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Application of an Improved Micro-amount of Virion Enrichment Technique (MiVET) for the Detection of Avian Influenza A Virus in Spiked Chicken Meat Samples

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Abstract

Highly sensitive detection of pathogens is effective for screening meat during quarantine inspection and export. The “micro-amount of virion enrichment technique” (MiVET) was recently developed, which is a new method combining virus concentration with immunomagnetic beads and simple RNA extraction with sodium dodecyl benzenesulfonate (SDBS) for the specific and sensitive detection of avian influenza viruses (AIVs). AIV subtypes H3N2 and H4N2 were used to spike the surface of chicken breast meat samples. The modified MiVET protocol was tested by comparing it against three different homogenate preparation conditions, as well as in samples with added α-amylase and collagenase to digest inhibitors. The performance of the modified MiVET was evaluated by real-time RT-PCR assay targeting the matrix gene. Compared with conventional RNA extraction, the modified MiVET reproducibly concentrated AIVs in chicken meat samples with 100–1000-fold improvement by 60 s-hand homogenization. The 30 s- and 60 s-

stomacher homogenizations resulted 100-fold and 10–100-fold improvement, respectively. The modified MiVET required < 60 min from homogenate preparation to final RNA elution. Further, use of the modified MiVET also decreased the rate of false-negative results. The modified MiVET is effective for the rapid and highly sensitive detection of AIVs in chicken meat samples, and can be applied to quarantine and export inspection at airports and seaports.

AQ1

AQ2

Keywords

MiVET
Micro-amount of virion enrichment technique
Influenza A virus
Chicken
Meat

Introduction

Transboundary animal diseases (TADs) cause serious harm to livestock and meat industries worldwide. Among these, the highly pathogenic avian influenza (HPAI) virus causes enormous economic damage due to the need for administrative measures such as culling to prevent the spread of outbreaks (Mase et al 2005; OIE 2018; Tumpey et al 2002; Tsukamoto et al. 2010). The acceleration of globalization and increases in international logistics and international flights exacerbate the risk of distributing pathogens worldwide (Masujin et al. 2019; Shibata et al. 2018a, b; Tumpey et al. 2002; Yamazaki et al. 2018, 2019). Viruses that cause TADs such as HPAI virus (HPAIV) and African swine fever virus (ASFVI) present in meat products and carcasses can be legally imported or exported or smuggled through shipping and air transportation (Beato et al. 2006; Masujin et al. 2019; Shibata et al. 2018a, b; Tumpey et al. 2002). It is therefore important to minimize the harm of these viruses by containing and preventing their spread through quarantine inspections at exit and entry points (Beato et al. 2006; Masujin et al. 2019; Shibata et al. 2018a, b).

AQ3

Achieving this goal requires that the sources and propagation routes of contaminated meat can be identified (Cottam et al. 2008; Yamazaki et al. 2018, 2019). Because they occur in small amounts and low concentrations, however, pathogenic viruses in the environment and in contaminated meat products are very difficult to detect using conventional techniques (Saito et al. 2015; Yamazaki et al. 2018, 2019). A survey of animal quarantine stations affiliated to international airports in Japan has isolated 3.7% (6/162)–5.9% (8/136) of influenza virus from poultry carcass illegally brought in from overseas (Shibata et al. 2018a, b). Although the EID_{50}/g of the poultry carcass was lower or slightly higher than the detection limit, inoculation of the isolates into chicken showed $10^{3.3}$–$10^{7.0}$ $TCID_{50}$/g of the skeletal muscle (Shibata et al. 2018a). Isolates from duck meat imported from China to Korea have also shown $10^{5.3}$–$10^{5.5}$ EID_{50}/g (Tumpey et al. 2002). Therefore, the introduction of contaminated poultry carcass and meat from abroad is an important risk factor for the spread of the transboundary animal diseases.

