

Etiologic and Immunologic Characteristics of Thoroughbred Horses with Bacterial Infectious Upper Respiratory Disease at the Seoul Race Park

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Received: December 12, 2008 / Revised: March 9, 2009 / Accepted: April 16, 2009

Equine respiratory disease is a common cause of poor performance and training interruptions. The higher incidence rate of infectious upper respiratory disease (IURD) in thoroughbred racehorses at the Seoul Race Park coincided with the frequent stabling season, shorter stabling periods, and younger ages in this study. Incidence rates were also correlated with significantly lower proportions of cells expressing MHC class II-, CD2 antigen-, CD4⁺- or CD8⁺-T lymphocyte-, and B lymphocyte in IURD patients compared with healthy control groups in the summer and fall and in 2- and 3-year-old groups. The data suggested that movement and new environments may have resulted in immunosuppression and inappropriate responses to respiratory pathogens in IURD patients. The IURD incidence decreased with age, perhaps by the acquisition of immunity, and study results suggested that immunologic protection was associated with IURD, particularly in young thoroughbred racehorses. Streptococci isolates were identified in 11 of 72 IURD horses, and 3 of these isolates were identified as *Streptococcus equi* subsp. *equi*. *S. equi* subsp. *zooepidemicus* was isolated from 2 of 23 IURD horses in the spring (8.7%), 5 of 23 in the summer (21.7%), and 1 of 6 in winter (16.7%). *S. equi* subsp. *zooepidemicus* (5%) was also identified in 3 of 61 isolates from clinically normal horses. Racetracks should implement anti-IURD protective measures by assessing the capacity of equine immunologic protection at the Park and by limiting the introduction of specific respiratory pathogens (such as *S. equi* subsp. *equi*) by preventing the access of infected but subclinical horses with a specified respiratory pathogen-free certification system prior to Park entry.

Keywords: Equine infectious upper respiratory disease, thoroughbred racehorses, immunologic protection, *Streptococcus equi* subsp. *equi*, Seoul Race Park

Equine infectious upper respiratory disease (IURD) is a common and frequently recurrent problem in horses worldwide. It is an acute clinical disease syndrome, and is caused by several respiratory viruses and bacteria including equine influenza virus, equine herpesvirus-1 and -4, equine arteritis virus, *Streptococcus equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* [17]. Viral IURDs are usually subclinical in racehorses (≥ 2 year old) secondary to immunologic exposure from previous infections. The exception is equine influenza [38].

The Seoul Race Park (Seoul, Korea) experienced yearly IURD epizootics with a 29.6% estimated incidence from 2001 to 2005 [Ryu S. H. *et al.* submitted for publication]. This rate was significantly higher than the 1.5% IURD incidence per quarter in non-racetrack populations in the United States [17] and the 8.9% inflammatory airway disease incidence per month in U.K. training yards [47]. The Seoul Race Park had an influenza epidemic in 1973 and an equine herpes virus outbreak in 1979, and no additional viral respiratory outbreaks have occurred since that time. Equine influenza or herpes virus infection have not circulated on a regular basis at the Seoul Race Park, because the majority of stabled racehorses originated at Korean breeding farms without any epidemic outbreaks with similar management systems to the Seoul Race Park. Horses infected with equine influenza or herpes virus were prevented from importation with positive test results. Moreover, compulsory pre-stabling veterinary inspection is conducted on all horses at arrival at the quarantine stable at the Seoul Race Park prior to entry into the park. Horses

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are all vaccinated with the EquivacS (CSL Veterinary, Parkville, Victoria, Australia) strangles bacterin vaccine and the Fluvac equine influenza Innovator (Fort Dodge Animal Health, Fort Dodge, Iowa, U.S.A.) vaccine twice yearly without exemption in the Park.

S. equi subsp. *equi* is a frequently encountered equine respiratory pathogen despite the widespread use of bacterin and M protein extract intramuscular vaccines [39, 40, 41, 42]. Vaccination results in high efficacy of the stimulation of specific bactericidal antibodies in the serum [35], although vaccines did not effectively stimulate SeM-specific IgA on the nasopharyngeal mucosa as did *S. equi* subsp. *equi* natural infection. The SeM gene is involved in host cell adherence on the tonsillar crypts and the ventral surface of the soft palate, and *S. equi* subsp. *equi* SeM-deficient mutants are not infectious. *S. equi* subsp. *equi* is difficult to detect on the mucosa a few hours after infection, but is detectable in the mandibular or retropharyngeal lymph nodes [40]. Therefore, culture generally provided only a minimal estimate of nasal *S. equi* subsp. *equi* because of inadequate numbers, the failure of transfer from swab to culture medium, or contaminant overgrowth [42].

The presence of persistent IURD patients who were negative on *S. equi* subsp. *equi* culture suggested that other infectious agents were involved. For example, *S. equi* subsp. *zooepidemicus* is known to be the most commonly isolated opportunistic bacterial pathogen of horses and results in uterine and respiratory tract disease in older mares [46].

