Pulsed-Field Gel Electrophoresis of *Staphylococcus hyicus* and *Staphylococcus chromogenes* Genomic DNA and Its Taxonomic, Epidemiologic and Ecologic Applications in Veterinary Medicine

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ABSTRACT. One hundred and thirty-eight strains of *Staphylococcus hyicus* and 21 strains of *S. chromogenes* isolated from animals were analyzed by pulsed-field gel electrophoresis (PFGE) after restriction endonuclease *Sma*I digestion of chromosomal DNA. Eighty-eight strains of *S. hyicus* from pigs with or without exudative epidermitis (EE) generated 16 to 26 fragments in the size range of <1 to 485 kb, and yielded 39 different patterns. With regard to the strains from pigs with EE, PFGE patterns differed according to the country of origin. Outbreaks of EE occurring on four separate pig farms in Japan involved *S. hyicus* with different PFGE patterns. The PFGE patterns shown by *S. hyicus* strains from 4 kinds of animals were compared. Strains from pigs differed from those isolated from chickens (n=45; 18 to 24 fragments of <1 to 425 kb), cows (n=3; 17 to 19 fragments of <1 to 475 kb), and goats (n=2; 16 or 17 fragments of <1 to 1,125 kb). Also, each of the chicken, cow and goat strains had a host-specific fragment. The results suggest that PFGE analysis might be a useful marker for distinguishing ecovars within *S. hyicus*. In contrast, strains of *S. chromogenes* from pigs and cows generated 17 to 24 fragments ranging from <1 to 545 kb. The PFGE patterns of *S. chromogenes* strains were more highly conserved than those of *S. hyicus*. *S. chromogenes* strains could be distinguished from *S. hyicus* strains by fragments within the range of 305 to 545 kb. The results indicate that PFGE analysis could be used to distinguish between *S. hyicus* and *S. chromogenes*. We conclude that PFGE analysis is a useful tool not only for species or strain identification but also for epidemiologic or ecologic studies of *S. hyicus* and *S. chromogenes*. — KEY WORDS: genomic DNA fingerprinting, pulsed-field gel electrophoresis, *Staphylococcus chromogenes, Staphylococcus hyicus*.

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The coagulase-variable species *Staphylococcus hyicus* has been implicated in exudative epidermitis (EE) [36] and septic polyarthritis [28] of pigs. This organism has also been occasionally isolated from cows with mastitis [4, 19, 25], horses with dermatitis [9], and chickens with exudative dermatitis [26] or tenosynovitis [21]. The coagulase-negative species *S. chromogenes* [15], originally described by Devriese *et al.* [10] as *S. hyicus* subsp. *chromogenes*, is also commonly isolated from the udders and milk of cows suffering from mastitis [4, 16, 19, 25] or bovine intramammary infections (IMI) [40, 41]. The two species are a common component of the skin, nasal or tonsil flora of normal pigs, cows and chickens [7, 22, 33–35, 37, 39].

Until recently, phenotypic methods of typing such as phage typing [14, 20, 32, 42, 43], biotyping with commercially available kit systems [24], serotyping [17], antimicrobial susceptibility testing [42, 43], zymotyping [38], and plasmid profile analysis [42, 43] have been used in ecological and epidemiological studies of *S. hyicus*. Typing methods have not been applied to *S. chromogenes*. However, simple phenotypic character analysis is often insufficient to differentiate these two species.

More recently, newly developed molecular typing techniques such as pulsed-field gel electrophoresis (PFGE) [3, 6, 29, 31], ribotyping [5, 29] and plasmid profile analysis [5, 18, 44] have been used extensively to distinguish different strains of *S. aureus* including methicillin-resistant *S. aureus* (MRSA). PFGE has been successfully applied to the epidemiological investigation of nosocomial infections caused by MRSA [18]. Recent studies [11–13, 23] indicated that PFGE analysis can also be used to support the classification and identification of coagulase-negative staphylococci such as *S. epidermidis, S. caprae, S. capitis* (subsp. *capitis*, subsp. *ureolyticus*), *S. haemolyticus, S. lugdunensis, S. schleiferi* and *S. warneri*. PFGE analysis of the related animal habitant species *S. hyicus* and *S. chromogenes* has not yet been reported.

The purpose of this study was to evaluate the potential usefulness of PFGE as a taxonomic tool in veterinary microbiology and as source of molecular markers for monitoring *S. hyicus* and *S. chromogenes* strains in animal infections.

