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Original Article

Antibody against synthetic peptide of prolactin-inducible protein homologue precursor (PIP-HP) of Bali Cattle (*Bos javanicus*) saliva as a potential biomarker for immunoassay development

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ABSTRACT

Objective: The present study aimed to produce and analyze antibody against a synthetic amino acid sequence fragment of PIP-HP of Bali cattle saliva.

Materials and Methods: The synthetic amino acid sequence of the PIP-HP (VIRELGICPDDWAVIPIKANRF) was developed, conjugated to bovine serum albumin and was used to immunize Indonesian local rabbits. Positive sera that specific against the PIP-HP were pooled and purified sequentially by implying ammonium sulfate precipitation and protein A affinity methods. Purified antibody was then employed to analyze of PIP-HP in the ruminants' saliva by means of westernblot assays.

Results: A polyclonal antibody specific to asynthetic amino acid sequence fragment of PIP-HP of Bali cattle saliva was successfully produced. Our results show that the antibody potentially to be used to develop an immuno-diagnostic kit. Furthermore, the antibody was also able to inhibit the growth of both *Escherichia coli* and *Staphylococcus aureus* cultures significantly ($P<0.001$) at concentration of 0.3 mg/mL.

Conclusions: In this study, antibody against synthetic peptide of PIP-HP (VIRELGICPDDWAVIPIKANRF) of Bali cattle saliva origin has been produced successfully. Further studies need to be carried out since the antibody has potential as biomarker for non-invasive immunoassays development.

KEYWORDS

Amino acid; Bactericidal; *E. coli*; Innate immunity; Lateral flow assay; *S. aureus*

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INTRODUCTION

The aim of this study was to follow up on the discovery we recently explored i.e. one of peptides in the saliva of Bali cattle, Prolactin-inducible Protein Homologue Precursor (PIP-HP) (Depamede, 2013). Lack information available about PIP-HP, most of the information is about Prolactin-inducible Protein. This peptide or protein is abundantly secreted in saliva, tears, and sweat (Myal et al., 1994). It is known that PIP as a product of normal apocrine lacrimal, axillar, salivary glands, eyelid, ear canal, bronchial submucosal, and seminal vesicle (Hassan et al., 2009). PIP has attracted the attention of researchers due to its vast function. Immunologically, PIP is considered to play a role in adaptive and innate immunities, *i.e.*, as a regulatory molecule (Umadat et al., 2013). From the pathological aspect, PIP is found to be expressed in cancerous tissue or tumor, as revealed in a breast tumor biopsy, hence PIP has been stated to be potential as a cancer biomarker (Clark et al., 1999; Baniwal et al., 2013). The physiological function of PIP especially PIP-HP in Bali cattle has not been investigated to date. We hypothesized that PIP-HP may contribute to mucosal host defense in cattle and may therefore be upregulated in response to an infection.

There are many differences in the genetics between Bali cattle (*Bos sondaicus/javanicus*), which is the predominant breed used in beef production in Indonesia, and Bos Taurus that are commonly found in the temperate area (Martojo, 2012; Purwantara et al., 2012). Some of these differences could result in differences in the expression of specific proteins, and consequently differences in their abundance in physiological secretions such as saliva. It is possible that this could include biomarkers for the nature or adaptability of survival in the less-conducive atmosphere for beef cattle, especially in the tropics.

It is therefore necessary to make antibodies against PIP-HP, which is not only important to study the presence and the role of PIP-HP in cattle, but also for the development of immunodiagnostics.

MATERIALS AND METHODS

Collection of saliva and gel analysis of salivary proteins: Saliva of Bali cattle was collected and treated according to Depamede (2013), with certain modification. Briefly mixed saliva was collected directly from oral cavity of healthy, non-pregnant and non-lactating Bali cows (*Bos javanicus*) using disposable plastic pipettes. Saliva was lyophilized and resuspended in distillated water to 2 mg/mL based on Bradford (1976). Saliva (20 µg each) was then subjected to electrophoresis on a discontinuous 12.5 % (w/w) SDS-PAGE (Laemmli, 1970) and stained with Commasie blue. The bands corresponding to about 14 kDa were excised for MALDI-TOF/TOF.MS treatment (Depamede, 2013).

