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Evaluation of Immunogenicity of *Pasteurella haemolytica* Serotypes in Experimental Models*

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Abstract: The immunogenicity of *Pasteurella haemolytica* serotypes was investigated in an experimental animal model. Rabbits were immunized with bacterins and leukotoxins of selected serotypes of *P. haemolytica*, and the IgG level and antileukotoxin activities of immune sera were measured. The highest IgG levels against A1, A2, A7 and T4 antigens were detected in rabbits receiving polyvalent combined (bacterin plus leukotoxin) immunogens. Monovalent combined immunogens induced high antibody titers against homologous serotypes, but low titers against heterologous serotypes. Serotype A1 and A7 were found to be more antigenic than A2 and T4. The highest antileukotoxin activity in monovalent groups was detected in A1 and A7 antisera. Immune sera were used for the protection of mice challenged with homologous and heterologous serotypes. Antisera to polyvalent combined immunogens protected 83-100% of mice against A1, A7 and T4 challenge, and 50% of mice against A2 challenge. The lowest protection (16-33%) was seen in serotype A6-infected mice, as heterologous challenge. In conclusion, marked differences were found in the immunogenicity of *P. haemolytica* serotypes and limited protection was observed against heterologous serotypes.

Key Words: *Pasteurella haemolytica*, serotype, immunity, immunogenicity.

Pasteurella haemolytica Serotiplerinin Immunojenitelerinin Deneysel Modeller ile Değerlendirilmesi

Özet: *Pasteurella haemolytica* serotiplerinin immunojeniteleri deneysel hayvan modelinde incelendi. Tavşanlar belirli *P. haemolytica* serotiplerinin bakterinleri ve leukotoksinleri ile bağışıklandıktan sonra, bağışık serumların IgG düzeyleri ve antileukotoksin aktiviteleri incelendi. A1, A2, A7 ve T4 antijenlerine karşı en yüksek IgG düzeyi, polivalan kombine immunojen verilen tavşanlarda saptandı. Monovalan kombine immunojenler, homolog serotipe karşı yüksek, heterolog serotipe karşı düşük titrede antikor oluşturdular. Serotip A1 ve A7, A2 ve T4'e göre daha antijenik bulundu. Monovalan gruplarda, en yüksek antileukotoksin aktivitesi A1 ve A7 antiserumlarında saptandı. İmmun serumlar, homolog ve heterolog serotiplerle infekte edilen farelerde pasif koruma amacıyla kullanıldı. Polivalan kombine immunojene karşı immün serum, farelerin %83-100'ünü A1, A7 ve T4 enfeksiyonuna, %50'sini A2 enfeksiyonuna karşı korudu. En düşük düzeyde koruma (%16-33), heterolog serotip olarak kullanılan A6 ile infekte farelerde görüldü. Sonuçta, *P. haemolytica* serotiplerinin immunojeniteleri arasında belirgin farklılıklar olduğu ve heterolog serotipe karşı yeterli koruma sağlanmadığı kanısına varıldı.

Anahtar Sözcükler: *Pasteurella haemolytica*, serotip, bağışıklık, immunojenite.

Introduction

Pneumonia is a major cause of economic losses in the sheep industry, and *Pasteurella haemolytica* is the infectious agent most frequently associated with pathologic damage of the ovine respiratory tract (1). *P. haemolytica* is divided into two biotypes based on carbohydrate fermentation patterns and 16 serotypes based on surface antigens (2). All serotypes can be involved in disease. However, there is a wide variation in the prevalence of individual serotypes isolated from

pneumonic pasteurellosis (3,4). *P. haemolytica* possesses several components that may function as virulence factors. Foremost among these is leukotoxin which is secreted during the logarithmic growth phase and is lethal to ovine leukocytes and lymphocytes (5,6). The capsular polysaccharide, as another virulence factor, also impairs the ability of neutrophils to ingest and kill *P. haemolytica* (7).

