Journal of Animal and Veterinary Advances 11 (24): 4563-4566, 2012
ISSN: 1680-5593

A Novel Low-Cost Sampler for Rapid Diagnosis of Infectious Agents in Tissue

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Abstract: Preparing biological tissue samples is an extremely tedious and time-consuming laboratory task, albeit one critical for rapid diagnoses of infectious diseases. Here, researchers reported a novel, low-cost sampling technique for rapid and accurate removal of minute tissue portions from various animal organs. Each sampled tissue portion could be easily dispersed and homogenized in storage solution in 2.0 mL microcentrifuge tube and was sufficient for the molecular diagnosis of the presence or absence of infectious disease using the Polymerase Chain Reaction (PCR) technique. This new sampling technique is beneficial as it provides both uniformity for high-throughput sampling and minimization of cross-contamination.

Key words: Sampler, tissue, PCR, molecular diagnosis, cross-contamination

INTRODUCTION

Molecular diagnostics of infectious diseases in particular, nucleic-acid-based methods are the fastest growing field in clinical laboratory diagnostics (Weile and Knabbe, 2009). Rapid, high-throughput, simple techniques of nucleic acid amplification and characterization have significantly broadened the diagnostic arsenal (Speers, 2006; Muldrew, 2009). With an acute awareness of the importance of optimal sample preparation, many companies have developed integrated processes to extract, purify and concentrate nucleic acids. However, most of these extraction processes are optimized for samples in suspension such as extracting RNA/DNA from blood and cerebrospinal fluid. Solid tissue samples have seen no corresponding ease of isolation. Although, mechanical techniques involving metallic extraction instruments followed by digestion can be successfully used for solid tissue samples, it nevertheless remains an extremely tedious and time-consuming laboratory task (Yu and Cohen, 2003). When conventional tools such as tweezers, scalpels, scissors, etc. are used to obtain and mince tissue samples for routine tests, this automatically incurs great costs in terms of labor to prepare and clean the tools in addition to the concerns of cross-contamination and bio-security risk during procedural removal of a minute tissue portion from each block of fresh or frozen tissue.

Innovative tools have been developed to facilitate the excision of tissue but they have their own shortcomings. A tissue needle can be safely employed in a biopsy for example but problematic sites within the organ in question can be missed (Baker *et al*., 2004). Needle biopsy samples also might be inadequate for reverse transcription followed by the Polymerase Chain Reaction (PCR) (Samija *et al*., 2008). Punches such as Harris Uni-Core™, etc. are convenient tools for cutting very small punch discs from specific animal tissues for molecular biology applications but these products are not intended for diagnostic use (www.qiagen.com/products/qiacardftaaccessories.aspx or www.sigmaal-drich.com/catalog/product/ALDRICH/Z747432?lang=zh&region=CN).

Subsequent to obtaining the tissue, the next concern involves extraction of the biological material. In general, mechanical homogenization is the simplest approach for individual and sporadic lab diagnosis and is considered the gold standard. However, when routine diagnostic tests are required to be performed on thousands of samples each year or when targeted surveillance and response testing are required to be performed under intense time pressure owing to a bio-event such as the SARS epidemic of 2003 in East Asia or a bioterrorism scare, an effective tissue preparation technique must be available.

Researchers report here a novel sample preparation method which integrates excision and homogenization for molecular diagnosis of infectious agents in particular bacterial or viral nucleic acids in tissue. A sampler made from commercially available parts was used to instantly

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remove appropriate tissue samples and to rapidly homogenize the sample by rotating the drill bit in the storage solution within the confined environment of a microcentrifuge tube. The sampler is inexpensive and disposable, so it would be ideal for reasonable uniformity of sampling with a minimal risk of cross-contamination between samples or contamination of the individuals conducting the assay. And the nucleic acids extracted from the tissue are of sufficient quality and quantity that routine laboratory assays can be conducted on them.