Synthesis of PIP-HP peptide: The amino acid sequence of the spot of 14 kDa Bali cattle saliva was analyzed against Ludwig database using Mascot software (Matrix Science, Boston, MA, USA) revealed three peptides, one of them was PIP-HP with a molecular weight around 22 kDa (Depamede, 2013). The peptide sequence was then used to synthesize PIP-HP peptide (Auspep, Australia).

Raising anti-PIP-HP antibody in rabbits: The process was carried out in two main steps i.e. vaccine preparation by conjugating the peptide with bovine serum albumin (BSA) as protein carrier according to Grant (2002) with slightly modification. The peptide (10 mg) was coupled with 3 mg of activated BSA with MBS (3-maleimidobenzoic acid N-hydroxy-succinimide ester) as a linking reagent.

Following vaccine preparation, the coupled peptides (100 µg/rabbit) were emulsified using complete Freund's adjuvant (1:1; v/v) and two rabbits were inoculated subcutaneously at a number of sites. Boosters were carried out three times using 50 µg of antigens/rabbit in incomplete Freund's adjuvant at intervals of 2 weeks. The antibodies produced were purified by means of ammonium sulfate precipitation and a protein A column affinity. The purified antibodies were then used to examine the existence of PIP-HP in the saliva of large ruminants using western blot.

Initial study on the development of lateral flow immunochromatographic assay using anti PIP-HP antibody as biomarker: A lateral flow immunochromatographic assay was developed according to Depamede (2011) with slight modification. One mg/mL of purified anti PIP-HP antibody in 5 mM Tris-HCl, pH 7.5 was added to 20 ml of pH-adjusted colloidal gold solution, and was agitated for 30 min at room temperature. Furthermore, 2 ml of 1% (w/v) BSA solution was added and followed by agitation for 15 min at room temperature. The mixture was then centrifuged at 4,800 x g for 30 min at 4°C. The gold pellets obtained were dissolved in 50 mM Tris/HCl buffer, and applied on to conjugate pad (glass fiber membrane). Subsequently on to the nitrocellulose membrane, closed to the