A number of risk factors for respiratory infection at the Seoul Race Park have been identified, including the frequent stabling season and the stabling period and age, which can affect host IURD immunity. Equine IURD treatment may be prolonged with great financial impacts. Seoul Race Park racehorse owners paid approximately 146,500 U.S. dollars per year corresponding to an average 11.8% of total veterinary fees from 2001 to 2005. Therefore, prevention through rational intervention strategies is an important veterinary and financial consideration [Ryu S. H. *et al.*, submitted].

The objectives of this study were to isolate and identify *S. equi* subsp. *equi* (the major bacterial pathogen of IURD) and *S. equi* subsp. *zooepidemicus* (the common pathogen of opportunistic respiratory infection) and determine their antibiograms. We also sought to characterize the association between equine capacity of immunologic protection and IURD occurrence in thoroughbred horses at the Seoul Race Park, Korea.

MATERIALS AND METHODS

Animals

Horses were selected from 54 trainer stables at the Seoul Race Park for inclusion into IURD and healthy control groups from March

2003 to January 2004. Trainers were queried regarding the presence of respiratory symptoms in their horses. Horses with increased cough frequency, mucopurulent nasal discharge, and at least one additional clinical symptom (fever, depression, decreased appetite, anorexia, poor exercise performance or enlarged lymph nodes of the head and neck) were diagnosed with IURD. A single member of the research team conducted all queries. A total of 72 IURD patient horses and 61 clinically healthy horses were included in the study. Clinical evaluation and nasal swab and blood sample collection were conducted in the spring (3/21/03-4/1/03), summer (7/21/03-7/28/03), fall (10/27/03-11/3/03), and winter (1/12/04).

Horses were initially held at the quarantine stable at the Park, and were transferred to trainer stables after normal physical examinations and negative laboratory results for equine infectious anemia and influenza. Wheat, barley, oats, molasses, mixed legume hay, and water sources were separated for each stable, although nose contact was possible during transfer. All horses received regular quarterly anthelmintic treatment (Oximinth Plus or Equimax, Virbac Pty Ltd., Peakhurst, NSW, Australia) after transfer to trainer stables. Horses were also vaccinated with the EquivacS bacterin (CSL Veterinary, Parkville, Victoria, Australia) for strangles and the Fluvac Innovator (Fort Dodge Animal Health) for equine influenza in early October. Vaccines were administered prior to nasal swab and blood sampling in the fall.

Clinical Evaluation and Bacteriologic Examination

Clinical observations and diagnostic sample collection were performed on suspect IURD and healthy control horses. Samples were stratified by age and stabling periods for statistical analysis. The number of clinical examinations was minimized to reduce handling-induced stress in the horses.

Nasal swab specimens were seasonally collected for bacterial culture from all horses in the Seoul Race Park stables. The nasal mucosa was swabbed a minimum of 10 cm from the external nares. Nasal specimens were chilled on ice, streaked on 5% sheep blood agar plates (Promed, Sungnam, Gyeonggi, Korea; n=3), and incubated at 37°C for 24 h. Beta-hemolytic colonies of varied morphologies from blood agar plates were Gram stained and subcultured at least 3 times in order to get a single pure colony. Pure colonies were tested for biochemical behavior (assessed by catalase, coagulase, esculin hydrolysis, and sodium hippurate tests), and for growth on Baird-Parker agar with 5% egg yolk tellurite (Becton Dickinson and Co., Sparks, MD, U.S.A.) and tryptic soy broth (TSB; Becton Dickinson and Co.) with 6.5 or 10% NaCl [30].

Mucoid beta-hemolytic colonies were initially designated as *Staphylococcus* spp. based on typical colony morphology, presence of Gram-positive colonies, catalase and coagulase positive status, and the Baird-Parker medium colony zone following enrichment of TSB with 10% NaCl. Beta-hemolytic *Streptococcus* spp. colonies exhibited no growth at TSB with 6.5% NaCl, were Gram-positive and catalase negative, did not exhibit esculin hydrolysis in the presence of bile, and were sodium hippurate negative. Further identification and confirmation were performed by the API 20 Strep, VITEK (bioMérieux, Marcy-l'Etoile, France), and polymerase chain reaction (PCR) amplification.

Identification of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* by PCR and Their Antibiotic Susceptibility

PCR identification of *S. equi* isolates. Isolates from equine nasal swab specimens and other reference strains (*S. equi* subsp. *equi*

