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Virology

Development of a one-run real-time PCR detection system for pathogens associated with porcine respiratory diseases

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ABSTRACT. The etiology of Porcine respiratory disease complex is complicated by infections with multiple pathogens, and multiple infections increase the difficulty in identifying the causal pathogen. In this present study, we developed a detection system of microbes from porcine respiratory by using TaqMan real-time PCR (referred to as Dempo-PCR) to screen a broad range of pathogens associated with porcine respiratory diseases in a single run. We selected 17 porcine respiratory pathogens (*Actinobacillus pleuropneumoniae*, *Boldetella bronchiseptica*, *Haemophilus parasuis*, *Pasteurella multocida*, *Pasteurella multocida* toxin, *Streptococcus suis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynovie*, porcine circovirus 2, pseudorabies virus, porcine cytomegalovirus, swine influenza A virus, porcine reproductive and respiratory virus US strain, EU strain, porcine respiratory coronavirus and porcine hemagglutinating encephalomyelitis virus) as detection targets and designed novel specific primer-probe sets for seven of them. In sensitivity test by using standard curves from synthesized DNA, all primer-probe sets showed high sensitivity. However, porcine reproductive and respiratory virus is known to have a high frequency of genetic mutations, and the primer and probe sequences will need to be checked at a considerable frequency when performing Dempo-PCR from field samples. A total of 30 lung samples from swine showing respiratory symptoms on six farms were tested by the Dempo-PCR to validate the assay's clinical performance. As the results, 12 pathogens (5 virus and 7 bacteria) were detected and porcine reproductive and respiratory virus US strain, *Mycoplasma hyorhinis*, *Haemophilus parasuis*, and porcine cytomegalovirus were detected at high frequency. These results suggest that Dempo-PCR assay can be applied as a screening system with wide detection targets.

KEY WORDS: diagnosis, porcine, respiratory disease, TaqMan real-time PCR

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Respiratory infections constitute some of the most important diseases of growing pigs and result in substantial economic losses [17]. Multiple pathogens contribute to a polymicrobial infection known as Porcine Respiratory Disease Complex (PRDC) [7, 9, 21]. The most commonly isolated pathogens are porcine reproductive and respiratory virus (PRRSV), swine influenza A virus (SIV), porcine circovirus 2 (PCV2), and *Mycoplasma hyopneumoniae*. The other pathogens associated with PRDC are *Streptococcus* suis, *Actinobacillus* pleuropneumoniae, *Pasteurella multocida*, *Pasteurella* multocida toxin, *Boldetella*

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bronchiseptica, *Haemophilus* parasuis, *Mycoplasma hyorhinis*, *Mycoplasma hyosynovie,* pseudorabies virus (PRV), porcine respiratory corona virus (PRCV), Porcine cytomegalovirus (PCMV) [7, 8, 15, 20]. Infection with each single pathogen does not necessarily result in appearance of symptoms, but complex infections with a variety of pathogens, including the indigenous agents, develop severe conditions. Infections with such multiple pathogens make it difficult to rapidly identify the etiology of PRDC. To adopt appropriate measures, such as vaccination or hygiene management, and to minimize the economic loss of PRDC, it is necessary to quickly, accurately and comprehensively detect multiple pathogens present in varying proportions in each herd. Serological tests [13], pathogen isolation [22] and PCR-based tests [1, 11] are currently available to diagnose PRDC in laboratories. Most tests are based on a one assay-one pathogen approach, and they are not enough for diagnosis of PRDC in terms of comprehensiveness and rapidity. Tsuchiaka *et al*. previously developed a system to detect microbes in bovine diarrhea by TaqMan real-time PCR, permitting the simultaneous screening of 19 pathogens associated with diarrhea [26]. TaqMan real-time PCR possesses the advantages of high sensitivity, high specificity, and simple operation.

The objective of this study is to develop a system based on TaqMan real-time PCR that can detect 17 pathogens, including viruses and bacteria, associated with porcine respiratory diseases in one run.