MATERIALS AND METHODS

Design of a novel tissue sampler: As shown in Fig. 1A (a, b) a tissue sampler (China Patent 201120108302.2) has a head in which a blade measuring 6 mm in length is cast and a wand to which a rapidly changeable connector is attached for quick substitution of new, sterile samplers between subjects. As shown in Fig. 1A (c), an electric or battery powered drill is attached. The head preferably includes an adapting sleeve which consists of a cylinder measuring 6-9 mm in length and 8 mm in diameter and a tube measuring 12 mm in length and 4 mm in diameter. Between the adapting sleeve and the end of the wand lies a spring which can make the cylinder hold the specimen and prevent the tissue from aerosolizing and contaminating either other samples or the individual obtaining the sample. The cylinder can be compressed to let the head move out of the adapting sleeve. More specifically, a screwdriver bit PH1-50H0 (Shanghai Tajima Tool Co., Ltd.) was used as the wand, an insulated cord end crimp terminal (Zhejiang, China) was used as the adapting sleeve, the head was self-made from a plastic ballpoint pen refill and fixed to the screwdriver bit and the blade was made from stainless steel scrap metal. When mass-production of the sampler is considered, the head or the ward can be made from plastic materials for easy injection molding processing, the blade can be designed and manufactured with a corresponding size and structure so that it can fit the head and the head and the wand may be fixed together by an adaptable structure. As pictured in the figures, the apparatus was made of easily available commercial parts with a total price of No. >3.5 CNY (Chinese Yuan Renminbi), corresponding to approximately 0.5 United States dollars. And even if the blade were to be made of hardened stainless steel or high carbon steel, the overall price of each sampler would be very low.

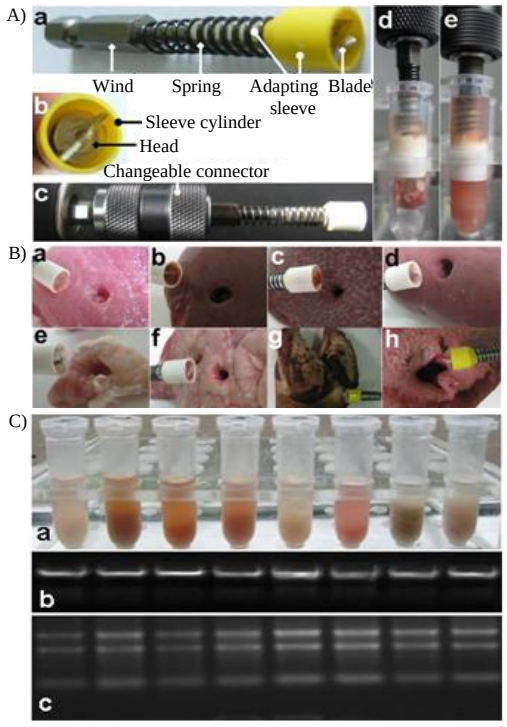

Fig. 1: A) Picture diagram of a sampler illustrating its structure and function; B) sampling operation on healthy porcine organs: a) Muscle; b) Liver; c) Spleen; d) Kidney; e) Lymph node; f) Lung; g) foot skin and h) snout skin; (C) prepared tissue homogenates; a) Electrophoretic patterns of total tissue genomic DNA; b) or RNA; c) from targeted organs in earlier-mentioned order

Collection of specimens from different type of tissues: Porcine organs such as muscle, liver, spleen, kidney, lymph node, lung, hoof, snout skin, etc. were collected freshly from a slaughterhouse. To operate the device as it is constructed and described in Fig. 1, the assayer must first open the keyless chuck, grip the rapidly changeable connector around the tip of the drill and rotate it counter-clockwise by hand then fix the sampler to the rapidly changeable connector, hold the drill and make the sampler head contact the specific sampling site, start the drill clockwise and stop it when the adapting sleeve of the head is just penetrating the tissue and then put the sampler into 1 mL storage solution (0.90% w/v NaCl) in a microcentrifuge tube shown in Fig. 1A (d). As the sample has been minced and scraped up into the adapting sleeve of the sampler, it is easy to release and homogenize each sampled tissue to a sufficient degree in the storage solution of sodium chloride by just having the drill bit rotate for optimal 25 seconds at a speed of 1800 rpm (Fig. 1A (e)). DNA was extracted with a commercial VetExtract (Column) kit (Hai Kang Life Co., Ltd. Hong Kong) and RNA was prepared with RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA was electrophoresed on a denaturing 1% (w/v) agarose gel.