MATERIALS AND METHODS

Bacterial strains: The number and origin of the two staphylococcal species used in this study are listed in Tables 1 through 3. One hundred and thirty-eight strains of *S. hyicus* were isolated from four different species of animals. The pig strains (n=88) were isolated from pigs affected with EE (n=40), and the skin, nares or tonsils of healthy pigs (n=48). The chicken strains (n=45) were isolated from the skin or nares of healthy chickens. The cow strains (n=3) were isolated from udder or milk samples of cows affected with mastitis. The goat strains (n=2) were isolated from milk samples of goats with mastitis. Twenty-one strains of

Table 1. PFGE patterns and sources of *S. hyicus* isolated from pigs

PFGE pattern no.	No. of strains	Source	Country	Year of isolation
1	8	EE[a] (A)	Japan	1991
2	2	EE (A)	Japan	1991
3	5	EE (B, D)	Japan	1984
4	8	EE (C)	Japan	1984
5	3	EE (D)	Japan	1984
6	3	EE (D)	Japan	1984
7	2	EE (D)	Japan	1984
8–12	5	EE[b]	U.S.A.	1975–1980
13	3	Skin[c]	U.K.	1986
14	2	Skin[c]	U.K.	1986
15–18	4	Skin[c]	U.K.	1986
19–21	3	Skin[d]	Belgium	1975
22	1	EE[e]	Czecho Republic	1975
23	1	Skin[d]	Belgium	1975
24	2	EE[e]	Czecho Republic	1975
25	1	EE[f]	Denmark	1953
26	2	Nare	Japan	1984
27	6	Nare	Japan	1984
28	3	Nare	Japan	1984
29	2	Nare	Japan	1984
30	1	Nare	Japan	1984
31	2	Nare	Japan	1980
32	2	Nare	Japan	1980
33	3	Tonsil	Japan	1993
34	3	Tonsil	Japan	1993
35	1	Tonsil	Japan	1993
36	3	Tonsil	Japan	1993
37	5	Tonsil	Japan	1993
38	1	Tonsil	Japan	1993
39	1	Tonsil	Japan	1993

a) Exudative epidermitis. A to D in parentheses shows outbreak farms. Twenty-one strains (B to D) were supplied by Dr. S. Takeuchi [37].
b) One strain was isolated from septic polyarthritis [28].
c) Supplied by Dr. W. C. Noble *et al.* [27].
d) Supplied by Dr. L. A. Devriese [7].
e) Supplied by Dr. L. A. Derviese *et al.* [10].
f) Type strain NCTC 10350 [10, 36].

Table 2. PFGE patterns and sources of *S. hyicus* isolated from chickens, cows and goats

PFGE pattern no.	No. of strains	Source	Country	Year of isolation
40	3	Chicken nare	Japan	1984
41	1	Chicken nare	Japan	1984
42	2	Chicken nare	Japan	1984
43	1	Chicken nare	Japan	1972
44	7	Chicken nare	Japan	1984
45	1	Chicken nare	Japan	1984
46	2	Chicken nare	Japan	1984
47	28[a]	Chicken nare, skin	Japan	1993
48	1	Cow mastitic udder	U.S.A.	1966
49	1	Cow mastitic milk	U.S.A.	1981
50	1	Cow mastitic milk	U.S.A.	1981
51	1	Goat mastitic milk	U.S.A.	1994
52	1	Goat mastitic milk	U.S.A.	1994

a) The 28 strains were isolated from 28 healthy chickens raised in a flock.

Table 3. PFGE patterns and sources of *S. chromogenes* isolated from pigs and cows

PFGE pattern no.	No. of strains	Source	Country	Year of isolation
1, 2	2	Pig skin	Japan	1991
3	1	Pig skin	Japan	1991
4	1	Pig skin	Japan	1984
5	1	Pig skin	Japan	1992
6	1	Pig[a]	U.K.	1963
7	1	Cow aborted fetus	Japan	1993
8–21	14	Cow mastitic milk	U.S.A.	1983

a) Type strain CBCC 1462^T [10].

S. chromogenes were isolated from the skin of healthy pigs (n=6), the milk samples of cows showing clinical signs of mastitis (n=14), and an aborted fetus from a cow (n=1).

