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Application of a Multiplex PCR Assay to Detect *Campylobacter fetus* subspecies *venerealis* from Imported Bovine Preputial Samples

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INTRODUCTION

Campylobacteriosis, caused by *Campylobacter spp.,* is of considerable economic importance to the cattle industry worldwide. *Campylobacter spp*. were recognized as etiological agents of abortion in sheep. *Campylobacter fetus* subsp. *fetus* (*Cff*) causes sporadic abortion in sheep, often late in gestation, while subspecies *venerealis* (*Cfv*) is a cause of sexually transmitted bovine infertility and sporadic abortion in cattle. Various investigations have been carried out in different countries to assess the prevalence and impact of this disease. Some published results surveys are outlined in Table 1.

Table 1. A summary of published data showing the prevalence of *C. fetus* in different countries

Study area	Sample type(s)	Sample size	Prevale nce of *C. fetus* (%)	Diagnostic method
Australia (1985-1986)	Bulls (preputi a suction)	1 008 animals 41 herds	87% herds positive	Serological (Fluorescen t antibody test)
Californi (United States of America)	Cows	400	47	Serological (ELISA)
New Zealand	Cows (vaginal mucous) and bulls (preputial wash)	1 230 cows (125 herds) 54 bulls	70% herds positive Cfv : 0 Cff/others	Serological (ELISA) Bacteriologi cal culture

Cff: *Campylobacter fetus* subsp. *fetus*, *Cfv*: *C. fetus* subsp. *venerealis*.

According to [1], *Bovine Genital Campylobacteriosis* (BGC) disease was classified as 1st Group of animal quarantine disease. It is an exotic disease that was not ever detected in Indonesia. However, large scale cattle importation to Indonesia from the countries which ever reported BGC prevalence in their territories, initiating and spreading BGC will be the major threat for feedlotter or dairy farm in Indonesia. Hence, we should apply diagnostic test to detect *Cfv* in order to prevent the introducing the BGC to Indonesia. The aim of this study was to verify that multiplex PCR assay applicative to detect *Campylobacter fetus* subsp. *venerealis* from field samples.

MATERIALS AND METHODS

This study was conducted between March and July 2016 in Center for Diagnostic Standard of Agricultural Quarantine (CDSAQ), Jakarta. Bovine preputial samples were obtained from the imported bulls from 4 animal quarantine installations of Tanjung Priok Agricultural Quarantine. Bulls were selected as the chance of a positive diagnosis is greater than in cows. Preputial samples collected by swabbing or scraping are more concentrated, less contaminated, and result in a higher percentage of positive diagnoses. *Campylobacter fetus* does not remain viable for more than 6 – 8 hours in preputial samples. Therefore, we used Clark's transport enrichment medium (TEM).

Preputial samples were collected to obtain 10-15 ml preputial wash. Supernatant of the preputial wash (2 ml) was injected to container contained 8 ml TEM, or inoculated directly 50-100 µl to selective blood agar. Supernatant were inoculated 50-100 µl to selective blood agar after a day (I), 3 days (II) or 6 days (III) in TEM. This trial was conducted to observe in case the *Campylobacter fetus* only survived in particular period in TEM. All samples were cultured, except samples from one farm, due to the unavailability of TEM and selective blood agar. The inoculated selective blood agar was incubated in a microaerophilic atmosphere (5% O_2, 5% CO_2 and 90% nitrogen) at 37°C and examined at 3-5 days. A suitable microaerophilic atmosphere can be achieved by the use of CampyGen™ in anaerobic jars.

Suspect Campylobacter colonies are 1

to 3 mm in diameter on blood agar, convex and raised (Fig 1). They are colorless, translucent, or grey to buff colored, and are non-hemolytic.

Fig. 1. Colonies growth of subculture of *Campylobacter fetus* subsp. *fetus*

Multiplex PCR

Multiplex PCR method was applied to identify and differentiate *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis* in a single step. Oligonucleotide sequences of the primers used in this study are shown in Table 2.

