusion

The use of direct PCR for touch DNA on bullet casings showed an increase in the number of alleles obtained. However, the difference between extraction and direct PCR is not as stark compared with earlier studies, probably due to the inhibitors and the low level of DNA present on the casings. Different gun types and ammunition types had no effect on the STR profiles, despite our initial hypothesis. Mock casework experiment, in which the guns were not cleaned between used to mimic real-world gun sharing, showed that direct PCR mainly picked up the alleles of the person who loaded the bullets. Continuum in shedder statuses among the

volunteers were observed. The high incidence of mixed STR profiles indicated that expert interpretation systems should be incorporated in laboratories that performed touch DNA typing. In conclusion, direct PCR should be considered for processing bullet casings, and bullet casings can provide a valuable source of DNA.

Acknowledgements

This work was supported by the Thailand Research Fund [grant numbers MRG5580105]. We acknowledge the help of Ms. Dalad Mattayat, Pol. Maj. Sukanya Phetpeng, and the DNA Analysis Center, Police Forensic Science Center 10, Royal Thai Police.

Conflict of interest

None

Reference

Allen, R. W., Pogemiller, J., Joslin, J., Gulick, M., & Pritchard, J. (2008). Identification Through Typing of DNA Recovered From Touch Transfer Evidence: Parameters Affecting Yield of Recovered Human DNA. *Journal of Forensic Identification, 58*(1), 33–41.

Blackie, R., Taylor, D., & Linacre, A. (2016). DNA profiles from clothing fibers using direct PCR. *Forensic Science, Medicine, and Pathology, 12*(3), 331-335. doi:10.1007/s12024-016-9784-y

Buckleton, J. S., Bright, J. A., Gittelson, S., Moretti, T. R., Onorato, A. J., Bieber, F. R., . . . Taylor, D. A. (2018). The Probabilistic Genotyping Software STRmix: Utility and Evidence for its Validity. *Journal of Forensic Sciences*. doi:10.1111/1556-4029.13898

Colussi, A., Viegas, M., Beltramo, J., & Lojo, M. (2009). Efficiency of DNA IQ System® in recovering semen DNA from cotton swabs. *Forensic Science International: Genetics Supplement Series, 2*(1), 87-88.

Combs, L. G., Warren, J. E., Huynh, V., Castaneda, J., Golden, T. D., & Roby, R. K. (2015). The effects of metal ion PCR inhibitors on results obtained with the Quantifiler ® Human DNA Quantification Kit. *Forensic Science International: Genetics, 19*, 180-189. doi:10.1016/j.fsigen.2015.06.013

Daly, D. J., Murphy, C., & McDermott, S. D. (2012). The transfer of touch DNA from hands to glass, fabric and wood. *Forensic Sci Int Genet, 6*(1), 41-46. doi:10.1016/j.fsigen.2010.12.016

Dieltjes, P., Mieremet, R., Zuniga, S., Kraaijenbrink, T., Pijpe, J., & de Knijff, P. (2011). A sensitive method to extract DNA from biological traces present on ammunition for the

purpose of genetic profiling. *International Journal of Legal Medicine, 125*, 597-602. doi:10.1007/s00414-010-0454-4

Fisher, B. A. J. (2004). Techniques of Crime Scene Investigation.

Fonneløp, A. E., Johannessen, H., & Gill, P. (2015). Persistence and secondary transfer of DNA from previous users of equipment. *Forensic Science International: Genetics Supplement Series, 5*, e191-e192. doi:10.1016/j.fsigss.2015.09.077

Fonnelop, A. E., Ramse, M., Egeland, T., & Gill, P. (2017). The implications of shedder status and background DNA on direct and secondary transfer in an attack scenario. *Forensic Sci Int Genet, 29*, 48-60. doi:10.1016/j.fsigen.2017.03.019

Horsman-Hall, K. M., Orihuela, Y., Karczynski, S. L., Davis, A. L., Ban, J. D., & Greenspoon, S. (2009). Development of STR profiles from firearms and fired cartridge cases. *Forensic science international. Genetics, 3*, 242-250. doi:10.1016/j.fsigen.2009.02.007

Kanokwongnuwut, P., Martin, B., Kirkbride, K. P., & Linacre, A. (2018). Shedding light on shedders. *Forensic Sci Int Genet, 36*, 20-25. doi:10.1016/j.fsigen.2018.06.004

Kishore, R., Reef Hardy, W., Anderson, V. J., Sanchez, N. A., & Buoncristiani, M. R. (2006). Optimization of DNA extraction from low-yield and degraded samples using the BioRobot EZ1 and BioRobot M48. *Journal of Forensic Sciences, 51*(5), 1055-1061. doi:10.1111/j.1556-4029.2006.00204.x

Kitpipit, T., Chotigeat, W., Linacre, A., & Thanakiatkrai, P. (2014). Forensic animal DNA analysis using economical two-step direct PCR. *Forensic Science, Medicine, and Pathology, 10*(1), 29-38. doi:10.1007/s12024-013-9521-8

Kitpipit, T., Sittichan, K., & Thanakiatkrai, P. (2014). Direct-multiplex PCR assay for meat species identification in food products. *Food Chemistry, 163*, 77-82. doi:10.1016/j.foodchem.2014.04.062

Kruschke, J. K. (2010). Bayesian data analysis. *Wiley Interdisciplinary Reviews: Cognitive Science, 1*(5), 658-676. doi:10.1002/wcs.72

Kruschke, J. K. (2013). Bayesian estimation supersedes the t test. *Journal of Experimental Psychology: General, 142*(2), 573-603. doi:10.1037/a0029146