Although several preliminary approaches have been reported to effectively concentrate and detect influenza virus in the samples (Dhumpa et al. 2011; Khalenkov et al. 2008), they are time-consuming, with relatively poor concentration performance at ≤ tenfold (Dhumpa et al. 2011) and limitation solely for water samples (Khalenkov et al. 2008). These difficulties lead to the underestimation of actual contamination levels, and thereby yield false-negative results at quarantine facilities (Yamazaki et al. 2018, 2019). Further, surveys to identify the sources and propagation routes of these infections rely on descriptive epidemiological studies, despite the fact that virus isolation is required to provide sufficient physical and forensic evidence to increase the accuracy of the surveys used in these studies (Cottam et al. 2008; Yamazaki et al. 2018, 2019). Moreover, virus isolation may take days. Accordingly, researchers have sought simple, highly sensitive, and rapid tests that do not rely on expensive and specialized equipment (Tsukamoto et al. 2010). Such detection techniques, applicable for routine inspections at general quarantine stations and meat-related facilities, would help strengthen efforts to reduce or eliminate the worldwide traffic of contaminated meat.

To address this problem, a new system for concentrating and detecting AIV called the Micro-amount of virion enrichment technique (MiVET) was developed. This technique provides by ≥ 10- and ≥ 100-fold higher analytical sensitivity in duck fecal and phosphate-buffered saline (PBS) samples than the conventional method (Yamazaki et al. 2019). Although MiVET using fluorescent probes (hereinafter referred to as the "probe system") is highly effective for testing artificially spiked samples of PBS, it is significantly inhibited by duck fecal materials (Yamazaki et al. 2019). SYBR Green I (hereinafter referred to as a "SYBR system" was previously used (Yamazaki et al. 2019). However, the SYBR system is problematic because of its lower diagnostic sensitivity and specificity compared with the probe system. Here, the MiVET protocol using the probe system was modified (modified MiVET system) and used this modified system to test optimized concentrations of AIV in artificially spiked skin of chicken breast meat samples (referred to as "meat samples").

Materials and Methods

Modified MiVET Protocol

The modified MiVET protocol includes incubation with α-amylase and collagenase at 35 °C for 30 min to replace the 15-min reaction step to isolate virions used in the previous MiVET protocol (Yamazaki et al. 2019). H3N2 and H4N2 AIV subtypes, serially diluted tenfold, were used to spike the skin of meat samples, and PBS was added to prepare a homogenate. The modified MiVET and conventional method (automatic nucleic acid extraction using the mag LEAD 6gc, Precision System Science, Co. Ltd, Matsudo, Japan) were simultaneously performed to compare their abilities to concentrate virus preparations. Three types of homogenate (gentle manual homogenization for 60 s and Stomacher homogenization for 30 s or 60 s) were compared.

AQ4

Preparation of Immunomagnetic Beads (IMBs)

IMBs were prepared according to the published protocol (Yamazaki et al. 2019). Briefly, a 2-mL microcentrifuge tube containing pG-MBs (Dynabeads Protein G, Magnetic Beads, Thermo Fisher Scientific Inc.,

Waltham, MA, USA) was placed on a Magnetic stand (Takara Bio, Kusatsu, Japan) for 2 min to remove the preservative solution according to the manufacturer's instructions. To conjugate virions to pG-MB, 1.2 mL of PBS containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MI, USA) and a polyclonal antibody against AIV (1:20; anti-influenza A antibody, 4–5 mg/mL concentration; AB1074, Merck Millipore Corp., Burlington, MA, USA), which broadly reacts with the surfaces of all influenza A virus subtypes, were added to the 2-mL microcentrifuge tube containing the pG-MBs. The mixture was incubated at 37 °C for 15 min on a rotator (20 rpm/min) (RT-30 mini, Taitec Co., Koshigaya, Japan). To remove supernatant containing unbound antibody after conjugation, the IMB was left on the magnetic stand for 2 min. After washing with 1.2 mL PBS containing 0.05% Tween 20 to completely remove unbound antibody, the prepared IMB were stored at 4 °C and used within 7 days.

Virus Strains

AIV strains H3N2 (A/duck/Hokkaido/5/77, GenBank accession numbers AB612883–AB612888 and AB277754–AB277755) and H4N2 (A/duck/CAD/1/2016, accession numbers LC415031–LC415038) were prepared by infecting 9- to 11-day old embryonated chicken eggs. The allantoic fluid containing AIV was harvested from the eggs and stored at − 80 °C. Copy numbers of the H3N2 and H4N2 calculated by using real-time RT-PCR were 2.4×10^3 and 2.5×10^3 copies/μL of allantoic fluid.

