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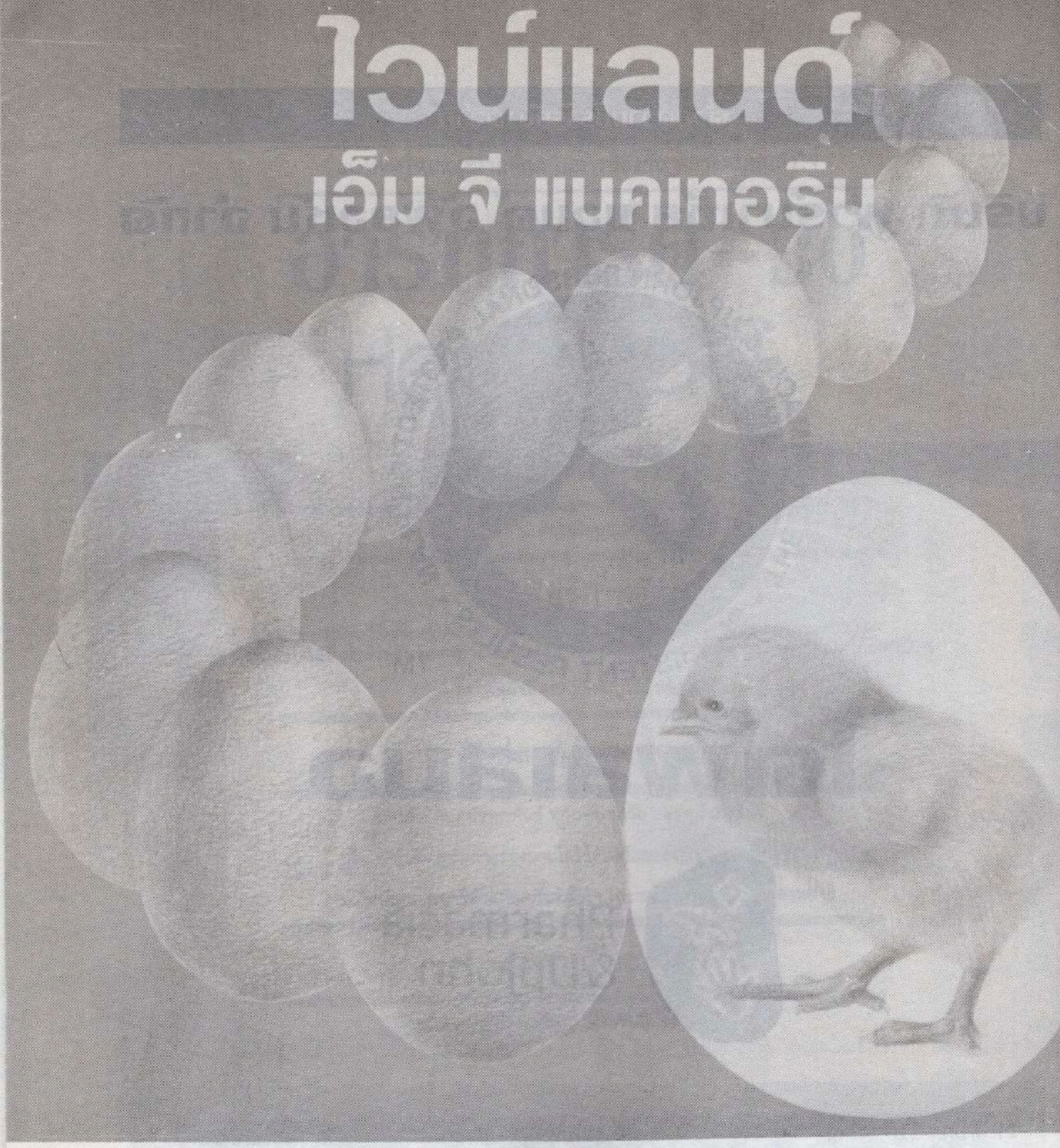