MATERIALS AND METHODS

Primer and probe design

A total of 17 primer-probe sets were used to detect pathogens that certainly or possibly cause respiratory diseases on porcine. Each primer-probe set was designed to detect a single target pathogen. New primer-probe sets were designed for *Pasteurella multocida* and toxin, *M. hyosynovie*, PCV2, PCMV, SIV and PHEV using the PrimerQuest software (Integrated DNA Technologies, Inc., Coralville, IA, USA) based on consensus sequences of each pathogen obtained from the GenBank database. Primer and probe information and their target pathogens are summarized in Table 1. GenBank accession numbers, the reference sequence, country, host and first deposited year used for primers and probes design of each pathogen were shown in Supplemental Table 1. Previously reported qPCR assays were used for 10 pathogen species, including RNA, DNA viruses and bacteria [1, 2, 12, 16, 18, 19, 25, 27, 28]. Furthermore, as an internal control within the Dempo-PCR reaction, primer-probe sets for β-actin were synthesized as previously reported [29]. All hydrolysis probes were labeled with the reporter dye FAM (6-carboxyfluorecein) at the 5′ end and the fluorescent dye TAMRA (6-carboxytetramethylrhodamine) at the 3′ end. Primers and probes were purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA), and probes containing the mixed base were produced at Integrated DNA Technologies (Integrated DNA Technologies, Inc.).

Real-time PCR

A One Step PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa Bio, Kusatsu, Japan) was used to detect viral RNA, and Premix Ex Taq (Perfect Real Time) (TaKaRa Bio) was used to detect viral and bacterial DNA. All reactions were performed in a total volume of 20 μl, which contained the sample nucleic acid, primers, probes (the final concentration of all primers and probes was 0.2 μM) and all other components included in the kits, according to the manufactures' protocols. Thermal cycling conditions were as follows: 45°C for 5 min and 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec, 55°C for 20 sec, and 72°C for 20 sec [26]. Fluorescent signal data were analyzed using an automatic quantification algorithm in LightCycler Nano Software 1.1 (Roche Diagnostics GmbH), and the parameters of analysis were as follows: exclude early cycle=7, minimum relative amplifications=0, and minimum amplification quality=5.

Validation of real-time PCR performance using synthesized DNA

To verify the sensitivity, linearity, and efficiency of the real-time PCR assay, the limit of detection (LOD), correlation coefficient (R^2) and PCR efficiency (E) were determined from standard curves. Standard curves were obtained, and the LOD, R^2 and E were calculated as described previously [11, 26].

Evaluation of real-time PCR performance using synthesized DNA

For the purpose of validation, real-time PCR reliability, sensitivity, and linearity of standard curves were verified by testing tenfold serial dilutions of synthesized DNA, including each target genome sequence (1×10^0 to 1×10^6 copies/reaction). The synthesized DNA was purchased from Integrated DNA Technologies (Integrated DNA Technologies, Inc.). Pathogen dilutions were repeated twice in separate runs, and a standard curve was constructed from the Cq values. The PCR efficiency (E) was calculated using the standard curve slope according to the following formula: $E=(10^{-1/\text{slope}\,(-1)})$. The correlation co- efficient (R^2) was also calculated. The limit of detection (LOD) was defined as the lowest concentration at which a fluorescent signal could be detected in all reactions. Reproducibility (inter-assay variance) was assessed using the coefficient value (CV) calculated based on quantification cycle (Cq) values.

Clinical samples and DNA and RNA extraction

The assay was applied to test clinical samples. A total of 30 samples of porcine lung tissue submitted in 2016–2018 to Azabu University for diagnosis of porcine respiratory pathogens were used to test. These pigs were 48 to 135 days old and belonged to 6 farms (A to F), all showing respiratory symptoms (Supplementary Table 3). Lung tissues were minced by scissors, diluted 1:10 in phosphate buffered saline (PBS, pH 7.4), homogenized for 20 sec at 3,200 rpm with the presence of three stainless steel beads