AIV-Spiked Meat Samples

Chicken meat samples with attached skin were purchased at supermarkets in Miyazaki and Kyoto between 2018 and 2019. Approximately 10 g was cut into pieces (approximately 2 cm^3) with a kitchen knife. To compare LODs with the modified MiVET and conventional RNA extraction methods for AIV in meat homogenates (described below), tenfold serial dilutions of the H3N2 and H4N2 AIV subtypes were prepared in PBS. 100 μL of each was spiked onto the skin of the meat samples in a sterilized plastic Stomacher bag, and 90 mL of PBS was added to prepare the homogenate.

Comparison of Homogenates for Use in the MiVET

To evaluate the effects of inhibitors of the MiVET present in meat samples, three different homogenate preparation conditions were compared as follows: (i) 60-s gentle manual homogenization (recovery of rinse solution from chicken meat surface), (ii) 30-s Stomacher homogenization, and (iii) and 60-s Stomacher homogenization. An Exnizer 400 homogenizer (Organo Co. Ltd., Tokyo, Japan) was used at 230 rpm/min. Approximately 50 mL of each homogenate was transferred to a 50-mL screw-capped tube, which was centrifuged at 20,000×*g* for 5 min. Approximately 40 mL of each supernatant was collected into new 50-mL screw-capped tubes, and 200 µL of the supernatant was used for conventional RNA extraction. The remaining supernatant was added to 50 µL of the IMBs to bind virions, to which 100 mg of α-amylase (Fujifilm Wako Pure Chemical Co. Ltd., Osaka, Japan) and 1 mL of 0.1% collagenase (Fujifilm Wako Pure Chemical) were added. The mixture was incubated at 35 °C for 30 min at 15–20 rpm/min in a rotator. All spike tests for the comparison of the three different homogenate preparation conditions were performed in duplicate each by using the H3N2 and H4N2 AIV subtypes.

RNA Extraction

Homogenates were placed on a magnetic stand for 50 mL tubes (DynaMag-50, Thermo Fisher Scientific) for 3 min, and the supernatant was subsequently removed using a 10 mL-graduated disposable dropper. After adding 1 mL of PBS to the tube, the mixture was transferred to a new 2-mL microcentrifuge tube and placed on a magnetic stand for 2 mL microcentrifuge tubes (Takara Bio) for 2 min before the supernatant was completely removed using a pipet and a cotton swab. Next, 0.1% dodecyl benzenesulfonate (8 µL) (SDBS, Tokyo Chemical Industry Co., Tokyo, Japan) was added down the walls of the tubes containing the IMB suspension. After quickly mixing with a pipet and vortexing the suspension, the tube was kept at room temperature during preparation of the rRT-PCR reaction mixture (within 15 min) and placed on the magnetic stand for 2 min. 4 µL of the supernatant was used as template for real-time RT-PCR. In parallel, 50 µL RNA was extracted from 200 µL of the supernatant using

automatic nucleic acid extractor (mag LEAD 6gc, Precision System Science).

Direct Real-Time RT-PCR Assay (rRT-PCR, Probe System)

Direct rRT-PCR targeting the matrix gene was performed using a Superscript III Platinum One-step qRT-PCR kit (Thermo Fisher Scientific) as previously described (Yamazaki et al. 2019). Briefly, the final rRT-PCR reaction mixture comprised 4 μL of crude RNA or 2 μL of automatically extracted RNA template, 0.2 μM of each primer (MP-39-67For: 5′-CCMAGGTCGAAACGTAYGTTCTCTCTATC-3′ and MP-183-153Rev: 5′-TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA-3′), 0.1 μM of MP-96-75ProbeAs: FAM-ATYTCGGCTTTGAGGGGGCCTG-BHQ, 1% Tween 20, and 0.4 μL of SuperScript III Reverse Transcriptase and Platinum Taq DNA Polymerase in 1 × reaction mix. The final volume was adjusted to 20 μL with distilled water. The rRT-PCR assay was performed using a Lightcycler 96 (Roche Diagnostics). The cycling conditions were as follows: one cycle at 50 °C for 15 min, 95 °C for 2 min, and 45 cycles each at 94 °C for 15 s and then 56 °C for 75 s.