Table 2. Primers used for the multiplex PCR assay

Name	Sequence	Amplicon size (bp)	Specificity
VenSF	5'-CTT AGC AGT TTG CGA TAT TGC CAT T-3'	142	*Cfv*
VenSR	5'-GCT TTT GAG ATA ACA ATA AGA GCT T-3'		
MG3F	5'-GGT AGC CGC AGC TGC TAA GAT-3'	764	*Cff*
MG4R	5'-TAG CTA CAA TAA CGA CAA CT-3'		

The VenSF, VenSR, MG3F, and MG4R, primer names and sequences were previously reported by [2]. *Cff*: *Campylobacter fetus* subsp. *fetus*, *Cfv*: *C. fetus* subsp. *venerealis*.

The PCR reactions were performed in a total volume of 20 µl consisting of the following: 11.5 µl of PCR-grade water (DNAse, RNAse free), 4.0 µl of 5X KAPA2G buffer (KAPA Biosystem, USA), 0.4 µl of dNTP (KAPA Biosystem, USA), 0.6 µl of each MG3F and MG4R primer, 0.4 µl of each VenSF and VenSR primer (XIDT), 0.1 µl of KAPA2G Fast DNA Polymerase (KAPA Biosystem, 5 U/µl) and 2 µl of template DNA. Positive and negative control should be included if ran any amplification. The positive controls for *Cfv* and *Cff* were obtained from the collections of the BBLITVET.

The cycling parameters were: initial denaturation at 95 °C for 3 min, followed by 35 three-step cycles, including denaturation at 95 °C for 15 sec, annealing at 54 °C for 15 sec, and extension at 72 °C for 15 sec, and a final extension at 72 °C for 5 min. The amplification products were analyzed by electrophoresis on 2% (w/v) agarose gel and visualized by ethidium bromide fluorescence. The unique amplification product should be at 764 bp (*Campylobacter fetus* subsp. *fetus*-specific) or 142 bp (*C. fetus* subsp. *venerealis*-specific)[2].

Each amplification product (5 µl) was mixed with 1 µl *loading dye* (Vivantis™). They were analyzed by electrophoresis on 2% (w/v) agarose gel (Promega™) and visualized by 0.5 µg/ml ethidium bromide (Applichem™) fluorescence, run on 120 V for 50 min, using DNA ladder 100 bp (Vivantis™). The electrophoresis result was visualized by UV-transilluminator.

RESULT AND DISCUSSION

Visual evaluation of inoculated plates indicated that microbial contaminants adversely affect the detection of *Campylobacter fetus*. It was the same as the result of [3] that showed the quickly growing, large, or swarming contaminants, such as *Pseudomonas,* or mold, have reduced the area of growth medium available for detection of *Campylobacter fetus*.

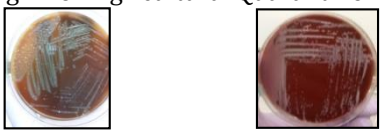
The subspecies of *Campylobacter fetus*, i.e. *Cff* and *Cfv*, are considered to be distinct in their habitats, the diseases they produce and biochemichal characteristic, but are closely related at the genomic level. Therefore, we can't differentiate them by identifying the colonies on selective blood agar. The bacteria culture and a trial with fresh preputial samples or after a day (I), 3 days (II) or 6 days (III) in TEM (Table 3) were conducted to ensure the *Campylobacter* grew, and evaluate its preference condition. Some samples showed suspect *Campylobacter* colonies Table 3, Figure 2 and 3, but some samples did not grow any colonies. To confirm the sample was positive of *Cff* or *Cfv*, or negative, we conducted PCR method. Almost all samples, including the scraping of preputium organ samples from one farm, were tested with PCR assay to ensure the existence of *Cfv*.

Table 3. Result of culture, subculture and Multiplex PCR detection

Farm	Sample no.	Culture				Sub culture		PCR typing				
		Fresh	I	II	III	I	Reculture	Fresh	I	II	III	Sub culture
P	1	Uncultured							Negative			
	2								Negative			
	3								Negative			
	4								Negative			
	5								Negative			
Q	1		C			C	C		N	N	N	N
	2			C					N	N	N	N
	3			C					N	N	N	N
	4			C			C		N	N	N	N
	5		C	C		C	C		N	N	N	N
R	1	C						N	N	N	N	N
	2							N	N	N		
	3		C					N	N	N		
	4	C						N	N	N		N
	5							N	N	N		
S	1	C	C					N	N	N	N	
	2	C						N	N	N	N	
	3	C				C		N	N	N		
	4					C		N	N	N		
	5	C	C			C		N	N	N		

Fresh: fresh sample, right after collected, cultured on blood agar; I : sample cultured a day after inside

transport enrichment media (TEM); II : sample cultured 3 days after inside TEM; III : sample cultured 6 days after inside TEM.
C = growth of suspected colonies, N = negative.
P, Q, R, and S = animal quarantine installations of Tanjung Priok Agricultural Quarantine.