Molecular diagnostic assay of mycoplasma bovis within lung specimen: Lung specimens collected during a necropsy of a young calf from a farm in Guizhou Province, China were kept at approximately 4°C and transported to the laboratory within a few hours of isolation and stored at -20°C (Hong *et al*., 2012). Upon examination in the lab, tissue scraps were obtained from the specimens using the present tissue sampler and simultaneously with traditional scissors and scalpels. After treatment either by the drill or by routine mortar and pestles homogenization or by a Bullet Blender® homogenizer (Next Advance, Inc., USA), the supernatant was divided into labeled aliquots for DNA extraction. A nested PCR was carried out using a GeneAmp® PCR System 9700 (Applied Biosystems, USA) using outside primers (PpMB920-1 and PpMB920-2) and inside primers (PpSM5-1 and PpSM5-2) as described (Pinnow *et al*., 2001). After 35 cycles of 94°C for 30 sec, 48°C for 60 sec and 72°C for 120 sec, the outside PCR product was diluted 1:100 as template for the inside reaction which was carried out under the same conditions except for the annealing temperature of 54°C. The 10 μL aliquots of the PCR product were separated by electrophoresis in a 1% (w/v) agarose gel and stained with ethidium bromide (0.5 μg mL^{-1}) for photography.

Diagnostic assay of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Viscera (e.g., lymph node, lung, liver and spleen) were obtained at necropsy of a pig with visible clinical symptoms of fatal Porcine Reproductive and Respiratory Syndrome (PRRS) (Tian *et al*., 2007). Fresh tissues were excised and homogenized using the present sampler, RNA was extracted with the VetExtract (Column) kit (Hai Kang Life Co., Ltd. Hong Kong) from 250 μL of each supernatant homogenate fraction. Reverse transcription PCR analysis was conducted utilizing a commercial RT-PCR kit (Anheal laboratories Co., Ltd. China) with the following conditions: 42°C for 45 min, 94°C for 3 min followed subsequently by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and then remaining at 4°C. Electrophoresis was carried out in a 2% (w/v) agarose gel.

RESULTS AND DISCUSSION

As shown in Fig. 1B, the sampler could be easily applied to different tissues for sample preparation. The total time for each sampling was 35±5 sec (n = 3) with the time for excision and homogenization as 10±3 and 25±4 sec, respectively. Nucleic acids extracted directly from supernatant homogenates showed sufficient quantity and high quality (Fig. 1C) which meant that total DNA or RNA was preserved and intact during the sampling procedure and suitable for further molecular diagnosis.

One issue concerns the safety of holding a microcentrifuge tube still with one hand while keeping an electric drill working with the other but this can be dealt with by adjusting the holding angle to keep the gap between drill bits and internal tube wall unchanged for just a few seconds, similar to an automated system. For large scale operation, an auto-adjustable bench drill and supporting plastic stands which could keep tubes still during the homogenizing process would make a perfect sampling platform in laboratories. Another issue is the adequateness of the sampled tissue and relevant concentration of infectious agents for it is well known that reproducibility of an analysis decreases disproportionately with decreasing concentration (Horwitz *et al*., 1980). In this study, researchers optimized the sampling capacity of the sampler to increase the concentration of infectious agents in the homogenate in each tube. The average weight for each organ sampled in triplicate was from 100-300 mg and the relative standard deviation varied from 5-10% such as the weight of muscle was 216.9±15.0 mg, kidney was 203.4±11.0 mg and snout skin 113.4±10.7 mg which indicated that sampling could be reproduced when a particular organ was targeted and the weighing step could be eliminated.