Statistical Analysis

The theoretical recovery rate of the modified MiVET was calculated as follows: $2^{-\Delta\Delta C_t} \times 4$. Multiplying by 4 reflects the reduction in sample volume from 200 to 50 μL in the conventional method. Next, it was compared the diluted samples that tested positive using the modified MiVET and conventional methods (10^{-3} and 10^{-4}) prepared using the three different homogenate condition to the previous data for the PBS spike test (Yamazaki et al. 2019). The significance of differences was evaluated using the Student *t* test ($P < 0.05$).

Preliminary Study to Determine Optimal α-Amylase and Collagenase Concentrations

Approximately 30 g of the H3N2 AIV-spiked meat samples were added to 270 mL PBS and homogenized for 120 s using a Stomacher homogenizer (Exnizer 400, Organo) to maximize the release of possible inhibitors from the meat samples. Homogenate was divided into 4 sets

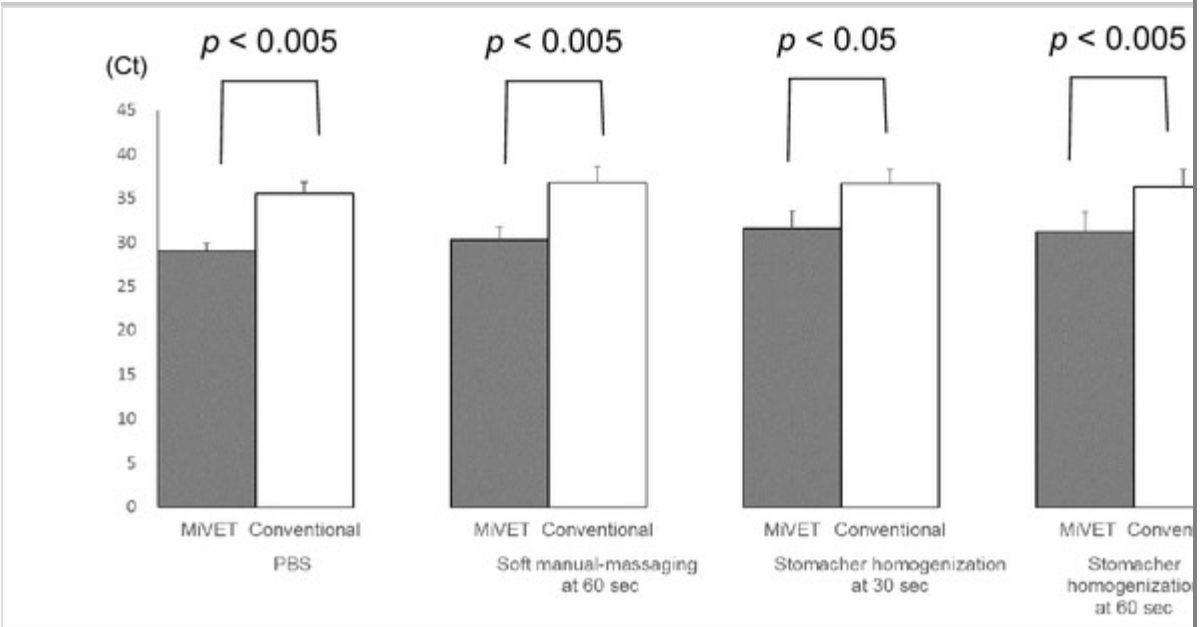
of approximately 50 mL each into 50-mL centrifuge tubes to obtain portions of supernatant containing 100 mg of α-amylase and 1 mL of 0.1% collagenase.