A B

Fig. 2. Suspect *Campylobacter* colonies, cultured a day after inside TEM (A), and 3 days after inside TEM (B), different samples from farm Q

A B

Fig. 3. Suspect *Campylobacter* colonies, cultured fresh (A), and a day after inside TEM (B), the same sample from farm S

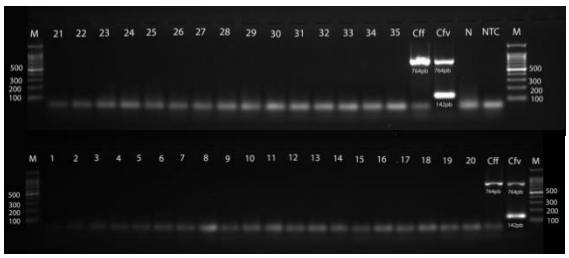


Fig. 4. 1-5: scrap of organ samples from farm P. 6-35: samples culture from farm Q, R and S. M = marker. Cff = positive control of *Campylobacter fetus* subsp. *fetus.* Cfv = positive control of *Campylobacter fetus* subsp. *venerealis.* N = negative control. NTC = no template control

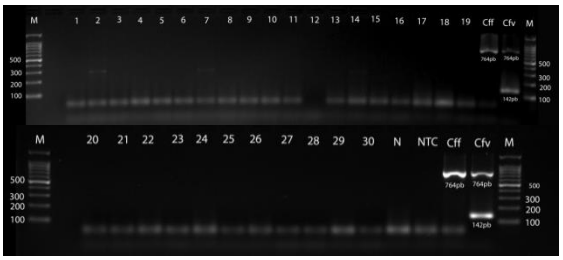


Fig. 5. 1-30: samples culture from farm Q, R and S. M = marker. Cff = positive control of *Campylobacter fetus* subsp. *fetus.* Cfv = positive control of *Campylobacter fetus* subsp. *venerealis.* N = negative control. NTC = no template control

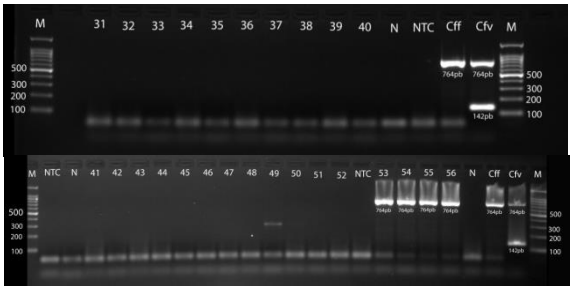


Fig 6. 31-40: samples culture from farm S and R. 41-52: samples subculture from farm S and Q. 53-56: subculture of *Campylobacter fetus* subsp. *fetus* isolate. M = marker. Cff = positive control of *Campylobacter fetus* subsp. *fetus.* Cfv = positive control of *Campylobacter fetus* subsp. *venerealis.* N = negative control. NTC = no template control

As described by [4], although some variability has been reported for traditional bacteriological methods (growth in 1% glycine and H_2S production) used to differentiate *C. fetus* subspecies, but the multiplex PCR assay remains a method which was able to correctly identify subspecies in all cases. In this study, we did not found any positive result of *Cff* nor *Cfv* from field samples by multiplex PCR assay (Fig 4, 5 and 6), but this method was applicative, as it showed the amplicons of 764 bp (*Cff*-specific) from all subcultures of *Cff* isolate (Fig 6). It also showed the positive control of *Cfv* and *Cff*.

CONCLUSION

Multiplex PCR assay was applicative to detect *Campylobacter fetus* subsp. *venerealis* from field samples. It is important to apply this method to test the imported bulls to prevent the introducing of Bovine Genital Campylobacteriosis to Indonesia.

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