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Application of Multiplex PCR Assay for *Campylobacter fetus Sub sp.* Detection and Differentiation in Bovine Preputial Wash

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ABSTRACT

The aim of this research was to evaluate the detection and differentiation of *C. fetus* subspecies through multiplex PCR (mPCR) method from the bovine preputial wash. The evaluation was done by comparing 2 extraction methods, which was by commercial kit extraction and boiling method, and also by measuring the effect of Transport Enrichment Media (TEM) addition as bacterial enrichment. Spiked bovine preputial washes with 2 extraction methods (commercial kit and boiling), with and without TEM addition were used as a sample. The results showed that the highest sensitivity/specificity was by boiling extraction without TEM addition, which reached 83.3%/100% for all or grouped sample. The results also supported by Poisson regression analysis which showed the comparison between sample with and without TEM addition which had 1.3 Incidence Rate Ratio (IRR). The obtained IRR score indicated that sample without TEM addition had 30% better result compared to the sample with TEM addition. Furthermore, a comparison between commercial kit and boiling extraction showed no significant difference (1.029 IRR). The research concluded that boiling extraction without TEM addition is the most accurate, fast and economical extraction method regarding its high sensitivity and specificity value, with less than 24 hours testing time and without any commercial kit requirements. However, a validation and verification with field sample were still needed before applied in the laboratory.

Keywords: Bovine genital campylobacteriosis, *Campylobacter fetus*, Cff, Cfv, Multiplex PCR

Introduction

Bovine genital campylobacteriosis disease (BCG) caused by *Campylobacter fetus* sub sp. *veneralis* (Cfv) is an infectious genital disease which could cause temporary infertility on cattle (Hum *et al.,* 2009), and also known to cause early embryonic death and abortus as well (Truyers *et al.,* 2014). A clinical symptom does not usually appear on cattle. However, the infected cattle still could act as a carrier to infect the cows (Eaglesome and Garcia, 1992). In addition, artificial insemination could also deliver the disease through infected frozen bovine semen (Modolo *et al*., 2000).

Another *C. fetus* subspecies which could cause a sporadic abortion in cattle is *C. fetus subspecies fetus* (Cff). Furthermore, Cff is also known to cause enteritis on cows and sheeps (Truyers *et al.,* 2014). Aside from Cfv, Cff is not correlated to the livestock infertility (Eaglesome and Garcia, 1992). Differentiation of the bacteria to the subspecies level then becomes essential for livestock diagnosis and treatment and for epidemiological surveillance (Hum *et al.*, 1997). The differentiation between subspecies with culture method utilizes the bacterial tolerance to 1% glycine, where Cff showed the capability to tolerate glycine, in which Cfv could not. However, Chang and Ogg (1971) successfully showed that there is a glycine tolerance Cfv, namely *Campylobacter fetus* subspecies *venerealis biovar intermedius.*

Indonesia still has no information status to the disease, in which means that there is still no report on BCG outbreak. The latest research to the disease was done by BBALITVET researchers in 1998, where Suprodjo Hardjoutomo observed 9 provinces in Indonesia and showed the negative Cfv result (Hardjoutomo, 1998). The national program for heifer breeding through artificial insemination (UPSUS SIWAB) program was launched by the government to achieve livestock self-sufficiency target in 2026, and the program focused on productive cows pregnancy, with the fulfillment of bulls and frozen semen availability as well (Kementan RI, 2016). An increase in livestock

and frozen semen importation would be possible to support the program. Thus a certain prevention of the disease outbreak should be done.

In 2016, 600 heifers from Australia were imported through Soekarno Hatta airport, while 302.167 cattle calves and 4 buffalo calves from Australia were imported through the port of Tanjung Priok (BBKP Tanjung Priok, 2017; BBKP Soekarno Hatta, 2017). The massive amount of calves importation to Indonesia would also increase the risk of BGC outbreak. According to *World Animal Health Information System* (WAHIS) by Office International des Epizooties (OIE), in 2016 Australia was not listed as BCG free country. According to the report, a Cfv test should be done as one of the importation requirement. Thus, a prepared BGC disease detection laboratory in Indonesia then become required.