DNA preparation: Chromosomal DNA was prepared by a modification of the procedure of George and Kloos [11]. Briefly, the cultures were grown overnight by shaking at 35°C in 5 m*l* of brain heart infusion broth (Difco Laboratories, Inc., Detroit, Mich., U.S.A.). Then, 700 μ*l* of the culture was harvested by centrifugation at 4,000 × g for 10 min, and the cell pellet was washed once in 1 m*l* of TEN buffer (0.1 M Tris, 0.15 M NaCl, 0.1 M EDTA, pH 7.5). The cells were centrifuged again at 4,000 × g for 10 min and then resuspended in 300 μ*l* of EC lysis buffer (6 mM Tris HCl, 1M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, pH 7.5). Then, 10 μ*l* of lysostaphin solution (1 mg/m*l* in 20 mM sodium acetate, pH 4.5) (recombinant lysostaphin [Ambicin L]; Applied Microbiology, Inc., New York, N.Y., U.S.A.) was added to the cell suspension, which was immediately vortexed and placed into 300 μ*l* of warm (55°C) 2% low-gelling-temperature agarose (SeaPlaque; FMC Bioproducts, Rockland, Maine, U.S.A.) prepared with EC lysis buffer. The agarose cell suspension was immediately vortexed and transferred into a well of a 10 well sample plug mold (Bio-Rad, Hercules, Calif., U.S.A.). The agarose plug was allowed to solidify at room temperature for 10 min. After solidification, the agarose plug was transferred to 3 m*l* of EC lysis buffer and incubated for approximately 1 to 2 hr at 37°C, until the plug was cleared (complete lysis). Following lysis, the EC lysis buffer was carefully decanted and replaced with 3 m*l* of TE buffer (10 mM Tris HCl, 1mM EDTA, pH 7.6). The agarose plug in TE buffer was incubated at 55°C for 1 hr. The TE buffer was then decanted and replaced with 3 m*l* of fresh TE buffer. The agarose plugs were stored at 4°C until use.

*Sma**I** digestion*: On the basis of the results of previous work with other *Staphylococcus* species [11], we used *Sma*I as the restriction endonuclease for digestion of *S. hyicus* and *S. chromogenes*. Digestion of total DNA by *Sma*I was performed in a 1.5 m*l* microcentrifuge tube by using a 125 μ*l* assay and 20 U of enzyme (New England BioLabs,

Beverly, Mass., U.S.A.) according to the instructions of the manufacturer. The assay tube was vortexed and briefly centrifuged. A small section (2 by 4 by 1.5 mm) of the agarose plug prepared as described above was transferred to the assay tube and then incubated with gentle shaking (130 rpm) for 2 hr at 25°C. After digestion, the agarose plug was carefully placed into 1 well of a 15 well 1% agarose (SeaKem GTG; FMC) gel slab (12.5 by 14 by 0.9 cm) prepared with 0.5 × TBE buffer (Tris base, boric acid, EDTA, pH 8.0). Then the plug was sealed in the well with 0.8% low-gelling-temperature agarose prepared with 0.5 × TBE buffer.

PFGE procedures and analysis: *Sma*I digest fragments were separated in the agarose gel slab by using a CHEF-DR II system (Bio-Rad). Electrophoresis was performed in 0.5 × TBE buffer maintained at 14°C. To identify the DNA fragments, which had a large range of sizes, each of two separate gels was run under different conditions. For the largest DNA fragments (≥100 kb), the gel was exposed to a pulse time of 5 to 40 sec at 200 V for 22 hr. To identify the smallest DNA fragments (4 to 50 kb), a second gel was exposed to a pulse time of 1 to 12 sec at 150 V for 20 hr. After electrophoresis, the gels were stained with ethidium bromide (2 μg/m*l*) for 25 min, washed with distilled water for 2 hr, and photographed under UV light. The lambda ladder DNA concatemers (New England BioLabs) were used as a reference for determining the sizes of the largest *Sma*I digest fragments. The sizes of the smaller *Sma*I digest fragments were determined with the Low Range PFG Marker (New England BioLabs).