Results

The modified MiVET concentrated and detected AIVs in spiked meat samples with 10^1–10^3-fold greater efficacy than the conventional RNA extraction method. Among the three conditions, 60 s of gentle manual homogenization achieved 10^2–10^3-fold greatest efficacy. Stomacher homogenization for 30 s or 60 s achieved 10^2-fold and 10^1–10^2-fold improvement compared with that of a conventional RNA extraction method, respectively. The modified MiVET was performed in < 60 min from the beginning of homogenate preparation to the final RNA elution. When diluted samples were positive in both the modified MiVET and conventional methods (10^{-3} and 10^{-4}, respectively), PBS and meat samples manually homogenized for 60 s showed equivalent efficacies for concentrating virions. Recovery rate with the modified MiVET was 90.5-fold greater than that of the conventional automated extraction platform, while the theoretical rate was 362.0-fold greater ($P < 0.005$) (Fig. 1). Stomacher homogenization for 30 s or 60 s decreased efficacies by 34.3-fold, and by 137.2-fold for the theoretical rate ($P < 0.05$) (Fig. 1).

Fig. 1

Detection of AIV-spiked breast meat samples using the MiVET. Data on PBS are taken from Yamazaki et al. (2019)



Pre treatment	PBS	Soft manual-massaging at 60 sec	Stomacher homogenization at 30 sec
MiVET	29.0	30.2	31.5
Conventional	35.5	36.7	36.6
Ct difference	6.5	6.5	5.1
MiVET vs. conventional	90.5	90.5	34.3
Theoretical recovery rate of MiVET	362.0	362.0	137.2

Combination of the absence of α-amylase and collagenase with prolonged 120 s Stomacher homogenization resulted in a decrease in MiVET efficacy to Ct 30.4 (Table 2). The combined use of 100 mg of α-amylase and 1 mL of 0.1% collagenase or the single use of 100 mg of α-amylase in the supernatant from 50 mL of the meat homogenate improved the efficacies of the MiVET to Ct 23.8 and Ct 25.1, respectively. The values theoretically correspond to 203.7-fold and 87.4-fold greater efficacies, respectively, compared with the conventional automated extraction platform. In particular, the improvement achieved using the two enzymes was equivalent to that of the spiked-PBS test (218.3-fold) shown in the previous report (Yamazaki et al. 2019). When Stomacher homogenization was performed for 120 s without adding α -amylase and collagenase, concentration improved 2.1-fold.

Discussion

Here it is described that the development of the modified MiVET, a rapid and highly sensitive virus concentration and detection system. IMB (a complex comprising a commercially available anti-influenza A virus polyclonal antibody and pG-MB) was used to capture micro-amounts of virus in the samples. Using a combination of simple incubation for virion capture, washing, and SDBS elution within 60 min, the modified MiVET concentrated virions with at least 10^2-fold greater efficacy from meat samples compared with a conventional RNA extraction method with 60 s of gentle manual homogenization or 30 s of Stomacher homogenization (Table 1). The differences at 10^2–10^3-fold in the former and at 10^2-fold in the latter are likely explained by the degree of release of meat-derived inhibitors during preparation of the homogenate. When the two AIV strains were simply used to spike PBS in the previous study, the efficacy of concentrating virions was reproducibly 10^2-fold greater than with the conventional RNA extraction method (Yamazaki et al. 2019). Use of crude RNA extracted using SDBS as a template enables direct detection using real-time PCR amplification without further steps to remove inhibitors.

Table 1

Comparison of the modified MiVET with a conventional RNA extraction method

Strains	**Homegenate preparation**	**Assays**	**Dilutions of viruses in spiked chicken meat homogenates**				
			10^{-3}	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
H3N2	(Expected copy number in the reaction tube by conventional assay)						
			(1.9×10^1)	(1.9×10^0)	(1.9×10^{-1})	(1.9×10^{-2})	(1.9×10^{-3})
	A	Modified MiVET	28.2	31.7	34.7	38.7	No. Ct
A: Hand homogenization at 60 s							
B: Stomacher homogenization at 30 s							
C: Stomacher homogenization at 60 s							
All three-digit numbers including one decimal place indicate threshold cycle values (Ct)							