Table 1. The nucleotide information of the primer-probe sets used for Dempo-PCR

Target pathogen	Target gene		Primer/Probe (FAM/TAMRA) sequence 5′-3′	Reference No.
Actinobacillus pleuropneumoniae	omlA	F	GGGGACGTAACTCGGTGATT	[1]
		R	GCTCACCAACGTTTGCTCAT	
		P	CGGTGCGGACACCTATATCT	
Boldetella bronchiseptica	Fla2	F	AGGCTCCCAAGAGAGAAAGGCTT	[24]
		R	AAACCTGCCGTAATCCAGGC	
		P	ACCGGGCAGCTAGGCCGC	
Haemophilus parasuis	CTinfF1	F	CGACTTACTTGAAGCCATTCTTCTT	[27]
		R	CCGCTTGCCATACCCTCTT	
		P	ATCGGAAGTATTAGAATTAAGTGC	
Pasteurella multocida	Kmt1	F	GGGCTTGTCGGTAGTCTTT	This study
		R	CGGCAAATAACAATAAGCTGAGTA	
		P	CGGCGCAACTGATTGGACGTTATT	
Pasteurella multocida toxin	toxA	F	GATACAGTAATTTCAGCGCCTTT	This study
		R	GCAGGAAGTTCCCAGTAATTTG	
		P	TGGTGCGATTCCAGAGGCAATAGA	
Streptococcus suis	16S RNA gene	F	AGAAGAGTGGAAAGTTTCTCA	[2]
		R	TCACAGTTTCCAAAGCGT	
		P	CAAACCGCCTGCGCTCGCTTTACG	
Mycoplasma hyopneumoniae	*p102*	F	GTCAAAGTCAAAGTCAGCAAAC	[18]
		R	AGCTGTTCAAATGCTTGTCC	
		P	ACCAGTTTCCACTTCATCGCCTCA	
Mycoplasma hyorhinis	*p37*	F	TATCTCATTGACCTTGACTAAC	[25]
		R	ATTTTCGCCAATAGCATTTG	
		P	CATCCTCTTGCTTGACTACTCCTG	
Mycoplasma hyosynovie	rpoB	F	GCTGATATTCCTAACGCATCAAAC	This study
		R	CACCTTTAGGGCTAGTTCTTCC	
		P	TGACCAAGGAATTGTTAGAGTTGGATCTGA	
Porcine circovirus 2	ORF2 (capsid protein)	F	CCATCTTGGCCAGATCCTC	This study
		R	AGGCGGGTGTTGAAGATG	
		P	CACCGTTACCGCTGGAGAAGGAAA	
Pseudorabies virus	gE	F	CTTCCACTCGCAGCTCTTCTC	[16]
		R	GTRAAGTTCTCGCGCGAGT	
		P	TTCGACCTGATGCCGC	
Porcine cytomegalovirus	gB	F	CTCTCAAGAAGATGCCGTCTG	This study
		R	CTGCTGATATTCCAAGTGACGTA	
		P	ACAAAGCCTAGCCCGAGCGTATT	
Swine influenza A virus	matrix (M) gene	F	GGCTCTCATGGAATGGCTAAA	This study
		R	TGCAGTCCTCGCTCACT	
		P	TTTGTGTTCACGCTCACCGTGC	
Porcine reproductive and respiratory virus US strain	3′UTR	F	ATRATGRGCTGGCATTC	[12, 28]
		R	ACACGGTCGCCCTAATTG	
		P	TGTGGTGAATGGCACTGATTGACA	
Porcine reproductive and respiratory virus EU strain	ORF7	F	GCACCACCTCACCCAGAC	[12, 28]
		R	CAGTTCCTGCGCCTTGAT	
		P	CCTCTGYYTGCAATCGATCCAGAC	
Porcine respiratory coronavirus	Nucleocapsid	F	AGCTATTGGACTTCAAAGGAAGATG	[19]
		R	CATAGGCATTAATCTGCTGAAGGAA	
		P	TCACGTTCACACACAAATACCACTTGCCA	
Porcine hemagglutinating encephalomyelitis virus	Spike protein	F	CAACCAGATCCTTCCACATATAAAG	This study
		R	GAGCAATCATCCTCCACAAGA	
		P	ATACAACCAGGTCAGCATTGCCCT	
β-actin	Actin	F	AGCGCAAGTACTCCGTGTG	[29]
		R	CGGACTCATCGTACTCCTGCTT	
		P	TCGCTGTCCACCTTCCAGCAGATGT	

F, Forward primer; R, Reverse primer; P, Probe.