RESULTS

PFGE patterns of pig S. hyicus strains: Of the 88 pig strains, 40 strains were isolated from pigs affected with EE (except for one strain from septic polyarthritis) in 4 countries. Forty-eight strains were isolated from the skin, nares and tonsils of healthy pigs in 3 countries (Table 1). A total of 16 to 26 fragments were detected. They ranged in size from about <1 to 485 kb, and most of the detectable fragments were less than 291 kb. Thirty-nine distinctive PFGE patterns were identified in the 88 pig strains examined (Fig. 1). There were differences in PFGE patterns among pigs from the different countries. Of special interest were the isolates from outbreaks occurring on farms designated A to D in Japan (Fig. 1, lanes 1–7 and Table 1). Isolates from the outbreaks designated A and D yielded either two or four kinds of PFGE patterns, respectively. In outbreaks designated B and C, the isolates were of a single PFGE pattern. Strains from healthy pigs showed some deviation from the EE strains with respect to their PFGE patterns (Fig. 1, lanes 13–21, 23, 26-39). Five fragments of 31 kb, 23 kb, 10 kb, 4 kb and 2 kb were shared by both EE pig and healthy carrier isolates (Table 4).

PFGE patterns of chicken, cow and goat S. hyicus strains: The chicken, cow and goat strains had a PFGE pattern different from that of the pig strains, but shared the 23 kb fragment with pig strains. Furthermore, the PFGE patterns of the chicken, cow and goat strains were quite different from each other (Fig. 2 and Table 4). The 45 chicken isolates generated 18 to 24 fragments in the size range of

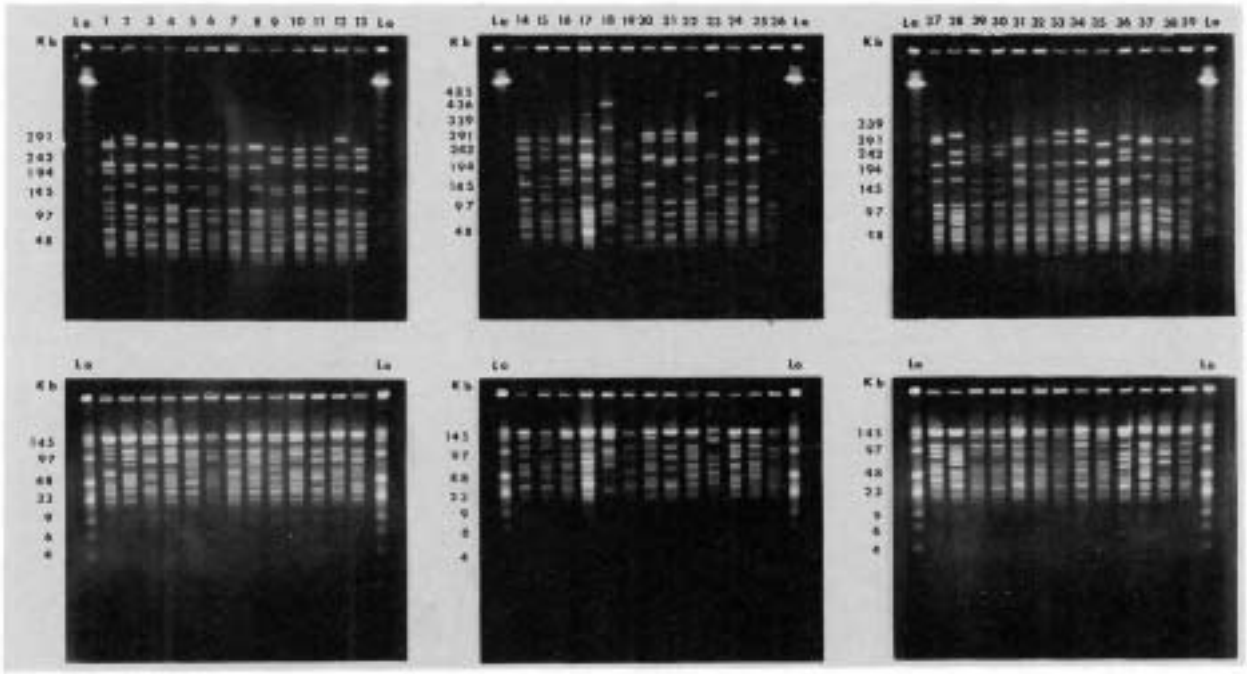

Fig. 1. PFGE of *Sma*I-digested, large (top) and small (bottom) genomic fragments of *S. hyicus* isolated from pigs. The number of isolates belonging to each pattern is listed in Table 1. Lanes 1–7, EE, Japan; lanes 8–12, EE, USA; lanes 22 and 24, EE, Czecho Republic; lane 25, EE, Type strain NCTC 10350, Denmark; lanes 13–18, normal skin, UK; lanes 19–21 and 23, normal skin, Belgium; lanes 26–32, nare, Japan; lanes 33–39, tonsil, Japan. La and Lo indicate the lambda ladder DNA concatemers and the Low Range PFG Marker, respectively, as size markers.