ATCC 33398, *S. equi* subsp. *zooepidemicus* ATCC 43079, and *S. aureus* ATCC 25923) were grown in 5% defibrinated sheep blood agar (Promed) for 24 h and then suspended in 1 ml of saline. Optical density values (OD) were measured using a spectrophotometer (Specgene, Techne Inc., Princeton, NJ, U.S.A.) for the estimation of appropriate concentrations (1.0×10^9 CFU) to increase DNA yield. Cell pellets were resuspended in 200 μ l of enzyme incubation buffer [20 mM Tris-HCl, 2 mM EDTA, pH 8.0, 1.2% Triton-X 100, 18 μ g/ml lysozyme (Sigma-Aldrich, St. Louis, MO, U.S.A.), and 2.5 U/ml mutanolysin (Sigma-Aldrich)] followed by an incubation at 37°C for 1 h [3]. Cell pellets from clinical isolates and *S. aureus* reference strains were resuspended in 500 μ l of 50 mM Tris-HCl buffer (pH 8.3) with 50 mM disodium EDTA and 20 U lysostaphin/ml (Sigma-Aldrich), and incubated at 37°C for 1 h. [34]. Cell solutions were treated with 200 μ l of AL buffer, and 25 μ l of proteinase K, and were incubated at 70°C for 30 min. This mixture was transferred to columns and eluted per the manufacturer's instructions [32].

Oligonucleotide primers were used for amplification of specific parts of the superoxide dismutase A encoding gene (*sodA*) and for further differentiation of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* by amplification of the *seel* gene encoding the enterotoxin *SeeI* (which is only specific for *S. equi* subsp. *equi*) [3]. Amplification of *S. aureus* was performed with 2 oligonucleotide primer sets, including the *S. aureus* specific primer set, Sa442 [26], and the *S. aureus nuc* primer set [6]. The PCR primers included pair 1 (235 bp, *sodA equi/zooep-F*: 5'-CAG CAT TCC TGC TGA CAT TCG TCA GG-3'; *sodA equi/zooep-R*: 5'-CTG ACC AGC CTT ATT CAC AAC CAG CC-3'), pair 2 (520 bp, *SeeI-F*: 5'-GAA GGT CCG CCA TTT TCA GGT AGT TTG-3'; *SeeI-R*: 5'-GCA TAC TCT CTC TGT CAC CAT GTC CTG-3'), pair 3 (108 bp, Sa442-F: 5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3'; Sa442-R: 5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3'), and pair 4 (270 bp, *nuc-F*: 5'-GCG ATT GAT GGT GAT ACG GTI-3'; *nuc-R*: 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3').

PCR was performed in a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). The PCR reaction had a final volume of 20 μ l with 0.4 μ l of each primer (10 pmol/ μ l), 1.6 μ l of 2.5 mM dNTP (Takara Bio Inc., Otsu, Shiga, Japan), 1.2 μ l of 25 mM MgCl₂ (Takara Bio Inc.), 2 μ l of 10 \times buffer (Takara Bio Inc.), 2 U of *Taq* polymerase (Takara Bio Inc.), and 1 μ l of template DNA [3]. A total of 30 PCR cycles were run under the following conditions: a pre-PCR step at 94°C for 5 min, a denaturation step at 94°C for 30 s, an annealing step at 59°C for 30 s, and an extension step at 72°C for 1 min. The preparation was kept at 72°C for 7 min to complete the reaction after the final cycle. The PCR conditions for *S. aureus* amplification were similar to previously described conditions except for an annealing temperature of 55°C. PCR products were stored at 4°C until analysis [32]. PCR products (10 μ l), molecular size markers, and a 100-bp, 1.5-kb DNA ladder (Takara Bio Inc.) were loaded onto 1.5% (w/v) agarose gels stained with 0.5% of ethidium bromide and were run in 0.5 \times TBE (0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0) at 135 V for 30 min followed by visualization under UV illumination.

Antimicrobial susceptibility tests. *In vitro* antimicrobial susceptibility testing was conducted by the disc-diffusion testing method with the antimicrobial Sensi-disc system (ampicillin, penicillin, ceftiofur, amikacin, gentamicin, kanamycin, neomycin, streptomycin, oxytetracycline, trimethoprim/sulfamethoxazole, bacitracin, polymyxin B; Becton, Dickinson and Co.) according to the guidelines of the

National Committee for Clinical Laboratory Standards (NCCLS) [28] and the Clinical and Laboratory Standards Institute (CLSI) [7, 8]. Isolates with concentrations at 10^8 CFU/ml were estimated by measuring the cellular OD by a spectrophotometer (Techne Inc.) and were swabbed on Mueller-Hinton with sheep blood (5% v/v). *S. equi* subsp. *equi* ATCC 33398, *S. equi* subsp. *zooepidemicus* ATCC 43079, and *S. aureus* 25923 were used as the quality control organisms. Determination of the zone diameters was followed by CLSI, NCCLS, and Food and Drug Administration (FDA) guidelines in the U.S. and France [10, 18].

Proportion of Equine Leukocyte Subpopulations

Approximately 40 ml of jugular venous blood was collected from each animal. A set of monoclonal antibodies (mAbs) specifically reactive with equine leukocyte differentiation antigens and flow cytometry were used to examine the proportions of leukocyte subpopulations in peripheral blood from each group of IURD patient or healthy control horses.