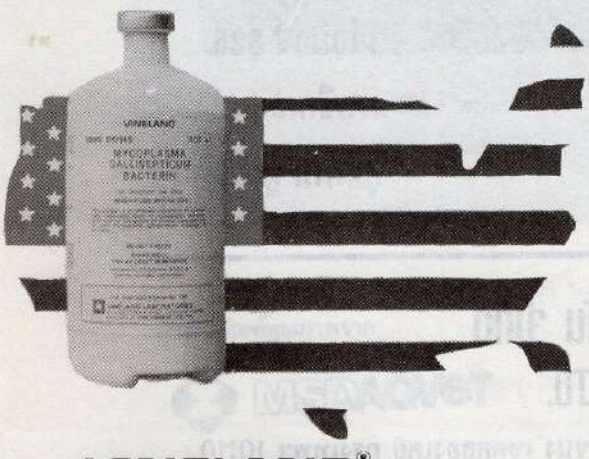

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องค์ประกอบของแอนติเจนที่มีผลในการสร้างความคุ้มโรคต่อ *Riemerella anatipestifer*

พรเพ็ญ พัฒนโสภณ* ทิพา ตันติเจริญยศ* ทากูโอะ ซาวาดะ**

บทคัดย่อ

ศึกษาความคุ้มของวัคซีนป้องกันโรคนิวดัคซินโดรม (Anatipestifer infection) ซึ่งเตรียมจากเชื้อ *Riemerella anatipestifer* สเตรน 1081 ซีโรไทป์ 1 ในเป็ด โดยใช้ส่วนโปรตีนกึ่งบริสุทธิ์ที่ตกตะกอนโดย ammonium sulfate และสกัดด้วย Sephadex G-200 gel filtration วัคซีนที่มีโปรตีน 100 μg และมีอลูมิเนียมไฮดรอกไซด์อยู่ 25% ทำหน้าที่เป็นแอ็ดจูแวนท์ สามารถให้ความคุ้มโรคในเป็ดอายุ 14 วันได้ 100% โดยการฉีดเข้าใต้ผิวหนังเพียงครั้งเดียว ต่อการฉีดพิษทับด้วยเชื้อดังกล่าว $5x10^9$ Colonyofarming Units หลังการฉีดวัคซีน 21 วัน เมื่อลดปริมาณโปรตีนลงเหลือ 50 μg สามารถคุ้มโรคได้ 50% ค่า median effective dose (ED_{50}) ของโปรตีนในวัคซีนได้จากการคำนวณเท่ากับ 43 μg

วัคซีนเชื้อตายชนิดเซลล์รวมน้ำเลี้ยงสามารถให้ความคุ้มได้สูงอย่างมีนัยสำคัญต่อการฉีดพิษทับ ด้วยเชื้อสเตรนเดียวกัน แต่จะให้ความคุ้มลดลงอย่างมีนัยสำคัญเมื่อทำให้วัคซีนร้อน 100 °C นาน 1 ชั่วโมง หรือย่อยด้วยเอ็นซายม์ trypsin ในขณะที่เอ็นซายม์ lipase ลดความคุ้มของวัคซีนได้ปานกลางอย่างมีนัยสำคัญ

จากผลดังกล่าวชี้ให้เห็นว่า โปรตีน หรือสารประกอบ peptide เป็นองค์ประกอบที่สำคัญที่สุดในการสร้างความคุ้มโรคต่อเชื้อ *Riemerella anatipestifer* ในลูกเป็ด ในขณะที่ lipid หรือสารประกอบของ lipid มีผลต่อการสร้างความคุ้มรองลงมา

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INTRODUCTION

Two types of vaccines, inactivated bacterin and live vaccine, were reported as successful prophylaxis against infection with virulent *R. anatipestifer* strains of the same serotype (Sandhu, 1979; Layton and Sandhu, 1984; Sandhu, 1991). However no information was reported sbout what component of the bacteria or vaccine is immunogenic. No research on the vaccine has also been done in Thailand.