Molecular analysis method which yields more accurate and sensitive results compared to culture method has been developed to differentiate *C. fetus*. *Polymerase Chain Reaction* (PCR) assay, *Pulse Field Gel Electrophoresis* (PFGE), *Multilocus Sequence Typing* (MLST), *Amplified Fragment Length Polymorphisms* (AFLP) has been developed for phenotype differences confirmation (Van Bergen *et al.,* 2005; Schulze *et al*., 2006; Van der Graaf-van Bloois *et al.,* 2013).

The mPCR assay for *C. fetus* subspecies detection and differentiation has been developed by Hum *et al.* (2009) and Iraola *et al*. (2012). The method is known to have shorter testing time, and the sequences amplification can be done simultaneously. Multiplex PCR required several sequences in a reaction so that mPCR could be faster testing method compared to single amplification through single PCR assay (Markoulatos *et al*., 2002).

Materials and Methods

Bacterial isolate and detection limit

This research used Cff dan Cfv isolate from Balai Besar Penelitian Veteriner (BBALITVET), *Brucella abortus* was collected from Balai Besar Uji Standard Karantina Pertanian (BBUSKP) and *Campylobacter jejuni* (ATCC®). The Cff dan Cfv isolate was measured by spectrophotometer at 620 nm until reached Optical Density (OD) 0.669 or equal to McFarland no. 4 standards (cell concentration 10^9 CFU/ml). The isolate was then diluted until reached 10^8 to 10^0 cfu/ml isolate concentration. The nine inoculum concentration then tested to observe the detection limit. The four lowest inoculum concentration from the detection limit was then used as a sample for the next measurement. Sample extraction method for detection limit was done by using the commercial kit and by boiling.

Preparation of spiked sample

Preputial wash samples which showed the negative result to Cff dan Cfv (BBUSKP sample archive) were spiked with Cff (4 samples), Cfv (4 samples), *B. abortus* (1 sample), *C. jejuni* (1 sample) and 1 negative sample (non-spiked), with a total of 11 samples. The preparation was done by mixing 10^x CFU/ml bacterial isolate with nonsterile preputial wash at the ratio of 1:9, so the preputial wash sample at the bacterial concentration of 10^{x-1} CFU/ml was achieved. The detection limit measurement for spiked samples was also done for the result comparison between pure isolate and the isolate in preputial wash under same concentration.

Weybridge transport enrichment media addition

Weybridge transport media was made based on Lander (1990) with modification on the usage of antibiotics. Spiked sample was taken at 1 ml, added with 9 ml Weybridge TEM, and then homogenized. The next step was 6 days incubation at 25^{o}C under aerobic condition before tested with mPCR. Bacterial growth was expected on TEM so that the result of mPCR would have higher sensitivity.

Sample extraction

Pure isolate and preputial wash samples were extracted with a commercial kit (QIAamp® DNA Mini Kit, Qiagen) and by boiling according to Hum *et al.* (2009). The commercial kit could purify total DNA from the various sample through provided reagents and by following the listed protocols from the producer. Sample extraction by boiling was started by taking 1 ml of preputial wash sample, centrifuged at 12,000 rpm for 5 minutes. The obtained precipitate then added with 1 ml of nuclease-free water and boiled in water bath at 95^{o}C for 10 minutes. The sample was then centrifuged at 10,000 rpm for 1 minute, and the obtained supernatant was collected for DNA template.

PCR primers

PCR primers for PCR assay were MG3F and MG4R primers, which were specifics to 2 C. *fetus* subspecies; and also VenSF and VenSR which were specifics to Cfv. The oligonucleotide primers used in this research can be seen in Table 1.