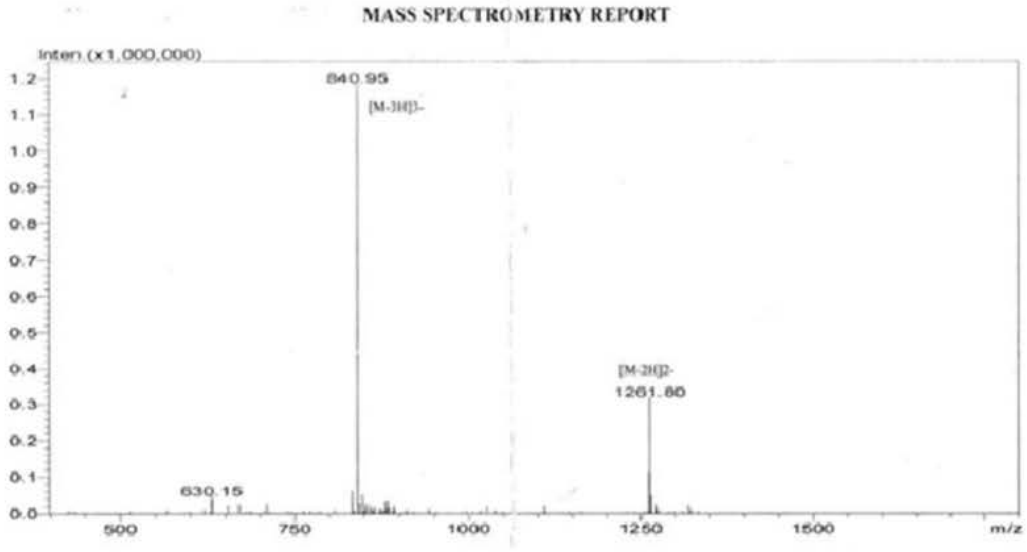

Figure 1. Mass-spectrometry analysis of the PIP-HP synthetic peptide

conjugate pad position, 20 µg/mL of purified anti-PIP-HP antibody, and closed to the absorbent pad, goat anti-mouse antibody (1.000 mg/mL) were applied as the test and control lines, respectively, using Isoflow dispenser (Imagine Technology, USA). All of the components were dehydrated in a controllable drying chamber (Heraeus Instruments, USA) at 35°C for 60 min.

Finally, after drying process all of the lateral flow components i.e. the glass fiber membrane, pretreated sample pad, nitrocellulose membrane that has test and control lanes, and absorption pad were assembled, then cut as the strip (6 cm by 4 mm) using Matrix 2360-programable Shear (Kinematic Automation, USA). The strips were then kept in sealed desiccated foils until used.

Effects of anti PIP-HP antibody on bacterial growth: Effects of anti PIP-HP antibody on the growth of *E. coli* and *S. aureus* were also studied as modified from Tobias et al. (2012) and Depamede et al. (2012). Briefly, each stock of *E. coli* and *S. aureus* were prepared in filtered sterile phosphate buffered saline containing 1% (w/v) skim milk (PBS-Skim) until the OD590 reached 0.6-0.8. Following this, bacterial samples (25 µL each) were cultured in 1 ml PBS-skim with or without addition of anti PIP-HP antibody in concentrations of 30 and 300 µg/mL. Penicillin and Streptomycin at a concentration of 1000 U/mL and 1000 µg/mL respectively were used as antibiotic controls. Bacterial samples were then cultured under a constant shaking for four hours at room temperature. Following the culture, bacterial growth was determined by photometric reading (OD590 nm).

Data analysis: Most data were analyzed descriptively. The Tukey-test was used to compare the effects of anti-PIP-HP antibody on bacterial growths. $P<0.05$ was considered significant based on One-way analysis of variance.

RESUTS AND DISCUSSION

Peptides with approximate size of 14-22 kDa isolated from the saliva of Bali cattle (*Bos javanicus*) have been reported to play an important role as innate immunity (Depamede et al., 2012). MALDITOF/TOF.MS analysis and Mascot search revealed that one of the peptides is identical to the sequence of prolactin inducible protein-homologue precursor (PIP-HP).

In this study, the amino acid sequences of PIP-HP (VIRELGICPDDWAVIPIKANRF) were synthesized and the result of mass-spectrometry analysis of the synthetic peptide is presented in **Figure 1**. The peptide was then used to develop peptide vaccine by conjugating it to BSA, then polyclonal antibodies were risen in Indonesian local rabbits. Purified antibodies were analyzed by means of western blots against the peptide vaccine, and used as a tool for analyzing PIP-HP in the large saliva of large ruminants. The results were presented in **Figure 2**.