Preparation of peripheral blood leukocytes. The separation of peripheral blood leukocytes was conducted by previously defined methods [9]. Briefly, collected blood was mixed with equal volumes of acid-citrate dextrose (ACD)-ethylenediamine tetraacetic acid (EDTA), and leukocytes were separated using density gradient centrifugation with Hypaque Ficoll (d: 1.086; Sigma-Aldrich) at 670 $\times g$ for 30 min. Live cells were counted by the trypan blue exclusion technique, and the final concentration was adjusted to 1×10^7 cells/ml.

mAbs specific to equine leukocyte differentiation antigens. The mAbs specific to major histocompatibility complex (MHC)-class II (TH81A5) [1, 2, 9, 33], CD2 (HB88A) [5, 25], CD4 (HB61A) [5, 25, 45], CD8 (HT14A) [5, 13, 25, 45], and surface IgM (B29A) [25, 44] were obtained from VMRD (Pullman, WA, U.S.A.) and were used to examine leukocyte subpopulation proportions [24].

Flow cytometry (FC) analysis. Approximately 50 μ l of mAbs (15 μ g/ml) was mixed with 100 μ l of cells at a 1×10^7 cells/ml concentration in a V-bottomed 96-well microplate. Plates were incubated on ice for 30 min, washed 3 times with the first washing buffer [450 ml of PBS, 50 ml of ACD, 5 ml of 20% NaN₃, 10 ml of gamma globulin-free horse serum (Sigma-Aldrich), 20 ml of 250 mM EDTA, and 1 ml of 0.5% phenol red], and centrifuged at 670 $\times g$ for 5 min. The pellet was disrupted by vortexing, mixed with 100 μ l of $\times 200$ dilution of FITC-conjugated goat anti-mouse IgG+IgM antibody (Caltag Lab., Burlingame, CA, U.S.A.), and incubated on ice for 30 min in the dark. Cells were then washed 3 times with the second washing buffer (450 ml of PBS, 50 ml of ACD, 5 ml of 20% NaN₃, 20 ml of 250 mM EDTA, and 1 ml of 0.5% phenol red) by centrifugation at 670 $\times g$ for 5 min. Cells were mixed with 200 μ l of 2% PBS-formaldehyde (38% formalin, 20 ml; PBS, 980 ml) after the final wash and refrigerated for FC analysis. Leukocytes were only stained with the secondary antibody [FITC-conjugated goat anti-mouse IgG+IgM antibody (Caltag Lab.)] for the negative control without any primary mAb specific to the equine leukocyte differentiation antigen. The leukocyte subpopulation proportions were determined by lymphocyte gating and FC analysis (FACSCalibur) using the CellQuest program (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) and FCS Express software (De Novo software, Thornton, Ontario, Canada) [24].

Statistical Analysis

The lymphocyte proportions expressing various peripheral blood antigens were compared between IURD patients and healthy control

groups using the Kruskal-Wallis one-way ANOVA by ranks. Statistical significance was defined at P -values <0.05 . Analyses were performed using the Analyse-it program (Analyse-it Software, Ltd., Leeds, England, U.K.).

RESULTS

Bacteriologic Findings in Association with Clinical Symptoms in Animals

Selected horses were categorized by age, season, and stabling period (Table 1). Streptococci isolates were identified in 11 of 72 IURD horses, and 3 of these isolates were identified as *S. equi* subsp. *equi* (Table 2 and Fig. 1). *S. equi* subsp. *zooepidemicus* was isolated from 2 of 23 IURD horses in the spring (8.7%), 5 of 23 in the summer (21.7%), 0 of 20 in the fall (0%), and 1 of 6 in winter (16.7%). *S. equi* subsp. *zooepidemicus* (5%) was also identified in 3

of 61 isolates from clinically normal horses. The antimicrobial susceptibilities of *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* isolates were 100% (14/14) for ceftiofur, 85.7% (12/14) for penicillin, 57.2% (8/14) for trimethoprim/sulfamethoxazole, 14.3% (2/14) for tetracycline, and 0% (0/14) for aminoglycoside. *Streptococcus acidominimus*, *Streptococcus constellatus*, *Streptococcus bovis*, *Streptococcus pyogenes*, *Streptococcus uberis*, *Streptococcus mitis*, *Streptococcus suis*, *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus capitis*, and *Staphylococcus sciuri* were also isolated from IURD patients (17 of 72, 23.6%) and from healthy controls (30 of 61, 49.2%) (data not shown).