Linacre, A., Pekarek, V., Swaran, Y. C., & Tobe, S. S. (2010). Generation of DNA profiles from fabrics without DNA extraction. *Forensic Sci Int Genet, 4*(2), 137-141. doi:10.1016/j.fsigen.2009.07.006

Manabe, S., Morimoto, C., Hamano, Y., Fujimoto, S., & Tamaki, K. (2017). Development and validation of open-source software for DNA mixture interpretation based on a quantitative continuous model. *PloS One, 12*(11), e0188183. doi:10.1371/journal.pone.0188183

Martin, B., Blackie, R., Taylor, D., & Linacre, A. (2018). DNA profiles generated from a range of touched sample types. *Forensic Sci Int Genet, 36*, 13-19. doi:10.1016/j.fsigen.2018.06.002

Meakin, G. E., Butcher, E. V., van Oorschot, R. A. H., & Morgan, R. M. (2017). Trace DNA evidence dynamics: An investigation into the deposition and persistence of directly- and indirectly-transferred DNA on regularly-used knives. *Forensic Sci Int Genet, 29*, 38-47. doi:10.1016/j.fsigen.2017.03.016

Montpetit, S., & O'Donnell, P. (2015). An optimized procedure for obtaining DNA from fired and unfired ammunition. *Forensic Sci Int Genet, 17*, 70-74. doi:10.1016/j.fsigen.2015.03.012

Phetpeng, S., Kitpipit, T., & Thanakiatkrai, P. (2015). Systematic study for DNA recovery and profiling from common IED substrates: From laboratory to casework. *Forensic Sci Int Genet, 17*, 53-60. doi:10.1016/j.fsigen.2015.03.007

R Core Team. (2018). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Raymond, J. J., van Oorschot, R. A., Gunn, P. R., Walsh, S. J., & Roux, C. (2009). Trace evidence characteristics of DNA: A preliminary investigation of the persistence of DNA at crime scenes. *Forensic Sci Int Genet, 4*(1), 26–33. doi:10.1016/j.fsigen.2009.04.002

Raymond, J. J., Walsh, S. J., van Oorschot, R. A. H., Gunn, P. R., Evans, L., & Roux, C. (2008). Assessing trace DNA evidence from a residential burglary: Abundance, transfer and persistence. *Forensic Science International: Genetics Supplement Series, 1*(1), 442–443. doi:10.1016/j.fsigss.2007.10.040

Szkuta, B., Ballantyne, K. N., Kokshoorn, B., & van Oorschot, R. A. H. (2018). Transfer and persistence of non-self DNA on hands over time: Using empirical data to evaluate DNA evidence given activity level propositions. *Forensic Sci Int Genet, 33*, 84-97. doi:10.1016/j.fsigen.2017.11.017

Szkuta, B., Ballantyne, K. N., & van Oorschot, R. A. H. (2017). Transfer and persistence of DNA on the hands and the influence of activities performed. *Forensic Sci Int Genet, 28*, 10-20. doi:10.1016/j.fsigen.2017.01.006

Taylor, D., Biedermann, A., Samie, L., Pun, K. M., Hicks, T., & Champod, C. (2017). Helping to distinguish primary from secondary transfer events for trace DNA. *Forensic Sci Int Genet, 28*, 155-177. doi:10.1016/j.fsigen.2017.02.008

Templeton, J. E., & Linacre, A. (2014). DNA profiles from fingermarks. *BioTechniques, 57*(5), 259-266. doi:10.2144/000114227

Templeton, J. E., Taylor, D., Handt, O., Skuza, P., & Linacre, A. (2015). Direct PCR Improves the Recovery of DNA from Various Substrates. *Journal of Forensic Sciences, 60*(6), 1558-1562. doi:10.1111/1556-4029.12843

Thanakiatkrai, P., Raham, K., Pradutkanchana, J., Sotthibandhu, S., & Kitpipit, T. (2017). Direct-STR typing from presumptively-tested and untreated body fluids. *Forensic Sci Int Genet, 30*, 1-9. doi:10.1016/j.fsigen.2017.06.001

Thanakiatkrai, P., & Rerkamnuaychoke, B. (2017). Direct STR typing from bullet casings. *Forensic Science International Genetics Supplement Series, 6*, E164-E166. doi:10.1016/j.fsigss.2017.09.058

van Oorschot, R. A., Ballantyne, K. N., & Mitchell, R. J. (2010). Forensic trace DNA: a review. *Investig Genet, 1*(1), 14. doi:10.1186/2041-2223-1-14

van Oorschot, R. A. H., Phelan, D. G., Furlong, S., Scarfo, G. M., Holding, N. L., & Cummins, M. J. (2003). Are you collecting all the available DNA from touched objects? *International Congress Series, 1239*, 803-807. doi:https://doi.org/10.1016/S0531-5131(02)00498-3

Verheij, S., Harteveld, J., & Sijen, T. (2012). A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples. *Forensic Sci Int Genet, 6*(2), 167-175. doi:10.1016/j.fsigen.2011.03.014

Wang, D. Y., Chang, C. W., Lagace, R. E., Calandro, L. M., & Hennessy, L. K. (2012). Developmental validation of the AmpFlSTR(R) Identifiler(R) Plus PCR Amplification Kit: an established multiplex assay with improved performance. *Journal of Forensic Sciences, 57*(2), 453-465. doi:10.1111/j.1556-4029.2011.01963.x

Wickenheiser, R. A. (2002). Trace DNA: A review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. *Journal of Forensic Sciences, 47*(3), 442–450.