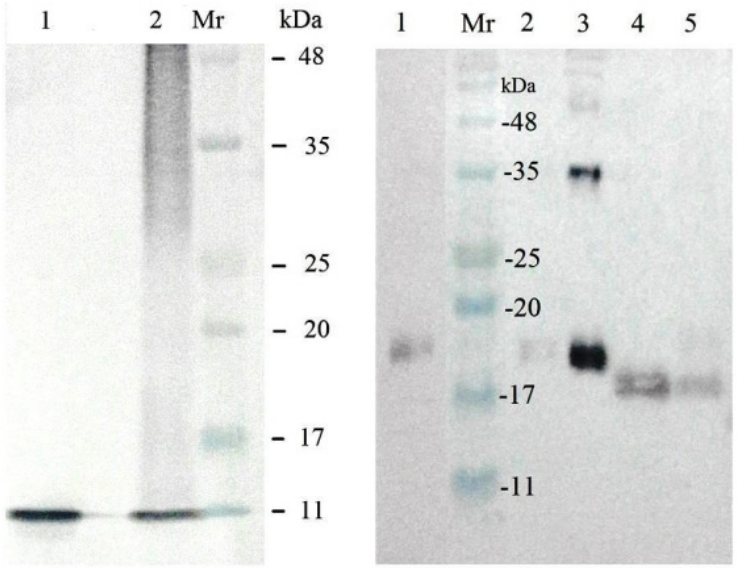

Figure 2. Representation of western blot results. (**a**) Detection of PIP-HP synthetic peptide (lane 1) or BSA conjugated PIP-HP vaccine (lane 2). (**b**). Detection of Bali cattle saliva (lanes 1, 2 and 3) and local buffalo saliva (lanes 4 and 5), about 20 µg each were subjected to western blotted against anti-PIP-HP antibody raised in this study. Antibody used was diluted 1 in 2000.

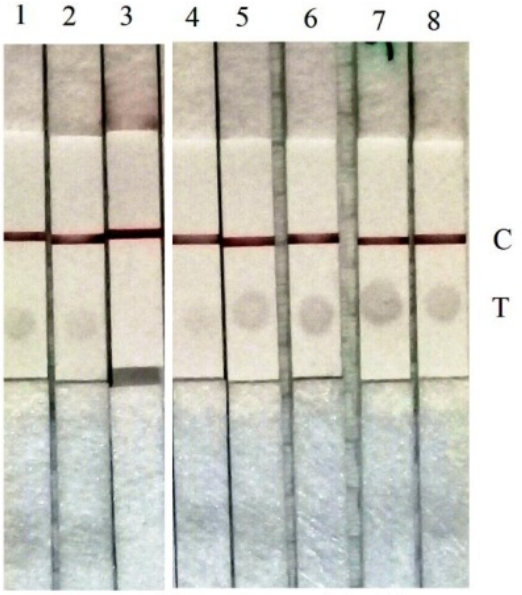

Figure 3. Representation of immune lateral flow assay results based on anti PIP-HP antibody as biomarker. Strips 1 and 2, *E. coli* and *S. aureus* extracts respectively; strip 3, negative control; strip 4, lactoferin; strip 5, Bali cattle saliva; strip 6 PIP-HP peptide; strip 7, buffalo saliva; and strip 8, testicular extracts of Bali cattle bull. C: control line, T: Test spot.

Western blot analysis (**Figure 2a**) revealed that antibody (1:2000 dilutions) was able to detect the synthetic PIP-HP without BSA (lane 1) or BSA conjugated PIP-HP vaccine (lane 2) with a molecular weight around 11 kDa. Myal and Shiu (2000) mentioned that PIP as a precursor polypeptide that synthesized as 12.5 kDa molecule, then its size is reduced to 11 kDa following the removal of signal peptide. We could then assume that the synthetic peptide we developed in this study was similar to the peptide mentioned by Myal and Shiu (2000). From the results, it is clear here that we have successfully developed a peptide vaccine using a sequence fragment of peptides found in the saliva of Bali cattle with molecular weights around 14 kDa, which was reported as PIP-HP based on Mascot search analysis (Depamede, 2013). Slightly differences were observed in Bali cattle and local buffalo cattle saliva, in term of their molecular weights. These might be related with the isoform nature of PIP with three molecular size *i.e.*, 11, 14 and 16 kDa as reviewed by Umadat et al. (2013). PIP with molecular weight around 15, 17 and 22 kDa has also been reported elsewhere. The ability of anti PIP-HP antibody to detect protein/peptide in the saliva of cattle and buffaloes around those molecular sizes (**Figure 2b**) has challenged us to observe if the antibody could be used to develop an immunoassay kit, as have been carried out by Priyadarsini et al. (2014). For this reason, we then carried out an initial