FC Analysis

Seasonal differences in the proportions of equine leukocyte subpopulations between IURD patients and the control group were investigated using mAbs and FC. No significant

Table 1. The number of IURD and healthy horses categorized by age, season, and stabled period

Age ^a (year old, yo)	Season ^b	Stabled period (P) ^c								Total	
		P≤3 months		3<P≤6 months		6<P≤12 months		P>12 months		IURD	Control
		IURD	Control	IURD	Control	IURD	Control	IURD	Control		
2 yo	Spring	9	0	0	0	0	0	0	0	9	0
	Summer	12	0	6	0	0	0	0	0	18	0
	Fall	15	0	2	0	0	0	0	0	17	0
	Winter	2	0	1	0	0	1	0	0	3	1
	Sum	38	0	9	0	0	1	0	0	47	1
3 yo	Spring	1	0	0	1	6	2	0	0	7	3
	Summer	1	0	0	0	2	3	0	5	3	8
	Fall	0	0	0	0	1	1	2	4	3	5
	Winter	0	1	1	0	0	0	1	3	2	4
	Sum	2	1	1	1	9	6	3	12	15	20
4 yo	Spring	1	0	0	0	0	0	2	6	3	6
	Summer	0	1	0	1	0	1	1	4	1	7
	Fall	0	0	0	0	0	0	0	4	0	4
	Winter	0	1	0	0	0	0	0	0	0	1
	Sum	1	2	0	1	0	1	3	14	4	18
5-9 yo	Spring	0	0	0	0	1	2	3	10	4	12
	Summer	0	0	0	0	0	1	1	3	1	4
	Fall	0	0	0	0	0	0	0	6	0	6
	Winter	0	0	0	0	0	0	1	0	1	0
	Sum	0	0	0	0	1	3	5	19	6	22
All ages	Spring	11	0	0	1	7	4	5	16	23	21
	Summer	13	1	6	1	2	5	2	12	23	19
	Fall	15	0	2	0	1	1	2	14	20	15
	Winter	2	2	2	0	0	1	2	3	6	6
	Total	41	3	10	2	10	11	11	45	72	61

^aAge proportions of selected IURD horses: 2 yo (65%), 3 yo (21%), 4 yo (6%), and 5-9 yo (8%).

^bSeasonal proportions of selected IURD horses: spring (32%), summer (32%), fall (28%), and winter (8%).

^cProportions of stabled periods of selected IURD horses (P, months): P≤3 (57%), 3<P≤6 (14%), 6<P≤12 (14%), and P>12 (15%).

Table 2. Clinical and bacteriologic findings of IURD patients and healthy controls

Season	Finding	IURD group ^{a, b}					Control group ^b	
		Nasal discharge	Cough	Fever	Lymph node abscess	<i>S. equi</i> ^c	<i>S. equi</i> ^c	
Spring		21/23 (91)	18/23 (78)	2/23 (9)	5/23 (22)	5/23 (22)	0/21 (0)	
Summer		22/23 (96)	23/23 (100)	1/23 (4)	0/23 (0)	5/23 (22)	1/19 (5)	
Fall		20/20 (100)	19/20 (95)	1/20 (5)	0/20 (0)	0/20 (0)	2/15 (13)	
Winter		6/6 (100)	6/6 (100)	0/6 (0)	0/6 (0)	1/6 (17)	0/6 (0)	
Total		69/72 (96)	66/72 (92)	4/72 (6)	5/72 (7)	11/72 (15)	3/61 (5)	

^aIURD: Infectious upper respiratory disease.

^bResults are expressed as the number of horses positive to each finding/the number of horses examined in each season or total horses, with percent of sensitivity of each finding shown in parentheses.

^cExcept for 3 cases of isolation of *Streptococcus equi* subsp. *equi* from the IURD patient group in the spring, all isolates obtained from the two groups were *Streptococcus equi* subsp. *zooepidemicus*.

differences were noted in cells collected from the 2 groups in winter ($p > 0.05$). However, the proportion of CD2 surface marker-expressing cells, which represents the total T lymphocyte population, from the IURD patient group was significantly lower than that from the control group from spring to fall ($p < 0.01$). Moreover, the proportion of cells expressing MHC class II, CD2 antigens, and CD8⁺ T lymphocytes, which participate in secondary defense mechanisms, and B lymphocytes was significantly lower in the IURD group than those in the control group in the summer and fall ($p < 0.05$) (Figs. 2A to 2D). The proportion of CD4⁺ T lymphocytes in the IURD group was also significantly lower than that in the control group in the summer ($p < 0.05$) (Figs. 2A to 2D). The proportion of cells expressing MHC class II, CD2 antigens, and CD4⁺ or CD8⁺ T lymphocytes, and B lymphocytes was significantly lower in the IURD group than those in the control group, particularly in the 2 to 3 year age group ($p < 0.01$) (Fig. 2E).



Fig. 1. *Streptococcus equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* PCR products. PCR products were approximately 230 bp with *sodA* specific oligonucleotide primers and were approximately 520 bp with *seeI* specific oligonucleotide primers. Note the negative reaction of *S. equi* subsp. *zooepidemicus* with the *seeI* oligonucleotide primers. Lanes: M, 100-bp DNA ladder (Takara Bio Inc., Otsu, Shiga, Japan); 1–5, *S. equi* isolates in the spring; 6–11, *S. equi* isolates in the summer; 12–13, *S. equi* isolates in the fall; 14, *S. equi* isolates in the winter; 15, *S. equi* subsp. *equi* ATCC 33398; 16, *S. equi* subsp. *zooepidemicus* ATCC 43079; 17, Negative control (distilled water).