Strains	Homegenate preparation	Assays	Dilutions of viruses in spiked chicken meat homogenates				
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		Conventional	34.5	39.1	No. Ct	No. Ct	No. Ct
	B	Modified MiVET	30.4	34.3	37.7	39.3	No. Ct
		Conventional	34.6	38.8	No. Ct	No. Ct	No. Ct
	C	Modified MiVET	29.9	34.4	39.1	No. Ct	No. Ct
		Conventional	33.9	38.8	No. Ct	No. Ct	No. Ct
H4N6	(Expected copy number in the reaction tube by conventional assay)						
			(2.0×10^1)	(2.0×10^0)	(2.0×10^{-1})	(2.0×10^{-2})	(2.0×10^{-3})
	A	Modified MiVET	30.7	33.3	37.8	38.7	No. Ct
		Conventional	36.6	No. Ct	No. Ct	No. Ct	No. Ct
	B	Modified MiVET	29.7	33.6	38.1	No. Ct	No. Ct
		Conventional	36.5	No. Ct	No. Ct	No. Ct	No. Ct
	C	Modified MiVET	29.4	33.2	36.7	39.0	No. Ct
		Conventional	36.2	38.8	No. Ct	No. Ct	No. Ct
A: Hand homogenization at 60 s							
B: Stomacher homogenization at 30 s							
C: Stomacher homogenization at 60 s							
All three-digit numbers including one decimal place indicate threshold cycle values (Ct)							

When virus contamination of chicken meat (carcass) originates from intestinal contents, the virus can be easily transferred into PBS by rinsing combined with 60 s of gentle manual homogenization, which achieves 10^2–10^3-fold improvement. Further, low pathogenic avian influenza (LPAI) H7N2 in carcass washings (Swayne and Beck 2005) indicates that the modified MiVET has the potential to improve the isolation rate using simple rinsing with 60 s of gentle manual homogenization. Conditions for preparing homogenates have been previously evaluated for detecting *Salmonella* in enrichment broth cultures of chicken meat and goblet samples using direct PCR screening without the addition of degradative enzymes (Kanki et al. 2009). Compared with 60-s Stomacher homogenization, 30-s gentle manual homogenization achieved positive detection rates of 57.1% (40/70) and 42.0% (34/81), respectively (Kanki et al. 2009). Frequent false-negative results were observed in the latter, possibly caused by chicken meat-derived inhibitors. Among data for ground chicken, possibly containing larger amounts of inhibitors, the ratio of positive samples was 48.0% (12/25) for manual homogenization and 21.7% (5/23) for Stomacher homogenization, and a larger number of false negatives caused by inhibitors (Kanki et al. 2009). The previous and present findings indicate that it is critically important to minimize the time required to prepare homogenates and to add digestive enzymes to prevent false negatives.

The effects of the meat-derived inhibitors can be sufficiently reduced to achieve a 10^2-fold improvement in detection when Stomacher homogenization is performed within 30 s. Swayne and Beck (2005) reported that they were unable to isolate viruses from samples of breast or thigh meat of chickens experimentally infected with two LPAIs (H7) but obtained a positive result with one HPAI (H5), whereas Mase et al. (2005) isolated HPAI H5N1 at a frequency of 21.4% (3/14) from lots of duck meat imported from China into Japan. The presence of AIVs in meat differs depending on avian species and the subtypes involved. For example, ducks are asymptomatic carriers, so both HPAI and LPAI are easily detected in duck meat contaminated with duck feces and cecal contents; in contrast, chickens die immediately with viremia on infection with HPAI, before the viral load in the meat sufficiently

increases, and is therefore relatively difficult to isolate. Especially, the latter case may require Stomacher homogenization so that the viruses with low concentrations present in meat are sufficiently extracted into PBS. Therefore, establishing a highly sensitive AIV detection method using optimized conditions in both hand- and Stomacher-homogenizations to prepare homogenates of avian meat samples is important for safeguarding veterinary and public health.