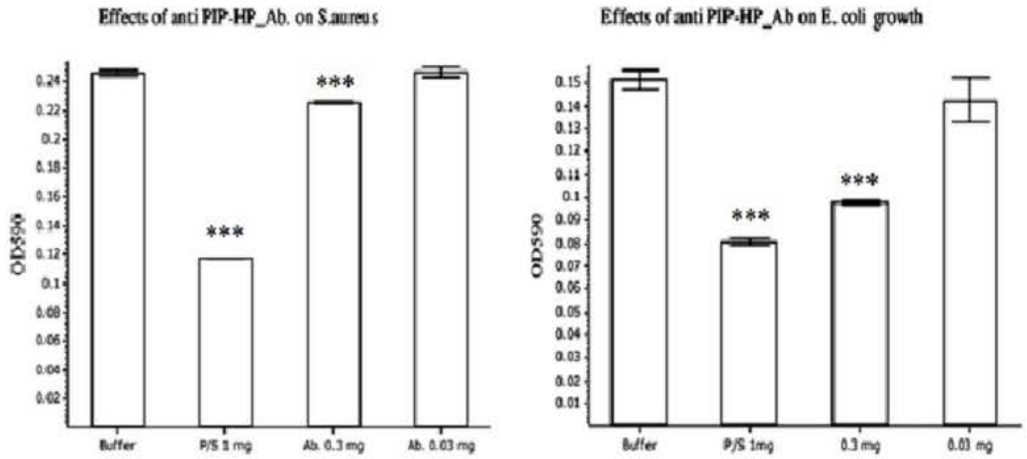

Figure 4. Effects of anti PIP-HP antibody on *S. aureus* (A) or *E. coli* (B) growth. S. aureus growth was not affected by anti PIP-HP antibody, the anti PIP-HP antibody inhibited *E. coli* growth significantly (***P<0.001) at concentration of 0.3 mg/mL. Penicillin/streptomycin (P/S) was used as antibiotics as inhibition control.

study on the development of immune-lateral flow assay. The results (**Figure 3**) show that the anti PIP-HP antibody was able to be used as a biomarker in immune-lateral flow assay. Interestingly, it was not only for detecting PIP-HP in the saliva of cattle and buffaloes but also in the extract of Bali cattle testis. The latter is quite challenging that whether PIP-HP is presence in the testis or not, needs to be proofed.

In this study, additional observation was also carried out on the effects of anti PIP-HP antibody on the bacterial growths. **Figure 4** shows that anti PIP-HP antibody able to inhibit both strains *S. aureus* and *E. coli* significantly (P<0.001) at concentration of 0.3 mg/mL, even though *E. coli* appear to be more susceptible than *S. aureus* against the antibody. This phenomenon is quite enigmatic while information on it is also limited. Interestingly, Tobias et al. (2012) reported similar results that egg yolk IgY of Ostriches was able to inhibit the proliferation of *S. aureus* and *E. coli* significantly (P<0.05). Furthermore some natural antibodies have also been reported elsewhere that able to inhibit microbes naturally (Ochsenbein et al., 1999; Zhou et al., 2013; Rothstein, 2016).

In regards to our study, whether the antibody is in line with the mode of action of natural antibody or it contributes to the antibacterial activity of the natural antibody repertoire as mentioned by Ochsenbein et al. (1999), Zhou et al. (2013) and Rothstein (2016) need to be elucidated further.

CONCLUSION

The results showed that antibody against synthetic peptide of PIP-HP (VIRELGICPDDWAVIPIKANRF) of Bali Cattle saliva was able to be produced. The antibody can be used to develop immune lateral flow assay, with a prospect to develop non-invasive rapid tests, based on salivary examination. Furthermore the antibody was also able to inhibit *E.Coli* and *S. aureus* comparable to those of penicillin and streptomycin commercial antibiotics.

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CONFLICT OF INTEREST

No potential conflicts of interest that was declared by the authors with respect to authorship, the research, and/or publication of this article.

AUTHORS' CONTRIBUTION

DK together with SND designed and carried out the experiments, sample collection and data analyzing. SND also supervised the research work especially for production and purification of antibodies, proteomic analysis, designed peptide,as well as drafted the manuscript. Both authors read and approved the manuscript before submission.

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