DISCUSSION

Several studies have highlighted the importance of host immunity-related susceptibility to infection in horses [14, 35, 39], but there have been limited findings regarding host immunity data in association with IURD risk factors. All stabling and clinical care data are electronically documented by a team of veterinary surgeons employed by the Korean Racing Authority in the registration and veterinary data management system, and this database can offer data for analysis. The primary purpose of this study was to improve the health of the racehorse population in Korea, but results could be relevant to the worldwide thoroughbred population. A high correlation between equine immunologic capability and IURD occurrence was identified in this study. Horses with IURD symptoms in the spring to fall (March to November) outnumbered those in the winter (December to February). Significantly lower proportions of cells expressing MHC class II, CD2 antigens, CD8⁺ T lymphocytes, and B lymphocytes, which mean a lower immunologic defense capacity, were observed in the IURD patients group compared with the healthy control group in the summer and fall. Horses may have been less exposed to IURD etiologic agents in the winter, since the number of newly stabled horses was significantly low (Figs. 2A to 2D).

The MHC class II molecule-expressing cells are significantly involved in bacterial defense mechanisms, phagocytosis, and antigen presentation. CD4⁺ and CD8⁺ T lymphocytes help and activate other antipathogen effector cells, and have a critical role in the elimination of viral and intracellular bacterial infection, respectively [15, 45]. This implied that the higher proportions of cells in the healthy control group was activated after IURD infection and were effectively involved in protection against IURD disease progression. Functional phagocytosis studies against *S.*

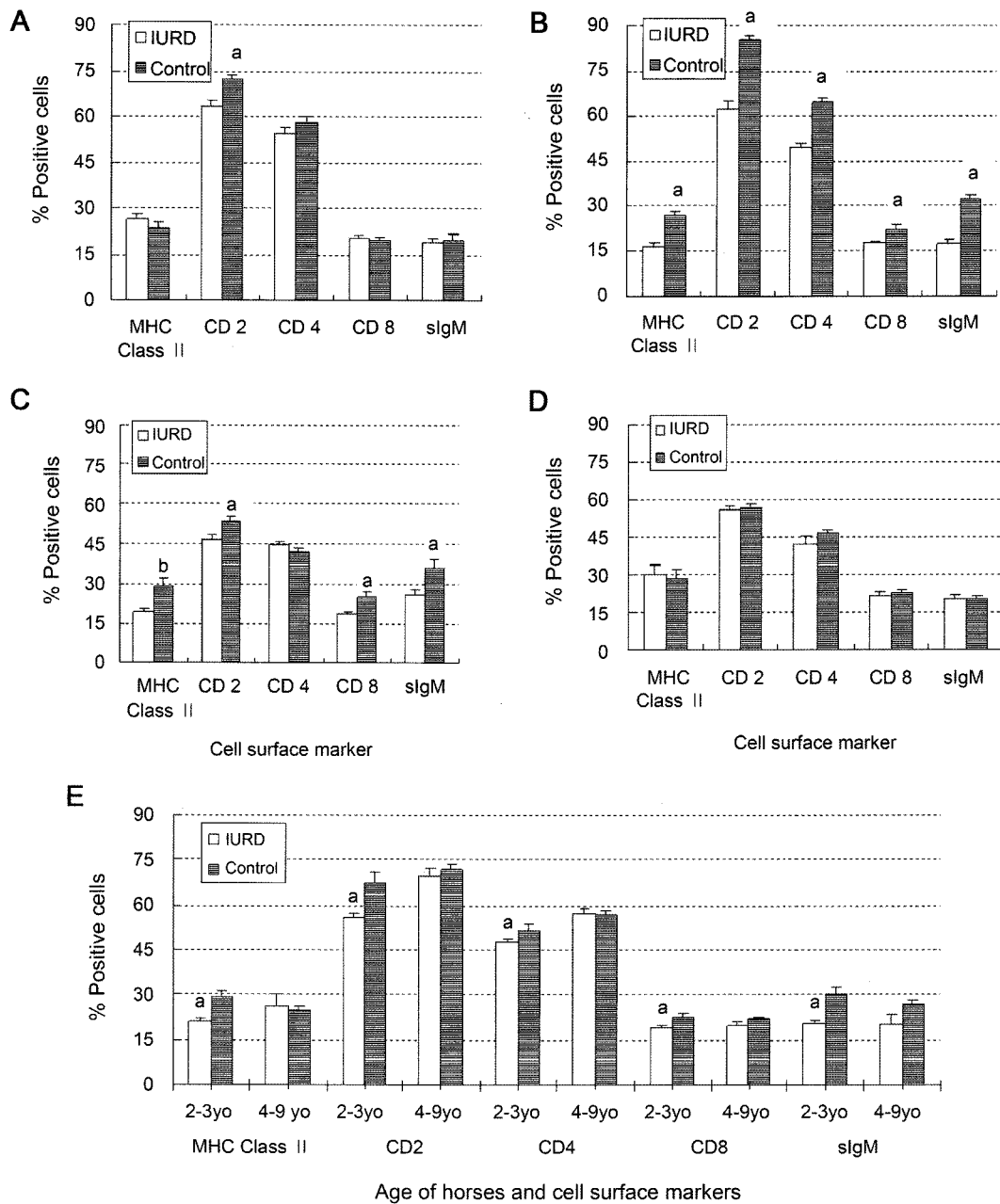


Fig. 2. Summary of flow cytometric analysis of cells expressing MHC class II, CD2, CD4, and CD8 T lymphocytes, and sIgM⁺ B lymphocytes in peripheral blood at time zero.