Table 1. PCR primers sequence (Hum *et al*., 2009)

Primers	Sequences	Product Size
VenSF	5'-CTT AGC AGT TTG CGA TAT TGC CAT T-3'	142 bp
VenSR	5'-GCT TTT GAG ATA ACA ATA AGA GCT T-3'	
MG3F	5'-GGT AGC CGC AGC TGC TAA GAT-3'	764 bp
MG4R	5'-TAG CTA CAA TAA CGA CAA CT-3'	

Multiplex PCR assay

The mPCR assay in this research is modified from BGC disease identification by OIE according to Hum *et al*. (2009), with minor modification on the used enzyme. The reaction consisted of 10 µl Kapa ReadyMix (Kapa Biosystem); 0.6 µl (10µM) for each MG3F and MG4R primers and 0.5 µl (10µM) for each VenSF dan VenSR primers; 5.8 µl nuclease free water and 2 µl DNA template with the total of 20 µl reaction volume. Positive Cff dan Cfv control, negative and no template control (NTC) should be included in each amplification.

PCR assay was done on 1 cycle at 95°C for 3 minutes on early denaturation, followed by 35 cycles at 95°C for 15 seconds on denaturation, 54°C for 15 seconds on annealing, and 72°C for 30 seconds on extension, then followed by 1 cycle at 72°C for 5 minutes on final extension. The amplification products were then analyzed with electrophoresis.

The PCR products were separated by using 2% agarose after added with 0.5 µg/ ml ethidium bromide at 120 V for 1 hour with 100 bp *DNA* ladder marker. The results of electrophoresis were then observed by using Gel Documentation. Specific Cff band would be seen at 764 bp, while Cfv at 142 bp (Hum *et al*., 2009).

Data analysis

The sensitivity and specificity evaluation were measured by comparing obtained mPCR result with references. The 4 sensitivity and specificity results from 2 extraction methods (boiling or kit), with or without TEM addition would be obtained as follows: (1) Spiked sample without TEM and by kit extraction; (2) Spiked sample with TEM and by kit extraction; (3) Spiked sample without TEM and by boiling extraction; and (4) Spiked sample with TEM and by boiling extraction. All of 11 samples were replicated 3 times and considered as different samples so that a total of 33 samples were measured.

Sensitivity and specificity evaluation would be measured by overall and grouped based on the bacteria (Cfv, Cff, and negative group). The Poisson regression statistic analysis was done to understand the ratio of each extraction methods. The ratio of 1 or close to 1 would show the nonsignificant difference between each method.

Result and Discussion

The mPCR assay according to Hum *et al*. (2009) with minor reagent modification was able to detect and differentiate *C. fetus* subspecies. The positive Cff result on agarose showed 1 DNA fragment at 764 bp, and the positive Cfv result showed 2 DNA fragments at 764 bp and 142 bp. The 764 bp fragment was MG3F/MG4R primers amplicon which showed *C. fetus* species, while 142 bp fragment was VenSF/VenSR specific primers for Cfv (Figure 1).

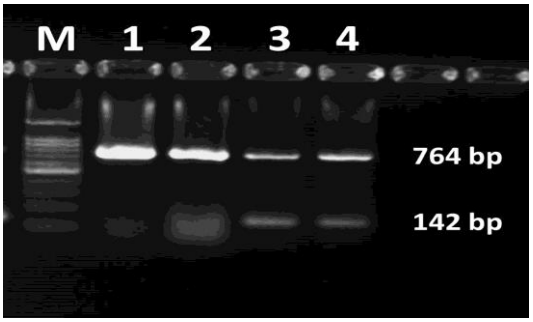

Figure 1. The result of referenced isolates Multiplex PCR. (M) marker100 Bp; (1) Cff; (2) ;Cff; (3) Cfv; (4) Cfv.

Determination of isolate detection limit

The result of detection limit on mPCR assay with pure isolate sample and spiked isolate sample on preputial wash can be seen in Table 2.