In one of the biological applications-preparing sample for detection of infectious bacteria in tissue, researchers compared the detection efficiency for *M. bovis* between the described method and routine methods (scissors plus a mortar and pestle or a homogenizer). The 1911 bp outside and 442 bp internal targeted DNA fragments (Pinnow *et al*., 2001) were specifically amplified from each of the three prepared samples (Fig. 2a) which showed that *M. bovis* within calf lung tissue could be as efficiently detected from the samples obtained by the described sampling method as from those obtained by mechanical scraping plus a mortar and pestle or a homogenizer treatment. In another application-detecting infectious virus in tissues, researchers applied the described method for detecting PRRS virus in different organs. Samples were prepared by the sampler and PRRS virus sequences were evident following RT-PCR assay of lymph node, lung, liver and spleen tissues. The signal was especially strong in samples taken from the lung as would be expected with a virus which is spread by inhalation (Fig. 2b). These results, together with the significantly reduced sampling time which was calculated typically as total time of 4 sampling (4×35 sec) compared with a Bullet Blender® homogenizer (4×1.0 min for excision plus 5 min for bead

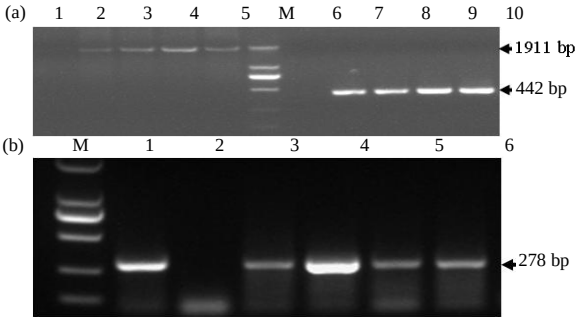

Fig. 2: a) Nested PCR-amplified *M. Bovis* specific products and b) PRRSV RNA targeted amplification and detection via RT-PCR. Panel a) Lane 1, 6: negative control; Lane 2, 7: excision and homogenization (E&H) using scissors and a mortar and pestle; Lane 3, 8: E&H scissors and a Bullet Blender® homogenizer; Lane 4, 9: E&H the present sampler; Lane 5, 10: frozen *M. bovis* culture as positive control and panel b) Lane 1: positive control; Lane 2: negative control; Lane 3: lymph node; Lane 4: lung; Lane 5: liver; Lane 6: spleen. Lane M: ML2000 Marker (TaKaRa)

beating), showed that the described sampling method with the novel sampler was ideal for molecular diagnostic application as it was faster but equally accurate.

The result of an experiment depends on the quality of the starting material. Thorough homogenization of tissues routinely gives high-quality DNA, RNA and protein for reliable and reproducible molecular diagnosis. Tissue samples although solid are highly aqueous in nature, a characteristic that can be exploited to rupture cells within the tissue matrix. Generally, scissors, scalpel, etc. are used to obtain and mince tissue samples from animal organs, the obtained samples should be processed to dissociate cells or release infectious agents for DNA/RNA extraction usually by mechanical homogenization or enzymatic digestion. In contrast, the present sampler can integrate sample excision and homogenization which reduces the total time for each sampling to approximately 30 sec and offers an innovative tool to facilitate optimal sample preparation and reduce the cross-contamination risks in molecular diagnostic assays of infectious agents within test specimens, especially when the number of samples could range from hundreds to thousands.

CONCLUSION

The study presented here demonstrates a novel sampler and its application to molecular diagnosis of infectious agents in tissue. Compared to conventional methods, the inexpensive sampler integrates rapid excision and easy homogenization for adequate and accurate sample preparation. With optimal uniformity of sampling and minimization of time, it could be applied to many biological sampling missions.

ACKNOWLEDGEMENTS

This research was supported by Guizhou Provincial Fund for Science and Technology (Grant No. 20102114). Researchers would like to thank Ruoming Huang, a retired school teacher for his help with the sampler design. Researchers would also like to thank Jianbo Sun and Gerson Rothschild (Columbia University Medical Center) for their suggestions on the manuscript. Researchers thank Jiangning Liu (Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences) for his early advice.

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