As shown in Table 2, chicken meat components in homogenates likely inhibited virion capture, real-time RT-PCR amplification, or both. Among potential PCR inhibitors (Wilson 1997; Saito et al. 2015; Schrader et al. 2012), carbohydrates interfere with the detection of viruses in food samples. This can be overcome by adding α-amylase (Saito et al. 2015), which is consistent with the present findings. Further, collagenase was added to remove the high levels of collagen in chicken skin. The frequency (approximately 0.5–1%) of nonspecific reactions using the ELISA test for bovine spongiform encephalopathy is reduced when collagens and non-prion protein are sufficiently digested by the addition of collagenase and proteinase K, respectively, to bovine medullary homogenates (Yamazaki, unpublished data). Therefore, nonspecific binding of antibodies to collagen will be reduced and digesting collagen will increase the efficiency of capturing virions. The present results were not as convincing as those for α-amylase, but the MiVET reaction was not inhibited. Therefore, collagenase was included in the MiVET protocol as a safeguard.

Table 2

Comparison of MiVET performance with or without degradative enzyme addition

Assays	**MiVET**			**Conventional (automated extraction)**
Enzyme	**100 mg of α-amylase and 1 mL of 0.1% collagenase**	**100 mg of α-amylase**	**Without enzyme**	
Ct	23.8	25.1	30.4	31.5
	7.7	6.5	1.1	

Ct threshold cycle values

Assays	MiVET			Conventional (automated extraction)
Enzyme	**100 mg of α-amylase and 1 mL of 0.1% collagenase**	**100 mg of α-amylase**	**Without enzyme**	
Ct difference from conventional				
MiVET vs. conventional	203.7	87.4	2.1	
Theoritical recovery rate of MiVET	814.6	349.7	8.4	
Ct threshold cycle values				

In previous reports, Saito et al. (2015) have developed an effective technique for concentrating norovirus from food samples using the Pan-trap method. However, the Pan-trap method has the disadvantage of using an inactivated *Staphylococcus aureus* as the ligand for the antibodies, requiring an extraction buffer included in expensive commercial column kit and time-consuming nucleic acid extraction step with phenol–chloroform (Saito et al. 2015). Dhumpa and collages have also tried to detect influenza virus concentration using IMBs (2011). However, the removal of inhibitors in the sample has not mentioned, and its relatively poor concentration performance has shown in equivalent to conventional RNA extraction method with column kit (Dhumpa et al. 2011). In contrast, the modified MiVET maintained the efficacy of virion concentration, thanks to the use of degradative enzymes and simple SDBS elusion, as well as gentle and brief homogenization that minimized the activities of inhibitors in meat.

The modifications to the MiVET reported here yielded highly increased virus concentrations from homogenates of meat samples. Detection from duck fecal samples was previously shown less reproducible with the probe system than with the SYBR system, with lower tolerance for feces-derived inhibitors (Yamazaki et al. 2019). In the present study, the modified MiVET using the probe system allows for the easy concentration of virions in meat samples with higher diagnostic

sensitivity and diagnostic specificity. The technique maintains efficacy with highly condensed virus concentrations using a one-step rRT-PCR method with supplemental use of 1% Tween 20 to sequester residual SDBS. Further, the addition of α-amylase and collagenase likely digested chicken meat-derived inhibitors and improved the affinity of the antibodies for binding virions in the supernatant of the meat samples. Further evaluation of the modified MiVET is essential. International collaborations to test for its ability to effectively detect AIVs, particularly HPAI subtypes such as H5 and H7, in naturally contaminated chicken meat samples in countries with endemic HPAI are now being planned.

In conclusion, a simple and highly sensitive virus concentration system (modified MiVET) was developed with a sensitivity $\geq 10^2$-fold higher than that of the conventional method for analyzing chicken breast meat samples. Although the detection of pathogenic viruses such as HPAIV and ASFV in meat products is frequently reported in quarantine inspections (Beato et al. 2006; Mase et al. 2005; Masujin et al. 2019; Shibata et al. 2018a, b; Tumpey et al. 2002), false-negative results may be reduced by applying the modified MiVET. Thus, the modified MiVET achieves simple and highly sensitive screening of HPAIV in meat samples and can be readily performed at quarantine stations and meat-processing facilities. Further, the modified MiVET is likely to enable the early diagnosis required to implement appropriate control measures and to determine the dynamics of virus distribution in the environment.

Publisher's Note

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by RM, YY, KN, FVA, HM, and WY. The first draft of the manuscript was written by WY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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