POLYMERASE CHAIN REACTION OPTIMIZATION FOR THE DETECTION OF *PASTEURELLA MULTOCIDA* B:2, THE CAUSATIVE AGENT OF HAEMORRHAGIC SEPTICAEMIA

LILY NATALIA and ADIN PRIADI

Balai Penelitian Veteriner
Jalan R.E. Martadinata 30, P.D. Box *151,* Bogar *16114,* Indonesia

(Diterima dewan redaksi 27 Oktober 2001)

ABSTRAK

NATALIA, L. dan A. PRIADI. 2001. Optimisasi Polymerase chain reaction untuk mendeteksi Pasteurella multocida B:2, penyebab penyakit Septicaemia epizootica. Jurnal Ilmu Ternak dan Veteriner 6(4): 280-284.

Suatu metode *polymerase chain reaction* (PCR) untuk mendeteksi *Pasteurella multocida* B:2 secara spesifik dengan menggunakan satu set *DNA primers* telah dioptimisasi. Dalam optimisasi ini, pengaruh penambahan ethylene diamine tetraacetic acid (EDT A) pada pelarut sampel, kontaminasi *Escherichia coli* dan jumlah *P. multocida* dalam sampel dievaluasi. Uji PCR ini juga dibandingkan dengan metode standar bakteriologis untuk mendeteksi *P. multocida* B:2 dalam sampel *tonsil swab* yang telah dikumpulkan dari rumah potong hewan di berbagai daerah di Indonesia. Penambahan 100 mM EDT A pada sampel *tonsil swab* yang telah diberi *P. multocida* B:2 temyata menghambat terbentuknya hasil PCR pada 350 *base pairs* (bp). Efek penghambatan oleh EDTA ini dapat dihilangkan dengan pencucian sebanyak 3 kali menggunakan air deionisasi. Uji PCR dapat mendeteksi *P. multocida* dengan jumlah 1 organisme dan pada keadaan sampel terkontaminasi dengan 100 *colony forming unit* (CFU) *E. coli.* Hasil pengamatan di atas menunjukkan bahwa *DNA primers* untuk *P. multocida* B:2 yang digunakan pada uji PCR temyata sensitif serta spesifik, dan efek hambatan oleh EDTA dapat dihilangkan dengan pencucian.

Kata kunci: PCR, EDTA, *Pasteurella multocida* B:2

ABSTRACT

NATALIA, L. and A. PRIADI. 2001. Polymerase chain reaction optimization for the detection of *Pasteurella multocida* B:2, the causative agent of *Haemorrhagic septicaemia. Jurnal Ilmu Ternak dan Veteriner* 6(4): 280-284.

Specific detection of *Pasteurella multocida* type B:2 by polymerase chain reaction (PCR), using a set of DNA primers was optimised. Effects of the addition of ethylene diamine tetra acetic acid (EDTA) to the sample preparation, *Escherichia coli* contamination and the number of *P. multocida* on the PCR product was assessed. The PCR test was compared to the standard bacteriological method for the detection of *P. multocida* B:2 in tonsillar swab samples collected from slaughter houses of various regions in Indonesia. Addition of 100 mM EDTA-saline to *P. multocida B:2* spiked tonsillar swab samples inhibits the production 350 base pairs (bp) PCR product. The inhibitory effect of the EDT A can be eliminated by three times washing with deionised water. The PCR can detect *P. multocida* as low as I organism and contanimation of 100 CFU of *E. coli* does not effect the PCR result. The results show that the DNA primers for *P. multocida* B:2 is sensitive and specific. The inhibitory effect of EDTA in PCR samples can be eliminated by washings.

Keywords: PCR, EDT A, *Pasteurella multocida* B:2 .

INTRODUCTION

Haemorrhagic Septicaemia (HS) is one of the most economically important livestock diseases in Indonesia, affecting both draught and production animals (PUTRA, 1992). Although control program through vaccination has been implemented in Indonesia, annual loses of 28 billion rupiahs caused by HS was still reported in 1997 (DIREKTORAT BINA KESEHATAN HEWAN, 1998). As the nature of the disease, in an endemic area there were always a small proportion of animals which harboured pasteurellae organisms in their nasopharynx and acted as carrier animals (DE ALWIS, 1993). The existence of these animals play an important role in the epidemiology of HS as they will provide a continuing source of infection for non immune animals. Thus, even in a population with at least 70 % of animals vaccinated against HS, there may be individual cases of HS which occur when carrier animals transmit organisms to succeptible animals (MORGAN, 1996). Continous annual vaccination of the new non-immune generation in a population will minimise the number of carrier animals hence will prevent the outbreak of HS.