Pure Cff isolate extraction with commercial kit showed detection limit at 10^4 CFU/ml concentration and reduced to 10^6 CFU/ml for isolate in the bovine preputial wash. On the Cff boiling extraction, the detection limit between pure isolate and isolate in bovine preputial wash showed the same result, which was at the concentration of 10^5 CFU/ml. Extraction of pure Cfv with commercial kit showed detection limit at the concentration of 10^4 CFU/ml and reduced to 10^7 CFU/ml for isolate in the bovine preputial wash. On the Cfv boiling extraction, the obtained detection limit on pure isolate was 10^4 CFU/ml and reduced to 10^6 CFU/ml for the sample with the bovine preputial wash. Furthermore, at the concentration of 10^3-10^0 CFU/ml, detection of Cff and Cfv could not be done (data not shown). From the detection limit test, it can be seen that

Table 2. Detection limit comparison between pure isolate sample and isolate sample in bovine preputial wash

No	Cff/Cfv (CFU/ml)	Pure Isolates Cff/Cfv		Isolates in bovine preputial wash Cff/Cfv	
		Kit Extraction	Boiling Extraction	Kit Extraction	Boiling Extraction
1	10^8	+ / +	+ / +	+ / +	+ / +
2	10^7	+ / +	+ / +	+ / +	+ / +
3	10^6	+ / +	+ / +	+ / -	+ / +
4	10^5	+ / +	+ / +	- / -	+ / -
5	10^4	+ / +	- / +	- / -	- /-

+ : Positive PCR results.
- : Negative PCR results.

even at four lowest concentration, which was 10^7, 10^6, 10^5 dan 10^4 CFU/ml, the bacteria were still able to be detected. It is assumed that in low concentration, the bacteria would be hard to be detected, but a positive result would still be able to be achieved with the right method.

The effect of preputial wash on detection limit measurement in this research reduced the sensitivity for bacterial detection even under same concentration with the pure isolate. The first affecting factor is a physical factor from the preputial wash, which contained tissues, blood, feces, and other contaminants (Rådström *et al*., 2004). Other elements like urine, semen, feces, and blood could disrupt the PCR assay sensitivity (Wilson, 1997; Rådström *et al*., 2004). The amount of excretes and proteins in preputial wash could also affect DNA isolation during extraction. DNA isolation aims to separate the DNA to other contents, such as proteins, fats, and carbohydrates. The main principals of DNA isolation are lysis, DNA extraction or separation from other solid contents like cellulose and proteins, and then DNA purification (Corkill dan Rapley, 2008). According to Surzycky (2000), there are several things to consider during DNA isolation, which are DNA should be free from contaminants like protein and RNA, the method should be effective and can be applied to all species, the method should not change the structure and the function of DNA molecules, and the method should be simple and fast. Any foreign substances, like debris, could give the false positive result to the PCR assay (Theophilus 2008).

The second affecting factor was the presence of contaminant bacteria. The prone characteristic of *C. fetus* could make other bacteria to grow in the preputial wash. Lander (1990) stated that other contaminants which could disrupt *C.fetus* isolation on the bovine preputial sample are *Pseudomonas spp*, *Proteus spp,* and fungi. Research by Chaban *et al.* (2012) detected *Pseudomonas aeruginosa* and fungi at 50% tested bacteria culture. Schmidt (2008) presumed that the presence of *Pseudomonas* and other contaminants on spiked preputial wash sample would contribute to the failure of PCR assay to detect Cfv. Other *Pseudomonas* strains, such as *P. aeruginosa* is known to produce *DNase* enzyme (Gilardi, 1985). The enzyme activity would degrade DNA on the sample, thus disrupt the PCR assay result.

Multiplex PCR assay sensitivity and specifitvy evaluation

Thirty-three samples consisted of 12 Cff samples, 12 Cfv samples, 3 *C. jejuni* samples, 3 *B. abortus* samples, and 3 nonspiked samples were extracted with 4 different extraction methods and then followed by mPCR assay (Table 3).

The overall samples measurement (Table 4) showed the highest sensitivity was in boiling extraction method without TEM addition, which was 83.3%, while the lowest sensitivity was in both commercial kit extraction and boiling extraction with TEM addition, which were 62.5%.