(φ4 mm) by using the bead crusher μT-12 (TAITEC, Inc.), and centrifuged at 12,000 g for 5 min to obtain the supernatant. Bacteria nucleic acids, viral DNA, and viral RNA were extracted from the supernatant using a QIAamp® cador® Pathogen Kit (Qiagen,

Hilden, Germany) with a sample volume of 200 μl and elution volume of 50 μl, as described by the manufacturer. The extracted DNA and RNA were stored at −80°C until examination. The extracted nucleic acids were evaluated in triplicated by targeting respiratory disease complex pathogens in a single run of Dempo-PCR. When the Cq values were calculated by algorithm described above in more than two out of three runs, the samples were considered positive. In order to compare Dempo-PCR assay with the classical method, the conventional PCR (cPCR) was performed under each condition using conventional primers (Supplementary Table 2). A PrimeScript™ RT Master Mix (TaKaRa Bio) and GoTaq® Green Master Mix (Promega) was used. All reactions were performed in a total volume of 25 μl, which contained the sample nucleic acid, primers (the final concentration of all primers was 0.4 μM) and all other components included in the kits, according to the manufactures' protocols. Amplicons were detected by electrophoresing. The samples which showed the results of the Dempo-PCR assay is not consistent with the cPCR assay did not match, were confirmed by direct sequencing of amplification products.

All the experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Azabu University.

RESULTS

Sensitivity, linearity, and efficiency evaluated with standard curves from synthesized DNA

To evaluate the sensitivity, linearity, and efficiency of the PCR, 10-fold serial dilutions of synthesized DNA were tested by real-time PCR. Standard curves were constructed from Cq values, then the LOD, R^2, and E were evaluated (Supplementary Fig. 1). Table 2 shows the results for LOD number and CVs of run-to-run variants. The LOD, based on DNA copy number, was ≤100 copies/reaction for all primer-probe sets. The CVs were at most 2.62%; this reproducibility was observed with PRCV testing. In addition, the calibration curves of all assays covered a linear dynamic range of more than five orders of magnitude and showed R^2 values of at least 0.9922. Although the PCR efficiency for PRRSV US strain and PRV was slightly low (81.6% and 88.6%, respectively), the PCR efficiency in all detection assays was more than 80%, which was enough to quantify the target copy number.

Dempo-PCR performance in clinical sample testing

A total of 30 lungs from different affected animals on six farms with respiratory disease outbreaks were applied to Dempo-PCR. In addition, cPCR assay were also performed to compare the sensitivities of Dempo-PCR assay. As the results, there were samples detected by Dempo-PCR but not detected by cPCR. The sequences of these samples proved to be identical to the sequence of the target pathogens by direct sequencing of amplification products. To the contrary, there were no samples detected by cPCR but not detected by Dempo-PCR (Supplementary Table 3).

The results are presented as the number and percentage of positive samples from each farm (Table 3). In samples from farm C and F, both viral and bacterial pathogens, including PCV2 (100% and 50%, respectively), PRRSV US strain (100% and 50%, respectively) and *M. hyopneumoniae* (85.7% and 50%, respectively), were detected at high frequency, whereas mainly bacterial pathogens, including *B. bronchiseptica*, *H. parasuis*, *P. multocida*, *S. suis* and *M. hyorhinis* were prevailed in farm A, and *A. pleuropneumoniae* and *S. suis* were prevailed in farm E. In samples from farms B, mixed infections of PRRSV US strain, SIV and bacterial pathogens; *H. parasuis*, *P. multocida*, *M. hyorhinis*, and *M. hyopneumoniae* were detected. PCMV was detected at high frequency from all farms, whereas *P. multocida* toxin, *M. hyosynooviae*, PRV, PRRSV EU strain, and PHEV were not detected.