Since carrier animals play an important role in the epidemiology of HS, control strategy through vaccination should be followed by bacteriological

surveillance to detennine the exsistence of those animals. Attempts to isolate HS causing pasteurellae by collecting tonsil swabs of cattle and buffalo nom Jakarta and Bogor slaughter houses by bacterial culture method has been reported (BALITVET-ACIAR, 1995). Of the 12 *Pasteurella multocida (P. multocida)* isolates obtained nom 209 tonsil samples, none was HS causing pasteurellae. Heavy contamination of other bacteria always hampers the isolation of *P. multocida.* Recent advances in recombinant DNA technology have provided a new approach to the development of rapid and sensitive diagnostic tools. The Polymerase Chain Reaction (PCR) test has come into increasing use for the diagnosis of infectious diseases (BELAK and BALLAGIPORDANY, 1993; KEE *et at.* 1994; BARKER 1994; SILVEIRA *et at.* 1996). BRICKELL *et at.* (1998) have identified a set of DNA primers specific for *P.multocida* type B:2, the causative agent ofHS. These primers fonn the basis for a highly specific and highly sensitive PCR test. This test can be us.ed to detect the presence of genetic material nom *P. multocida* type B:2, even in samples where the organism is no longer viable or where other contaminating bacteria make culture of *P. multocida* impossible. Therefore, by using PCR, which is theoretically more sensitive than culture method, the presence of the HS causing pasteurella in the tonsils of cattle and buffalo in various provinces of Indonesia have been investigated since the year 1996. This test was also expected to improve our understanding of the epidemiology of HS. This report describes the optimization of PCR test for the detection of HS causing *P. multocida*

MATERIALS AND METHODS

Primer set

A set of primers that amplify the gene region which highly conserved among pathogenic isolates of *P. multocida* B:2 was developed by Victorian Institute of Animal Science, Australia (BRICKELL *et at.* 1998). These primers have the following sequence: 5'GAAAGAAACCCAAGGCGAA-3' and 5'ACAATCGAATAACCGTGAGAC-3' resulted in an amplified of ˜ 350 base pairs (bp).

Sample preparation

To assess the effect of ethylene diamine tetra acetic-acid (EDTA) on the PCR process, EDTA containing samples and samples without EDTA were tested. Therefore, 100 III saline and 100 III saline containing 200 mM EDTA were spiked with *P. multocida* B:2. EDTA containing samples were then washed 3 times with deionised water to eliminate the effect of EDTA. All samples preserved in saline/EDTA were also washed. These samples were boiled for 10 minutes and subjected to PCR.

DNA amplication

Amplication of the DNA was perfonned in 20 µ of a reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl2. 1.5 mM MgClz, 2 mM of each dNTP, 5 µ M of each of the primers and 2 U/ml Tth polymerase (Promega). Reaction mixtures were overlaid with a drop of paraffin oil and were subjected to 35 PCR cycles of 30 seconds at 94°C, 30 seconds at 65°C, 30 seconds at noc in a thennal cycler (Hybaid Omnigene). After amplification, 12 µl of the amplification products were separated in 1% agarose gel stained with ethidium bromide, and the DNA fragment was visualized by UV transilluminator.

Sensitivity of PCR amplification

To determine the sensitivity of the PCR test, bacterial suspension of *P. multocida* type B:2 were prepared and counted microscopically using a counting chamber. The bacterial suspension was then serially diluted from 10^8 organisms to 1 organism. Each of the dilution were boiled for 10 minutes and amplified.

Specificity of PCR amplification

To assess the specificity of the PCR test, the primers were used to amplify DNA from *P. multocida* B:2 and other than B:2 which are positive and negative in specific B:2 antigen ELISA respectively. Bacteria used were listed in the Table 1.

The influence of contaminating bacteria on samples for PCR was also examined. *Escherichia coli (E. coli)* was the one of the contaminating bacteria nequently, found in the abattoir samples (BALlTVET-ACIAR *PROJECT REPORT,* 1995). Therefore, in this study, each dilution of *P. multocida* was mixed with 10 or 100 organisms of *E. coli* and amplified.