The grouped samples measurement (Table 5), showed the highest sensitivity was on Cff group for the sample with TEM addition, which reached 100%. The result indicated that all of Cff sample with TEM addition, extracted both with commercial kit and boiling, gave positive result under mPCR assay.

Table 3. Multiplex PCR test on bovine preputial wash samples

No	Samples (CFU/ml)	Preputial wash Without/with TEM	
		Kit	Boiling
	Cff		
1	10^7	+ / +	+ / +
2	10^7	+ / +	+ / +
3	10^7	+ / +	+ / +
4	10^6	+ / +	+ / +
5	10^6	+ / +	+ / +
6	10^6	+ / +	+ / +
7	10^5	+ / +	+ / +
8	10^5	+ / +	+ / +
9	10^5	- / +	+ / +
10	10^4	+ / +	- / +
11	10^4	- / +	- / +
12	10^4	- / +	+ / +
	Cfv		
13	10^7	+ / +	+ / +
14	10^7	+ / +	+ / +
15	10^7	+ / -	+ / -
16	10^6	+ / -	+ / -
17	10^6	+ / +	+ / +
18	10^6	+ / -	+ / -
19	10^5	+ / -	+ / -
20	10^5	+ / -	+ / -
21	10^5	+ / -	+ / -
22	10^4	+ / -	- / -
23	10^4	- / -	+ / -
24	10^4	- / -	- / -
	Negative		
25	*C.jejuni*	- / -	- / -
26	*C.jejuni*	- / -	- / -
27	*C.jejuni*	- / -	- / -
28	*B.abortus*	- / -	- / -
29	*B.abortus*	- / -	- / -
30	*B.abortus*	- / -	- / -
31	non *spike*	- / -	- / -
32	non *spike*	- / -	- / -
33	non *spike*	- / -	- / -

+ : positive PCR results.
- : negative PCR results.

The result is in contrary to Cfv group with the same method, which showed the lowest sensitivity (25%). The result of specificity measurement showed 100% on all method, which

indicated that there was no false positive result on other bacteria sample and a sample without bacteria.

The result of Poisson regression analysis showed close to 1 Incidence Rate Ratio (IRR), which was 1.029 with confidence interval (CI) at 95%. The result indicated that there was no significant difference between commercial kit extraction and boiling extraction. However, the comparison between TEM addition and without TEM addition showed 1.3 IRR, which indicated that extraction without TEM addition gave 30% better result. The Poisson regression analysis can be seen in Table 6.

The result of sensitivity measurement (Table 4) showed that boiling extraction without TEM addition had the highest sensitivity compared to other 3 methods. The result also supported by Poisson regression analysis which showed no significant difference between commercial kit extraction to the comparison between with and without TEM addition, which showed that extraction without TEM addition yield better result. However, in the economic perspective, boiling extraction had better economical value compared to commercial kit extraction, regarding that commercial kit would require additional cost. Furthermore, boiling extraction had faster and more simple method, yet further studies should be done to determine the efficiency of the method.

Based on the sensitivity measurement (Table 5), where the bacteria were grouped, there were significant differences between Cff and Cfv. The Cff sensitivity for the sample with TEM addition reached 100%, which means that all Cff sample yield positive Cff result when tested with mPCR. The result was different with Cfv which only reached 25% sensitivity, far lower compared to sample without TEM addition which reached 83% whether on commercial kit or boiling extraction.

The different extraction result between Cff and Cfv with TEM addition could be caused by several reasons, such as different testing time, in which extractions without TEM addition were done first. Even though originated from same culture stock, the bacterial sub-culture factor or differences in a bacterial generation could alter the bacterial characters. The decrease in spiked Cfv sensitivity on preputial wash with TEM addition was different with Beniawan (2017) on the mPCR assay of a pure Cfv isolate with TEM addition. The result of mPCR assay on pure isolate with TEM addition showed an increase in detection limit from 10^4 CFU/ml (without TEM addition) to 10^1 CFU/ml (with TEM addition).