Patient group lymphocytes were derived from IURD patients and healthy controls in 4 seasons [spring (A), summer (B), fall (C), and winter (D)] and were categorized by age group (E). Significant differences between the two groups (A to D, IURD patient versus healthy group; E, 2 to 3 versus 4 to 9 year old group) are indicated (a, $p < 0.01$; b, $0.01 < p < 0.05$). yo: year old.

equi subsp. *equi* and *S. aureus*, and FC with mAbs against equine immunomodulating cells were previously performed by our group to elucidate the IURD immunosurveillance mechanisms [24].

Young horses (2 to 3 years old) and horses with a stabling period of less than 3 months were more likely to develop IURD (Table 1). This could be associated with the frequent movement of horses in and out of the Seoul Race Park

from the spring to fall, the limited movement in the winter, and the age (2 to 3 years) of the main populations of newly stabled horses [Ryu S. H. *et al.*, submitted]. The increased chances of contact with possible pathogen-carrying horses, the stress of transportation, immunosuppression caused by the new environment, and the incubation period resulted in a greater IURD susceptibility of newly stabled horses compared with other resident horses [4, 11, 12, 22, 29, 31,

37, 43]. Significantly lower proportions of cells expressing MHC class II, CD2 antigens, CD4⁺ and CD8⁺ T lymphocytes, and B lymphocytes were present in 2 to 3 year old IURD patients compared with the healthy control group, but not in IURD patients older than 3 years of age (Fig. 2E). It is impossible to determine the exposure status of horses involved in natural epizootics, but it is unlikely that all the adult horses examined at the Seoul Race Park from March 2003 to January 2004 were naïve to IURD pathogen exposure because of the mobility seen in these equine populations.

IURD cases in older horses may result after immunity from past exposures waned and allowed susceptibility of horses to re-infection and disease recurrence. The clinical signs of IURD in older horses are usually milder than in younger horses. These cases may also be the origin of clinically severe cases in younger horses. Other factors involved in IURD cases in older horses included individual variation in immune responses, stress caused by other diseases, and the environment. For example, iatrogenic immunosuppression can be caused by corticosteroid administration, and may preferentially depress IgG_a and IgG_b responses [36]. Exercise is another stressor that modulates immune responses [19]. Moderate exercise has minimal effects on the severity of infectious diseases [16], but intense exercise in unconditioned horses can have profound effects [21].

S. equi subsp. *equi* and *S. equi* subsp. *zooepidemicus* infections were reported to comprise approximately 15% of IURD cases observed in this study (Table 2). This is comparable to the 11% prevalence of *S. equi* subsp. *equi* infection in IURD cases at a thoroughbred racetrack in Canada during epidemics from 1990 to 1992 [27], the 20% of occurrences of strangles out of IURD cases in non-racetrack populations in the United States without laboratory confirmation from 1998 to 1999 [17], and the 25.5% prevalence of *S. equi* subsp. *equi* by PCR on 6 farms with endemic strangles [20]. The difference in identification rates is likely due to diagnostic methods, geography, and stabling management.

In this study, 14 strains were cultured as *S. equi* subsp. *equi*, although PCR demonstrated that 3 of the cases were *S. equi* subsp. *equi* and 11 were *S. equi* subsp. *zooepidemicus*. Culture provides only minimal estimates of *S. equi* subsp. *equi* prevalence in the nose because of inadequate numbers, the failure of transfer from swab to culture medium and overgrowth of contaminants. Cultures may be unsuccessful during incubation and the early clinical phases of *S. equi* subsp. *equi* infection. *S. equi* subsp. *equi* is not normally present in the mucosa until 24 to 48 h after the fever onset, and it is therefore prudent to monitor the temperatures of in-contact horses during an outbreak in order to recognize disease onset and to isolate carrier horses prior to engagement

in the further transmission of *S. equi* subsp. *equi* [42]. PCR was shown to be a much more sensitive diagnostic method than standard bacterial culturing in a previous study, and the suggested protocol for strangles control on an infected premise is based on the PCR diagnosis of subclinical cases, prompt isolation of infected horses, immediate treatment with local and systemic antibiotics, and careful environmental sanitation [20].