P. haemolytica has numerous potential immunogens, such as capsular polysaccharide, LPS, OMPs, fimbriae,

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iron-regulated proteins and leukotoxin (7). Although a number of bacterins, live and extract vaccines containing such potential immunogens have been developed, their efficacy in field trials has been variable (7-11). In addition, differences have been found in the immunogenicity of particular serotypes (12,13). These are major problems in the development of effective vaccines against pneumonic pasteurellosis. The aim of this study was to compare the ability to produce protective immunity of *Pasteurella haemolytica* serotypes to be included in different vaccine preparations for ovine pneumonic pasteurellosis.

Materials and Methods

P. haemolytica Strains and Serotypes

P. haemolytica serotype A1, A2, A7, T4 and A6 strains used in the study were selected from 52 *P. haemolytica* isolates from pneumonic ovine lungs, described elsewhere (3). Isolates were identified and biotyped according to their phenotypical characteristics (2). Serotyping of *P. haemolytica* strains was performed by an indirect hemagglutination test (2). Representative strains of 16 *P. haemolytica* serotypes were kindly provided by Dr. Sanchis (CNEVA, Unite Petits Ruminants, France) and Dr. Fodor (Department of Epizootiology, University of Veterinary Science, Hungary).

Production of Immunogens

Immunogens were prepared using *P. haemolytica* serotype A1, A2, A7 and T4 isolates. To produce bacterins, 24 h Brain Heart Infusion (BHI) agar cultures of selected strains were collected in PBS and washed three times. Cell suspension was adjusted to 10^9 cfu/ml in PBS and formalin was added to give a final concentration of 0.3%.

To produce leukotoxin, selected strains were cultured in 250 ml of BHI broth by shaking for 4 h and centrifugated. Supernatant was filtered through 0.2 μ m membrane filter and dialyzed in a cellulose dialysis tube (Sigma, D9652) against distilled water overnight. Resultant fluid was stored at -70°C and used as leukotoxin.

To prepare combined immunogens, whole cell bacterin was pelleted by centrifugation and suspended in leukotoxin fluid to give 10^9 cell/ml.

Immunization of Rabbits

The following monovalent and polyvalent immunogens in one ml were administered intramuscularly to seven groups of New Zealand white rabbits (n=2-4),

two times with a 15 day interval. Two weeks after the last injection, sera were obtained from rabbits and stored at -20°C until used.

Group 1. Polyvalent bacterin, containing serotype A1, A2, A7 and T4 bacterins pooled in equal volumes.

Group 2. Polyvalent leukotoxin, containing serotype A1, A2, A7 and T4 leukotoxins pooled in equal volumes.

Group 3. Combined polyvalent immunogen, containing serotype A1, A2, A7 and T4 combined immunogens pooled in equal volumes.

Group 4. Combined monovalent immunogen of serotype A1.

Group 5. Combined monovalent immunogen of serotype A2.

Group 6. Combined monovalent immunogen of serotype A7.

Group 7. Combined monovalent immunogen of serotype T4.

Measurement of Serum IgG Levels

Serum IgG levels of immunized rabbits were measured by ELISA. In ELISA, sera from each immunization group were tested with whole cell antigens of A1, A2, A7 and T4 serotypes. Anti-rabbit IgG peroxidase conjugate (Sigma, A1949) and O-phenyldiamine (Sigma, P8287) plus H₂O₂ substrate were used in the test. The reaction was measured in an ELISA reader. Sera collected from each group prior to immunization were used as negative controls.

Measurement of Serum Anti-Leukotoxin Activity

Peripheral blood neutrophils to be used in the leukotoxin assay were prepared using a previously described method (14). The leukotoxin activity of *P. haemolytica* strains was detected by trypan blue dye exclusion assay (15). In short, two-fold serial dilutions of leukotoxin preparations of serotypes A1, A2, A7 and T4 were added to equal volumes of neutrophil cell suspensions (10^5 cell/ml). After 3 h incubation at 37°C, an equal volume of 0.4% trypan blue dye solution was added and cell viability was assayed microscopically within 30 min. To measure anti-leukotoxin levels of immune sera, sera of each group were diluted two-fold and mixed with an equal volume of leukotoxin preparations from each serotype. After 15 min of incubation, trypan blue dye exclusion test was performed and the dilution causing 50% toxin neutralization was determined. Sera collected from each group prior to immunization were used as negative controls.