Aside from preputial wash factors, which described above, it is suspected that several substances in TEM composition could disrupt the PCR, like charcoal, iron (Thunberg *et al.,* 2000) and blood (Al-Soud and Rådström, 2001). The Weybridge TEM media developed by Lander (1990), made from Mueller Hinton broth, charcoal, horse/sheep blood as a protein source, antibiotics (vancomycin, polymixin B, and trimethoprim), and FBP supplement (Ferrous Sulphate, Sodium meta-Bisulphite, and Sodium Pyruvate). In

Table 4. Sensitivity and specificity calculations of bovine preputial wash sample on all samples (n = 33)

Methods	True +[a]	False -[b]	True -[c]	False +[d]	Sensitivity value[e] (%)	Specificity value[f] (%)
Kit without TEM	19	5	9	0	79.17	100
Boiling without TEM	20	4	9	0	83.33	100
Kit with TEM	15	9	9	0	62.50	100
Boiling with TEM	15	9	9	0	62.50	100

[a] Positive PCR result from positive isolates reference; [b] negative PCR results from positive isolates reference; [c] negative PCR result from negative isolates reference; [d] positive PCR result from negative isolate reference; [e] the result of a/a+b (%); [f] the result of c/c+d (%).

Table 5. Sensitivity and specificity measurement of bovine preputial wash sample on grouped samples: Cff (n = 12); Cfv (n = 12) and negative (n = 9)

Methods	True Positive[a]		Sensitivity (%)[b]		Specifity (%)[c]	
	Cff	Cfv	Cff	Cfv	Cff	Cfv
Kit without TEM	9	10	75	83.3	100	100
Boiling without TEM	10	10	83.3	83.3	100	100
Kit with TEM	12	3	100	25	100	100
Boiling with TEM	12	3	100	25	100	100

[a]positive PCR results from positive isolate reference: [b] the result of a/12 (%); [c] all negative isolates gave a negative result on overall PCR assay.

Tabel 6. Poisson regression calculation

Result	IRR[a]	Std Err.[b]	Z[c]	P>|Z|[d]	[95% Conf. Interval][e]	
Boiling vs Kit	1.029412	.2478793	0.12	0.904	.6421291	1.650273
Without vs With TEM	1.3	.3157003	1.08	0.280	.8076666	2.092447
_Cons	.4479578	.0984003	-3.66	0.000	.2912451	.6889943

[a] Incidence Rate Ratio; [b] Standard Error ; [c] Normal distribution ; [d] Probability; [e] Confidence Interval

general, field sample in which will be tested in the laboratory is mixed with TEM to improve bacteria lifespan regarding that bacterial isolation with culture method is still considered as gold standard for *C. fetus* detection. However, the result of this research showed that TEM addition was not needed for molecular *C. fetus* detection. Chaban *et al.* (2012) stated that TEM addition did not affect the contamination reduction of *P.aeruginosa*. The *Pseudomonas* colony is also resistance to antibiotics on Weybridge TEM and Skirrow's media (Schmidt, 2008) so that TEM addition for molecular detection would possibly not yield a better result.

PCR assay is still used for confirmation to the bacterial isolation with culture method (OIE, 2017), but it is expected that PCR assay could also be used as a standard test for detection and differentiation of *C. fetus* subspecies. Aside from faster testing time compared to culture method, PCR assay could also differentiate *C. fetus* subspecies, in which it is hard to differentiate based on phenotype properties (van der Graaf-van Bloois *et al.*, 2014). Hence, validation and verification of the method with field sample are required to prove that the method fulfilled the aim and utilization factors, while also applicable in the laboratory. A faster and accurate test result is needed in international trading, in this case, a cattle importation from Australia to Indonesia. One deciding factor for cattle import permission is from laboratory test result. It is expected that accurate laboratory test would prevent BGC disease to hit and spread in Indonesia.

Conclusion

The most accurate, quick and economical extraction method on preputial wash sample is by boiling without TEM addition. The method showed 83.3% sensitivity and 100% specificity wether on overall samples or in the grouped samples (Cff, Cfv, and negative group). The method only requires less than 24 hours testing time without any commercial kit.

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