Riemerella anatipestifer has been once nominated as *Pasteurella anatipestifer,* species incertae sedis in Burgey's Manual of Systemic Bacteriology (Mannheim, 1984). *R. anatipestifer* and *P. multocida* have enough characteristic similarities including morphology and high pathogenicity in ducks. Immunity induced by the strains of *P. multocida* and *R. anatipestifer* is reportedly type specific (Rebers and Heddleston, 1974; Sandhu, 1979). Their serotypes have been determined by the gel-diffusion precipitin test with heat stable extract (100 to 121 °C, 1 h). Lipopolysaccharides (LPS) of *P. multocida* are major antigens reponsible for the type specificity (Rebers and Heddleston, 1974; Rebers *et al.,* 1980; Rimler and Brown, 1982), but purified polysaccharide antigen by cetylpyridinium chloride failed to provide protection (Kodama *et al.,* 1981). Therefore protein integrated in the basic LPS structure was essential for immunogenicity, because the treatment of the complex with phenol abolished its property (Rebers and Heddleston, 1974). An immunogenic fraction of *P. multocida* consisted mainly of a high-molecular weight protein-polysaccharide complex containing 25-27% protein and 10.7% carbo-hydrate (Ganfield *et al.,* 1976). Highly protective antigens (LPS-protein antigen complex) have been found in soluble fraction of *P. multocida* extracted by heating at 56 °C in 2.5% NaCl or prolonged stirring in formalin saline (Kodama *et al.,* 1982) or potassium thiocyanate extraction followed by ultracen-trifugation at 105,000 x g for recovering the antigen (McKinney *et al.,* 1982).

In this study a local isolate of serotype 1, which is the most prevalent serotype in relation to outbreaks in Thailand (Pathanasopahon *et al.,* 1995) was selected as the representative strain for characterization of antigenicity. This report is also documented the first attempt in the world to prepare sub-unit *Riemerella anatipestifer* vaccine which yielded high protection in ducks.

MATERIALS AND METHODS

Bacterial strains

R. anatipestifer strain 1081 of serothpe 1, which is highly lethal for ducks and gave the highest protection against homologous challenge, was selected from the local isolates and used as the representative strain for characterization of immunogenicity.

Partial purification of protein antigen

The lyophilized culture was reconstituted in 0.3% yeast extract added tryptic soy broth (Difco) (TSBY) and streaked on tryptic soy agar (Difco) with 5% defibrinated sheep blood (TSAB). The agar plates were incubated in 5% CO_2 incubator. One or two colonies of the culture were inoculated into 10 ml of TSBY and incubated in shaker bath at 37 °C for 24 h. The broth

culture was transferred to 1 litre of TSBY medium in 2 litre-flask and incubated as mentioned above. Purity was checked by Gram-staining and plating on TSAB. Bacterial cells were sedimented by centrifugation at 12,000 x g for 20 min at 4°C and washed 3 times in PBS, and finally resuspended in 25 ml of Tris-EDTA. The cell suspension was sonicated for 15 min in ice box. The extract was designated as crude extract (CE). The CE was centrifuged to seperate cell debris as above mentioned. Protein antigen in the supernatant was precipitated by slow adding of saturated ammonium sulfate at an equal volume with continuous gentle stirring on ice box. The precipitated material was harvested by centrifugation at 5,000 x g for 30 min at 4°C, resuspended in 5 ml of Tris-EDTA and dialyzed against PBS (pH 7.2) at 4°C for 3 days. Finally 7 ml of remaining non-dialyzable component (ammonium precipitated fraction : APF) was used for gel filtration. Gel filtration of the sample by Sephadex G-200 (Pharmacia) column of 2.5 x 48 cm was performed as described by Sawada *et al.* (1983). After elution by Tris-EDTA at a flow rate of 20 ml/h for 12 h, two peaks were obrained. The first fraction (fraction A) with a total volume of 37.5 ml of high molecular weight protein peak (partially purified fraction : PPF) was collected and concentrated by centrifugation in Centriprep-10 concentrator (Amicon, Inc.). Protein estination wasdone using Bio-Rad protein assay based on the method of Bradford (1976). Finally the protein concentration of PPF was adjusted to 50, 100, 200 and 400 μg/dose and adsorbed onto aluminium hydroxide gel (25% of $Al(OH)_3$ v/v). The adjuvant was provided by Foot and Mounth Disease Center, Veterinary Biologic Products Division, Department of Livestock Development, Thailand.