Passive Protection of Mice

The protective ability of immune sera against *P. haemolytica* challenge was investigated in Swiss albino mice. *P. haemolytica* A1, A2, A7, T4 and A6 serotypes were used as challenge strains. To determine the MLD₅₀ dose, serial ten-fold dilutions were prepared from BHI culture of each strain, and 0.2 ml of each dilution was given intraperitoneally (i.p) to mice.

To determine the protective ability of rabbit immune sera, 0.5 ml of sera from groups 1, 2 and 3 were injected to three groups of mice intraperitoneally, each consisting of 30 mice. Five hours after injection, 6 mice from each group were challenged with 10 x MLD₅₀ of *P. haemolytica* serotypes in 0.2 ml. The deaths within 24-72 h were recorded.

Results

Serum IgG levels of seven groups of rabbits immunized with various preparations are shown in Table 1. The highest IgG levels against A1, A2, A7 and T4 antigens were detected in group 3 rabbits receiving polyvalent combined immunogens. In polyvalent immunogen groups, the lowest IgG levels were detected in leukotoxin-immunized ones. Monovalent combined

immunogens induced high antibody titers against homologous serotypes, but low titers against heterologous serotypes. Serotype A1 and A7 were found to be more antigenic than A2 and T4. The IgG titers of rabbit sera prior to immunization were 1:2 or less.

Antileukotoxin activity of rabbit sera immunized with various preparations are shown in Table 2. Sera of rabbits immunized with leukotoxin-containing preparations (groups 2-7) showed more antileukotoxin activity than those that received bacterin (group 1). In monovalent groups, the highest antileukotoxin activity was detected in A7 and A1 antisera. Antileukotoxic activity of A1 and A7 sera against cross-serotype was also high. No antileukotoxin activity was detected in rabbit sera prior to immunization.

The protective ability of polyvalent immune sera in mice infected with challenge strains is shown in Table 3. The highest protection was detected in mice treated with antisera against polyvalent combined immunogen. Antisera against polyvalent combined immunogen protected 83-100% of mice against A1, A7 and T4 challenge, and 50% of mice against A2 challenge. The lowest protection (16-33%) was seen in serotype A6 infected mice, as heterologous challenge.

Rabbits	Antigen			
	Serotype A1	Serotype A2	Serotype A7	Serotype T4
Group 1	1:256-512	1:32-128	1:256-1024	1:128-512
Group 2	1:128-512	1:32-64	1:128-512	1:128-256
Group 3	1:128-512	1:256-512	1:512-1024	1:256-512
Group 4	1:1024	1:8-16	1:64-128	1:8
Group 5	1:4-8	1:256-512	1:4-16	1:4-8
Group 6	1:128	1:8	1:1024-2048	1:64
Group 7	1:8-16	1:4-16	1:16-32	1:256-512

Table 1. Serum IgG titers of immunization groups of rabbits to *P. haemolytica*. Serotype A1, A2, A7 and T4 antigens were used in ELISA.

Rabbits	Serotype A1	Serotype A2	Serotype A7	Serotype T4
Group 1	1:16-32	1:4-16	1:32-64	1:4-16
Group 2	1:128-256	1:64-128	1:256-1028	1:64-128
Group 3	1:128-256	1:16-32	1:256-512	1:64-128
Group 4	1:256	1:4	1:128	1:2-4
Group 5	1:2-4	1:128	1:4	1:2
Group 6	1:128	1:4	1:512	1:2
Group 7	1:4	1:2	1:2	1:128

Table 2. Serum anti-leukotoxin titers of immunization groups of rabbits. Serotype A1, A2, A7 and T4 leukotoxins were used in toxin-neutralization test.

Challenge strain	dose	Source of antiserum					
		Group 1		Group 2		Group3	
		death	protection	death	protection	death	protection
A1	2.0x10 ⁷	2/6	66%	4/6	33%	1/6	83%
A2	2.0x10 ⁶	4/6	33%	5/6	16%	3/6	50%
A7	2.0x10 ⁷	2/6	66%	4/6	33%	1/6	83%
T4	2.0x10 ⁸	1/6	83%	3/6	50%	0/6	100
A6	2.0x10 ⁷	5/6	16%	5/6	16%	4/6	33

Table 3. Passive protection of mice by immune sera from three immunization groups. Each challenge group contained six mice. Challenge doses show 10 X MLD50.