Other *Streptococcus* species (*S. acidominimus*, *S. constellatus*, *S. bovis*, *S. pyogenes*, *S. uberis*, *S. mitis*, and *S. suis*) and *Staphylococcus* species (*S. aureus*, *S. hyicus*, *S. capitis*, and *S. sciuri*) were isolated from both IURD patients and clinically normal control horses in this study. Further studies are required to determine the effects of this infection or of other respiratory viruses on local nasopharyngeal immune responses to *S. equi* subsp. *equi*.

Streptococcal isolates included in this study were highly susceptible to penicillin and ceftiofur antibiotics (Table 3). Doping control systems have prohibited the preventive use of antimicrobial feed additives, veterinarians have exercised judicious antibiotic use, and antibiotics are not frequently used in the breeding farm. *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* isolates had lower susceptibility values to aminoglycoside, oxytetracycline, trimethoprim/sulfamethoxazole, and polymyxin B in this study. This was similar to the antibiotic susceptibility pattern in a similar study [23]. Barodon, the anionic alkali mineral complex recommended both as an immunostimulator and an effective alternative to antimicrobial feed additives in our other study [24], may help prevent infection with multidrug-resistant *S. equi* subsp. *equi* and *S. aureus* and enhance associated equine immune responses.

This study revealed the critical role of host factors in IURD development. Racetrack regulatory veterinarians should segregate horses at great IURD risk, design effective and efficient preventative measures, including *S. equi* subsp. *equi* free certificate systems prior to arrival in the Park, and remove *S. equi* subsp. *equi*-carrying resident horses from the Park based on accurate (PCR) diagnoses of Park populations. Compulsory environment sanitation systems should be implemented in association with comparative equine lymphocyte subpopulation analyses, particularly in the peak seasons of IURD occurrence. Such cellular analysis would allow predictions of host capabilities of anti-IURD immune protection. Field trials of the recently marketed intranasal strangles vaccine at the Seoul Race Park will assess its efficacy compared with the questionable efficacies of currently administered intramuscular vaccines. Intramuscular vaccines do not efficiently stimulate SeM-specific IgA on the nasopharyngeal mucosa, but induce specific bactericidal antibodies in serum. Those suggestions for IURD protection will provide major economic benefits for the racing industry and will improve

Table 3. Antimicrobial susceptibility of *Streptococcus equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* isolates from IURD patients and healthy controls.

Group	Season ^a	Isolates	Antimicrobials ^b													
			Penicillins		Cephalosporin		Aminoglycosides				Tetracyclines		Sulfonamides		Polypeptides	
			AM	P	CEF	AN	GM	K	N	S	T	SXT	B	PB		
IURD	Spring	No. of susceptible <i>S. equi</i> spp. <i>equi</i> isolates (total n=3)	3/3	2/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	3/3	1/3	
		Susceptible percentage (%)	(100)	(66.67)	(100)	(0)	(0)	(0)	(0)	(0)	(0)	(33.33)	(33.33)	(100)	(33.33)	
	Spring to Winter	No. of susceptible <i>S. equi</i> spp. <i>zooepidemicus</i> isolates (total n=8)	8/8	7/8	8/8	0/8	0/8	0/8	0/8	0/8	0/8	1/8	6/8	8/8	5/8	
		Susceptible percentage (%)	(100)	(87.50)	(100)	(0)	(0)	(0)	(0)	(0)	(5.55)	(75.00)	(100)	(62.5)		
Control	Summer	No. of total susceptible isolates	11/11	9/11	11/11	0/11	0/11	0/11	0/11	0/11	2/11	7/11	11/11	6/11		
	to fall	Susceptible percentage (%)	(100)	(81.82)	(100)	(0)	(0)	(0)	(0)	(0)	(18.18)	(63.64)	(100)	(54.55)		
IURD	Spring	No. of susceptible <i>S. equi</i> spp. <i>zooepidemicus</i> isolates (total n=3)	3/3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	1/3		
		Susceptible percentage (%)	(100)	(100)	(100)	(0)	(0)	(0)	(0)	(0)	(0)	(33.33)	(66.67)	(33.33)		

^aOnly seasons with isolated *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* are described.

^bAbbreviation of antimicrobials (Becton, Dickinson and Company, Sparks, MD, U.S.A.): AM, ampicillin; P, penicillin; CEF, ceftiofur; AN, amikacin; GM, gentamicin; K, kanamycin; N, neomycin; S, streptomycin; T, oxytetracycline; SXT, trimethoprim/sulfamethoxazole; B, bacitracin; PB, polymyxin B.

equine welfare in Korea. They will also provide relevant information to other worldwide racetracks with similar operational environments.

Acknowledgments

We would like to thank Dr. Hyung-Seon Jeon and Dr. Byung-Hyun Kim of the Korea Racing Authority for helping to collect samples and check clinical observations during this study, as well as Dr. Nam Hoon Kwon and Ms. Sook Shin for outstanding technical assistance. This study was supported in part by the Research Institute of Veterinary Science, Department of Veterinary Microbiology, College of Veterinary Medicine, and BK21 Program for Veterinary Science, Seoul National University. Further support was also provided by the Korea Research Foundation Grants (KRF-2007-331-E00254).

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