Heat or enzyme treatment

One litre of broth of strain 1081 was prepared as mentioned above. Part of the broth culture was diluted and plated on TSAB to check purity and to determine colony-forming units (CFU/ml). The broth culture was inactivated by addition of 0.3% formalin (v/v) and stirrd at room temperature overnight. Optical density was checked by transferring a part of the sample to a cuvette to determine the absorbance at 540 nm (Spectronic 20 A, Shimadzu, Millon Roy Company). The whole broth colture (WC) was standardized the concentration to 2 x 10^{10} CFU/ml (double concentrated) by centrifugation at 4,000 x g for 20 min at 4°C and removing a part of supernatant. Protein determination was made by the same method as mentioned above.

Enzyme treatment was done by incubating the WC with equal volumes of the following enzymes (Sigma) at 37°C for 2 h : 0.9 units of wheat germ lipase per ml in 0.5 M phosphate buffer (PB), pH 7.5; 1,000 units of muramidase per ml in 0.05 M PB, pH 6.5; 6,000 units of trypsin per ml in 0.05 M Tris-buffer, pH 7.5.

Heat treatment was done by incubating the standardized WC, which was diluted with equal volume of CF to obtain the same concentration of bacterial cell as that in the enzyme treatment, at various degrees of temperature (37, 56 or 100°C) for 1 or 2 h in water bath.

Vaccination and challenge exposure

One-day-old male Khakhi campbel ducklings, free from *R. anatipestifer* infection, which were bred at Bangprakong Livestock Breeding Station, raised in the experimental animal house of Nationalinstitute of Animal Health were vaccinated at 2 weeks of age subcutaneously at the feather line of the neck with 0.5 ml of immunogen. Challenge exposure was done intramuscularly at the thigh with 0.5 ml of 10^{10} viable organisms/ml of strain 1081 three weeks after immunization. The control birds were raised in the same condition. The ducks were observed each day for 14 days after the vaccination and the challenge. Responses of the ducks to challenge exposure were determined by quantal (live-dead) method. The median effective dose (ED_{50}) was determined by the method of Spearman and Kärber (Finney, 1964). Before the challenge experiments, the median lethal dose (LD_{50}) of *R. anatipestifer* strains for ducks was determined. Concentrations of the bacterial suspensions were adjusted by spectrophotometer and CFU was measured by direct agar plate counting. The LD_{50} of strain 1081, which is one of the highly pathogenic strains, was more than 5×10^8 but less than 5×10^9 CFU. While LD_{100} was more than 5×10^9 CFU. Therefore 0.5 ml of 10^{10} CFU dose was used for challenge.

Statistics

Since there were variable mortalities in control ducks after challenge exposure, the protection was evaluated with a protective index calculated as described by Samdhu (1979):

$$\text{Protective index (PI)} = \frac{\text{\%MORTALITY IN CONTROLS-\%MORTALITY IN VACCINATED}}{\text{\%MORTALITY IN CONTROLS}} \times 100$$

A chi-square test was performed to compare immunized and control groups.

RESULTS

Gel filtration

Crude protein extract was precipitated by saturated ammonium sulfate and separated through the Sephadex G-200 column. Two fractions (A and B) corresponding to two protein peaks were each pooled. Fraction A (1 st peak) contained 172.67 μg/ml of protein while fraction B (2 nd peak) contained 2.3 μg/ml. Therefore only fraction A was studied further for protection test in ducklings. The efficacy of protein inoculums in ducks is shown in Table 1. A single subcutaneous inoculation of 100 μg protein of fraction A induced 100% protection against challenge exposure with 5×10^9 CFU of strain 1081 which killed all the unvaccinated control birds, while 50 μg protein inoculum protected a half of them. The ED_{50} was 43 μg of protein per dose.

Heat or enzyme treatments

Influence of heat or enzyme treatments of WC on protective activities in ducks is shown in Table 2. Heating at 37°C and 56°C did not have a noticeable effect on the immunogenicity.

However heating at 100°C for 1 h significantly reduced the protective effect. Muramidase had mild effect but not significant, while the treatment with lipase yielded moderate effect with significant reduction of the protection ($P<0.05$). Treatment with trypsin gave highly significant reduction of protective activity.