Discussion

P. haemolytica is the organism most commonly associated with ovine pneumonia in Turkey (1). Various serotypes may be involved, but serotypes A2, A1, A7 and T4 are more frequently isolated from the cases in Turkey, at least in central Anatolia (3,4). Despite intense efforts to treat and control the infection using antimicrobial drugs (16) and immunoprophylactic methods (7), *P. haemolytica* continues to be an important pathogen in sheep. A major problem in the control of ovine pneumonic pasteurellosis is the lack of a vaccine, that consistently induces protective immunity. Although a number of live and killed vaccines have been developed and used, their efficacy in field trials have been variable, ranging from no effect to reduced or even increased morbidity and mortality (7,9). These facts lead us to investigate the immunogenicity of common serotypes and the effectiveness of several formulations of immunogens in laboratory test systems.

P. haemolytica possesses a number of characteristics that should be taken into account when the production and formulation of a vaccine are contemplated. We chose the whole cell, which contains most of the potential antigens, and leukotoxin, which is the most important virulence factor in the pathogenesis of disease, for immunogen in this experiment.

Differences were observed in the immunogenicity of whole cell preparations of *P. haemolytica* serotypes used in this study. Relatively low antibody titers against serotypes A2 and T4 were remarkable. Several researchers have also reported antigenic differences among serotypes (8) and pointed out the low immunogenicity of serotype 2 (9). Jones et al. (9) have shown that A2 serotype was poorly immunogenic in lambs, mice and rabbits. The immunogenicity of leukotoxin was associated with its activity on target cells, except serotype A2. Although A2 had the most potent

leukotoxin activity, its immunogenicity was relatively low. Burrows et al. (17) have found at least seven different variations of leukotoxin determinants among 16 serotypes of *P. haemolytica*. This means that leukotoxins produced by *P. haemolytica* serotypes are not completely identical, and may explain the differences in the immunogenicity of leukotoxins from different serotypes.

A low degree of cross-reaction was detected among different serotypes used in this study and was evident in both whole cell and leukotoxin preparations. This was not surprising for whole cell immunogens, since differences in surface antigens are the basis of serotypes (2). Shewen and Wilkie (18) have reported that neutralization of toxin by type-specific antisera is more effective than that of heterologous antisera.

We performed challenge experiments in mice as a low-cost alternative model, since mice have been used as experimental animal in protection studies of *P. haemolytica* (19,20). Jones et al. (21) used passively transferred immune serum to protect experimentally infected lambs and concluded that systemic humoral immunity alone can prevent pneumonic pasteurellosis. In our study, serum IgG levels appeared to be related to protection, except serotype T4. The reason for this contradictory finding with T4 could not be explained properly, but it was assumed that a different mechanism might be involved in the in vivo susceptibility of serotype T4 to immune sera. Among three groups of immune sera, sera against polyvalent combined immunogen achieved the highest protection to all challenge serotypes. Sutherland et al. (10) have reported that cytotoxin is an essential component of a protective vaccine and protection correlates with cytotoxin-neutralising titer of serum. In our study, injected immunoglobulins may have been bactericidal with complement, and/or neutralized metabolic function or products.

The low degree of cross-protection among different serotypes was evident in mice challenged with serotype A6. Since immunogens did not contain serotype A6, none of the immune sera gave passive protection in mice challenged with this serotype. Purdy et al. (13) observed a limited degree of cross-protection against heterologous serotypes in an experimental goat model. Chandrasekaran et al. (22) have reported that vaccine containing local strains was more effective than imported commercial vaccine to prevent pneumonic pasteurellosis.

In conclusion, marked differences in the immunogenicity of particular serotypes and the limited protection against heterologous serotypes may cause vaccine failures. For these reasons, to control ovine pneumonic pasteurellosis, the prevalence of the different local serotypes should be continually monitored, and the pathogenicity and immunogenicity of new serotypes should be investigated, so that the appropriate strains can be incorporated in the vaccine.

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