Table l. The identification number and history of the *P. multocida* strains tested in the PCR assay

Lab. No.	Antigen		Origin	
	ELISA	Type	Animal	Location
111A	+	B:2	Bovine	Bali
111B	+	B:2	Bovine	Bali
0755	+	B:2	Bovine	Sumbawa
105	-	A:I,2,3	Chicken	Bali
0756	+	B:2	Bovine	Sumbawa
0758	+	B:2	Bovine	Sumbawa
0759	+	B:2	Bovine	Sumbawa
KBI5	+	B:2	Buffalo *	Bogor
0140	-	lIB	Bovine	Australia
P-4675	-	B:3,4	Elk	UK
1266A	-	A	Bovine	DIC-Yogyakarta

* Reisolated from heart blood of experimentally infected buffalo

RESULTS AND DISCUSSION

Sample preparation in PCR test is of paramount importance. There are substances that may interfere with PCR processes. Our results proved that EDTA saline has an inhibitory effect on PCR process in tonsil samples spiked with *P. multocida* B:2. The inhibitory effect of EDTA can be eliminated by washing the samples 3 times with deionised water. Amplification of the washed samples resulted in an amplified fragment of 350 bp. The failure to amplify EDTA-added spiked samples may be caused by the high concentration of EDTA (200 mM) chelating magnesium ions which are critical bivalent cations and present in quite low concentration (1,5 mM) in the PCR process, since the PCR positive reactions were recovered by three times washing of these samples. SMITH, *et at.* (1996) demonstrated that reaction components, including template DNA, chelating agents present in the sample *(e.g.* EDT A), dNTPs and proteins, all affect free magnesium concentration. It seems that the EDTA binds the free magnesium in the reaction mixture. In the absence of adequate free magnesium, DNA polymerase is inactive (SMITH *et al.,* 1996).

This study also showed that preparation of bacterial samples for PCR do not require DNA extraction. Sample for PCR can be as simple and rapid as boiling the bacterial organisms and adding directly to the PCR. With this method, the easiest way to increase the number of templates available to PCR should be to increase the number of cells added to the reaction. Pre-enrichment of sample using *P. multocida* B:2 selective broth (PRIADL and NATALIA, 2000) before the PCR test should minimised the inconsistent results. However, SAIKI *et at.* (1988) stated that as number of cells added goes above 600, the yield of product does not increase with the addition of more cells, and begins to decline at 4.800 cells. This is also happened with this PCR test for *P. multocida* B:2. In this test, the OD of the bacterial samples should be standardised for the PCR to get the consistent results. The addition of more cells in the samples will make more "cell debris" and more DNA is being trapped and inhibition of the PCR process is occurring (ERLICH, 1992).

Figure 1 shows that the PCR has a sensitivity to amplify single cell of *P. multocida* B:2 to give positive result. The 350 bp PCR products were shown by samples containing I to 10^8 bacteria. However, there were differences in the intensity of PCR bands of these samples (Figure 1). This sensitivity was 100-fold compared to the PCR assay for the detection of toxigenic *P. multocida* in pigs reported by KAMP *et al.* (1996). This highly sensitive PCR is also superior to the standard culture method for the identification of *P. multocida* B:2 especially in contaminated samples (PRIADI and NATALIA, 2000). However, there were inconsistency PCR results in samples containing 10 and 1 cfu of *P. multocida* B:2 were found. The small volume (1µl) of sample suspension taken from 100 µl preparation for the PCR mixture may have failed to pick up the bacterial DNA.

The specificity of the PCR test for *P. multocida* B:2 was tested using *P. multocida* of different capsular and somatic antigens. Figure 2. showes that the PCR resulted positive bands only from *P. multocida* 8:2 samples in lanes 2, 3, 4, 6, 7, 8, and 9, whereas sample

in lanes 5, 10, 11, 12 which were not P. multocida B:2 no positive bands were observed. The specificity of 100% was also reported by BRICKELL *et al.* (1998). This optimised PCR procedure for *P. mu/tocida* B:2 can be used as a molecular epidemiological tool for the better understanding of HS in cattle and buffalo especially for the detection of carrier animals in the endemic areas.