Table 1. Protection induced with a gel-filtrated antigen from *R. anatipestifer* strain 1081 against homologous challenge in Khakhi Campbel ducks

Protein antigen[a] (μg/dose)	Mortality (No. dead/No.tested)	% mortality	PI[b]
50	5/10	50	50
100	0/10	0	100
200	2/10	20	80
400	1/7	14	86
Nonimmune	10/10	100	0

[a] All the antigens as inoculum were adsorbed onto aluminium hydroxide to 25% (v/v) and injected once subcutaneously into 14-day-old ducklings.

[b] PI : protective index

Table 2 Influence of various treatments on immunogeniaty of broth culture bacterin prepand from strain 1081 in Khakhi Compbel ducks

Treatment	Protein content (μg/ml)	Mortality (No. dead/No. tested)	% mortality	PI[c]
Heat[a]				
37°C, 2h	1,312.5	5/18	27.8	72.2[e]
56°C, 1h	1,208.3	4/20	20	80[e]
100°C, 1h	750	13/20	65	35[d]
Enzyme[b]				
Lipase	1,293	8/18	44.4	55.6[d]
Muramidase	1,125	5/18	27.8	72.2[e]
Trypsin	1,292	12/19	63.2	36.8[d]
Control				
Untreated-vaccine (WC)[f]	1,395	4/20	20	80
Nonimmune		20/20	100	0

[a] Heat treatment was done in water-bath.

[b] Enzyme treatment was done at 37°C for 2 h in water-bath.

[c] PI : protective index

[d] Significantly different ($P<0.05$)

[e] Significantly not different

[f] WC : whole culture

DISCUSSION

In partial purification by gel filtration, the protein content, which was necessary for protection of ducks from challenge exposure was more than 6 times different between the WC and fraction A. As shown in Table2, the WC vaccine containing 1,395 μg/ml protein protected 80% while 100 μg/dose (0.5 ml) of fraction A protected 100% (Table 1). The quantitative difference was clearly noticed and showed that partial purified sub-unit vaccine was highly immunogenic. Commercial production of such a vaccine may be possible when industrial high technologies are employed

Results of the heat or trypsin treatment and ammonium sulfate precipitation indicated that protein or peptid moiety was an essential factor in the protective antigen (s) of *R. anatipestifer.* Decreasing of PI by lipase treatment was approximately half of that by heat or trypsin (24.4, 45 and 43.2 respectively). Therefore protective antigen (s) of *R. anatipestifer* is belived to consist approximately of lipid or lipid aggregated substance/protein or peptide moiety ratio of 0.5 by activity.

It is not known whether the protein component (s) alone is immunogenic or protein integrated in basic LPS structure is essential for immuogenicity similar to that described in *P. multocida* (Rebers and Heddleston, 1974). A direct comparison of these antigens in terms of their chemical compositon is possible only when they are purified from an identical method.

COMCLUSION

The sub-unit vaccine prepared from partially purified protein fraction containing 100 μg protein was highly efficacious. Protein or peptide moiety antigen played most important role of immunogenicity of *R. anatipestifer* in ducklings while lipid or lipid aggregated substant (s) had minor responsibility.

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Immunogenic Component of *Riemerella anatipestifer*

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Abstract

Immunogenicity of partially purified protein fraction obtained from the local strain 1081 of *Riemerella anatipestifer* serotype 1 by ammonium sulfate precipitation and Sephadex G-200 gel filtration was determined in Khaki Campbell ducklings. One subcutaneous injection with 100 μg of the protein adsorbed onto aluminium hydroxide gel into 14-day-old ducklings gave 100% protection against challenge exposure with $5x10^9$ Colony-forming units of the honologous strain 21 days after immunization. While inoculum containing 50 μg protein yielded 50% protection. The median effective dose (ED_{50}) was 43 μg of the protein.

Efficacy test of 0.3% formalin added whole broth culture bacterin gave highly significant protection with only singly inoculation against challenge with homologous strain but significantly lost its immunogenicity after treatment with heat (100°C, 1h) or trypsin while treatment with lipase gave moderately significant effect.

These results indicated that protein or peptide moiety antigen played most important role of immunogenicity of *R. anatipestifer* in ducklings while lipid or lipid aggregated substant (s) had minor responsibility.

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