Table 2. Performance of sensitivity tests

Type of materials	Pathogens	LOD (/reaction)	Reproducibility CV (%)
DNA (copy number)	*Actinobacillus pleuropneumoniae*	10	0.27–1.67
	Boldetella bronchiseptica	10	0.00–1.10
	Haemophilus parasuis	10	0.10–0.91
	Pasteurella multocida	10	0.07–0.83
	Pasteurella multocida toxin	10	0.01–0.71
	Streptococcus suis	100	0.02–0.51
	Mycoplasma hyopneumoniae	10	0.08–1.51
	Mycoplasma hyorhinis	10	0.25–1.60
	Mycoplasma hyosynoviae	10	0.04–0.37
	Porcine circovirus 2	100	0.18–2.43
	Pseudorabies virus	100	0.56–1.47
	Porcine cytomegalovirus	10	0.12–0.53
	Swine influenza A virus	10	0.22–1.52
	Porcine reproductive and respiratory virus US strain	100	0.73–1.96
	Porcine reproductive and respiratory virus EU strain	100	0.06–1.11
	Porcine respiratory coronavirus	10	0.05–2.62
	Porcine hemagglutinating encephalomyelitis virus	10	0.03–0.63

LOD, Limit of detection; CV, Coefficient of variation.

Table 3. Detection of targets in lung tissue from clinical cases by Dempo-PCR

Pathogens	Positive samples in Dempo-PCR						
	Farm A N=4 n (%)	Farm B N=8 n (%)	Farm C N=7 n (%)	Farm D N=2 n (%)	Farm E N=5 n (%)	Farm F N=4 n (%)	Total N=30 n (%)
Actinobacillus pleuropneumoniae	-	-	-	1 (50.0)	2 (40.0)	2 (50.0)	5 (16.7)
Boldetella bronchiseptica	1 (25.0)	-	-	1 (50.0)	-	1 (25.0)	3 (10.0)
Haemophilus parasuis	4 (100)	4 (50.0)	6 (85.7)	-	-	-	14 (46.7)
Pasteurella multocida	3 (75.0)	1 (12.5)	1 (14.3)	2 (100)	-	1 (25.0)	8 (26.7)
Pasteurella multocida toxin	-	-	-	-	-	-	-
Streptococcus suis	1 (25.0)	-	-	-	2 (40.0)	-	3 (10.0)
Mycoplasma hyopneumoniae	-	2 (25.0)	6 (85.7)	2 (100)	-	2 (50.0)	12 (40.0)
Mycoplasma hyorhinis	4 (100)	6 (75.0)	7 (100)	-	-	2 (50.0)	19 (63.3)
Mycoplasma hyosynoviae	-	-	-	-	-	-	-
Porcine circovirus 2	-	-	7 (100)	2 (100)	-	2 (50)	11 (36.6)
Pseudorabies virus	-	-	-	-	-	-	-
Porcine cytomegalovirus	4 (100)	5 (62.5)	5 (71.4)	1 (50.0)	3 (60.0)	4 (100)	22 (73.3)
Swine influenza A virus	-	3 (37.5)	-	-	-	-	3 (10.0)
Porcine reproductive and respiratory virus US strain	3 (75.0)	7 (87.5)	7 (100)	-	-	2 (50.0)	19 (63.3)
Porcine reproductive and respiratory virus EU strain	-	-	-	-	-	-	-
Porcine respiratory coronavirus	-	-	-	-	-	2 (50.0)	2 (6.7)
Porcine hemagglutinating encephalomyelitis virus	-	-	-	-	-	-	-

DISCUSSION

PRDC is one of the most important health concerns for pig producers and involves multiple viral and bacterial pathogens. PRDC is multifactorial, with both infectious and non-infectious factors contributing to respiratory disease seen in pigs between the ages of 3 and 6 months. The interaction of viral and bacterial pathogens, environmental factors, pig-specific factors and management conditions all contribute to the development and impact the severity of PRDC [20]. The most commonly isolated pathogens are PRRSV, SIV, PCV2, and *M. hyopneumoniae*. The other pathogens associated with PRDC are *S. suis, A. pleuropneumoniae*, *P. multocida, P. multocida* toxin, *B. bronchiseptica*, *H. parasuis*, *M. hyorhinis, M. hyosynovie,* PRV, PRCV, PCMV [7, 8, 15, 20]. However, no single-reaction diagnostic test currently exists for the simultaneous detection of major pathogens commonly associated with PRDC. Routine diagnostic methods for detection of viruses implicated in PRDC include virus isolation in cell culture, antigen detection by direct fluorescent antibody staining, and enzyme immunoassay [5] and culture-based methods for bacteria [23]. These methods are time-consuming and require independent tests for each pathogen. Furthermore, the detection of bacterial pathogens typically depends on culture-based methods that can take several days to obtain results. Due to their high sensitivity and ease of use, PCR and real-time PCR tests have been developed for several agents implicated in the PRDC; however, these tests typically target single pathogens [24]. A multiplex PCR assay capable of detecting five porcine viruses including two porcine respiratory viruses was developed [4]. However, to date, there are no diagnostic tests capable of simultaneous detection of multiple major viral and bacterial porcine respiratory pathogens in a single reaction.