ACKNOWLEDGMENTS

The author would like to thank the Director of the Research Institute for Veterinary Science (Balitvet), Bogor, Indonesia, the Director of the Australian Centre for International Agricultural Research (ACIAR), and the Director and staff of the Victorian Institute of Animal Science for providing facilities and supports. The assistances of Dinas Peternakan staffs from Kupang district of Nusa Tenggara Timur Province, Dinas Peternakan staffs from Tuban (East Java), Balai penyidikan Penyakit Hewan Wilayah VI, Bali, Balai Penyidikan Penyakit Hewan Wilayah III, Lampung are also gratefully acknowledged. M.Syafarudin is also acknowledged for the technical assistance. The work was supported by the Australian Centre for International Agricultural Research (Project No. 9202).

REFERENCES

BALITVET-ACIAR *PRO.IECT REPORT.* 1995. Badan Litbang Pertanian. Departemen Pertanian (unpublished)

BARKER. JR. R.H. 1994 Use of PCR in the Field. *Parasitol. Today*. 10: 117-119.

BELAK. S and A. BALLAGL-PORDANY. 1993. Application of thr polymerase chain reaction (PCR) in veterinary diagnostic virology. *Vet. Res. Comm.* 17: 55-72.

BRICKELL. S.K., L. M. THOMAS. K. A LONG. M. PANACCIO, and P.R. WIDDERS. 1998. Development of a PCR test based on a gene region associated with the pathogenicity of *Pasteurella multocida* serotype B:2. the causal agent of Haemorrhagic Septicaemia in Asia. *Vet. Microbial.* 59 (4): 294-307.

DE ALWLS. M.C.L. 1993. *Pasteurelosis in Production Animals:* A Review. 1993. AClAR Proceedings no. 43. Eds. *B.E. PATTEN et al*.. Watson Ferguson Pty Ltd. Brisbane, Australia.

DIREKTORAT BINA KESEHATAN HEWAN (1998) Laporan situasi penyakit hewan strategis tahun 1997. *Buletin Pelayanan Kesehatan Hewan.* Edisi No. 3/1998. 74.

ERLICH, H.A 1992. *PCR Technology. Principles and Applications for DNA Amplification.* Oxford University Press. New York, Oxford.

KAMP. E. M., G.C.AM. BOKKEN, T.M.M. VERMEULEN. M.F. DE JONG, H.E.C.M. BUYS, F.H. REEK. and M.A. SMITS. 1996. A specific and sensitive PCR assay suitable for large-scale detection of toxigenic *Pasteurella multocida* in nasal and tonsillar swabs specimens of pigs. 1. *Vet. Diagn. Invest.* 8: 304-309.

KEE. S.. I. KIM. M. CHOI and W. CHANG. 1994. Detection of Leptospiral DNA by PCR. 1. *Clin. Microbial.* 32 (4): 1035-1039.

MORGAN. I. 1996. Report a visit to Indonesia for AClARProject PN 9202. The diagnosis and control of haemorrhagic epticaemia in Indonesia. (Unpublished).

NAGAI, S., S. SOMENO. and T. Y AGIHASHI. 1994. Differentiation of toxigenic from nontoxigenic isolates of *Pasteurella multocida* by PCR. 1. *Clin. Microbial.* 32 (4): 1004-1010.

PRIADL. A. dan NATALIA, L. 2000. Patogenesis septicaemia epizootica pada sapi/kerbau: Gejala klinis. perubahan patologis, reisolasi, deteksi *Pasteurella multocida* dengan media kultur dan *polymerase chain reaction* (PCR). *Jurnal Ilmu Ternak dan Veteriner.* 5: 65-71

PUTRA. AA. G. 1992. Country Reports: Indonesia. Pasteurellosis In Production Animals. AClAR Proceedings no. 43. Eds. B.E. *PATTEN et al.*. Watson Ferguson Pty Ltd. Brisbane. Australia.

SAIIKI, RK., D.H. GELFAND. S. STOFFEL. SJ. SCHARF. R. HIGUCHI, G.T. HORN. K.B. MULLIS, and H.A. ERLICH. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239-491.

SILVEIRA. RM.. FIORENTIN. L. and E.K MARQUES. 1996. Polymerase chain reaction optimalization for *Mycoplasma gallisepticum* and *M. synoviae* diagnosis. *Avian Dis.,* 40: 218-222.

SMITH, R; K. MILLER, and D.R. STORTS. 1996. Performance optimization of Promega's Taq DNA polymerase in PCR. *Promega Notes Magazine* 56: 24-26.