Recently Lung *et al*. [15]reported a novel prototype automated microarray that integrates and automates all steps of post-PCR microarray processing for the simultaneous detection and typing of four bacteria (*M. hyopneumoniae, P. multocida, S. enterica serovar Choleraesuis, S. suis*) and four viruses (PRRSV, SIV, PCV2, PRCV) differentiation of the two PRRSV genotypes and pathogenic versus non-pathogenic *P. multocida* strains. This electronic microarray assay can be completed in less than 4 hr with little user handling plus approximately 1.5 hr for the RT-PCR. These methods are highly specific and sensitive, and easy to operate, but these are expensive to run and requires expensive equipment. On the other hand, Dempo-PCR assay can be completed in less than 3 hr, and easy operate.

In this study, Dempo-PCR has been developed, following the methods of diagnosis of bovine diarrhea developed by Tsuchiaka *et al.* [26]. Since all primer-probe sets were optimized in the same temperature conditions, Dempo-PCR can detect a total of 17 pathogens, including 8 viruses, 8 bacteria, and 1 toxin, in a single run of TaqMan real-time PCR. In sensitivity test by using standard curves from synthesized DNA, all primer-probe sets showed high sensitivity. Furthermore, the results of detection of target pathogens from clinical samples using this method showed similar results to the respective conventional PCR method. However, PRRS virus is known to have a high frequency of genetic mutations, and the primer and probe sequences will need to be checked at a considerable frequency when performing Dempo-PCR from field samples. The type of pathogens involved in PRDC is specific to the regions and countries where production occurs [20]. Therefore, it may be necessary to change the inspect pathogens according to the regions. However, Dempo-PCR is possible to detect many types of pathogens simultaneously.

By Dempo-PCR assay, multiple PRDC pathogens can be detected comprehensively and simultaneously. This assay can quickly elucidate existence of pathogens in a sample. In this study, Multiple viral and bacterial porcine respiratory pathogens were detected

from pigs of all farms examined. Especially, five bacteria pathogens (*A. pleuropneumoniae*, *B. bronchiseptica*, *P. multocida*, *M. hyopneumoniae*, *M. hyorhinis*) and four viruses (PCV2, PCMV, PRRS US strain, PRCV) were detected on pigs of farm F. In this study, PCMV was detected in high proportion from pigs of all farms, and mixed infection with multiple pathogens was observed. PCMV has been documented worldwide, and shows high infection rates on pig farms in Japan, Europe, North America, and China [3, 6]. Hansen *et al.*, reported that a significant association between PCMV and PCV2 was only found in the cases of PRDC, and the role of PCMV in PRDC needs to be elucidated [7]. It is necessary to elucidate the combination of multiple pathogens for the elucidation of the etiology of PRDC, and Dempo -PCR will be a useful tool for that. PHEV is a subclinical infection, but its role as a respiratory pathogen was suggested since it was isolated from the acute respiratory disease in pigs in Michigan in 2015 [14]. The swine serological survey of PHEV also showed that it is widely and highly distributed in Japan [10]. Therefore, PHEV was added to the target pathogens of Dempo-PCR, but it was not detected from these samples.

In conclusion, Dempo-PCR can identify a wider range of existing pathogens quickly and easily compared to one assay-one pathogen test. Considering multiple etiology of PRDC, screening by Dempo-PCR would help us determine treatment and prevention measures. This detection system may provide an alternative testing method that is simpler, faster, and more comprehensive than existing assays.

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