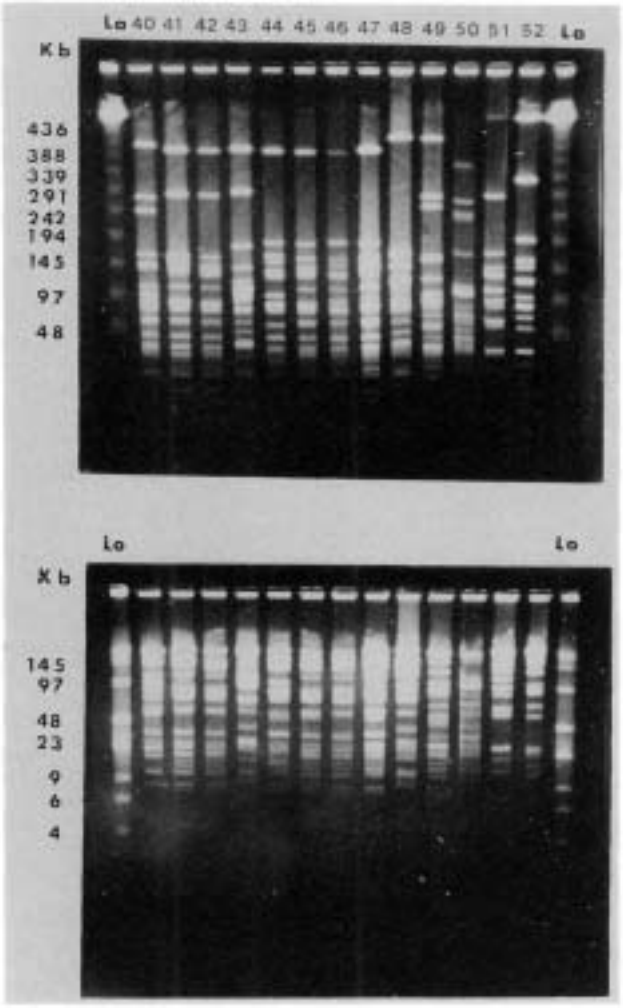

Fig. 2. PFGE of *Sma*I-digested, large (top) and small (bottom) genomic fragments of *S. hyicus* isolated from chickens, cows and goats. The number of isolates belonging to each pattern is listed in Table 2. Lanes 40–47, chicken nare or skin; lanes 48–50, cow udder or mastitic milk; lanes 51 and 52, goat mastitic milk. See Fig. 1 for key. In order to determine the molecular size of the uppermost band in lanes 51 and 52, the run was performed with a pulse time of 50 to 90 sec at 200 V for 24 hr. *Saccharomyces cerevisiae* genomes was used as size standard.

about <1 to 425 kb, and most of the detectable fragments were less than 185 kb. They also had a fragment of either 415 kb or 425 kb in size which was not present in the pig strains. The 3 cow isolates generated 17 to 19 fragments in the size range of about <1 to 475 kb, and had a fragment of either 370 kb or 475 kb in size which was not seen in the chicken strains. The 2 goat isolates generated 16 or 17 fragments in the size range of about <1 to 1,125 kb, and had a 1,125 kb fragment which was not seen in the chicken or cow strains.

Interestingly, all 28 isolates from the skin or nares of 28 different chickens (4-week-old) obtained from a single flock had the same pattern (Fig. 2, lane 47 and Table 2), suggesting that they may represent a single strain or clone.

PFGE patterns of S. chromogenes strains: The twenty-one isolates examined were obtained from the skin of healthy pigs, milk samples of cows with mastitis, and the aborted fetus of a cow (Table 3). These isolates generated 17 to 24 fragments in the size range of about <1 to 545 kb, and most of the detectable fragments were below 210 kb. Eight fragments of 210 kb, 115 kb, 86 kb, 68 kb, 51 kb, 26 kb, 18 kb and 8 kb were shared by all or almost all of the isolates examined (Fig. 3 and Table 4). The PFGE pattern of *S. chromogenes* strains was more highly conserved than that of *S. hyicus*.

Differentiation of S. hyicus and S. chromogenes by PFGE: *S. chromogenes* strains could be distinguished from *S. hyicus* strains by 14 fragments ranging in size from 305 kb to 545 kb. Only three fragments of 210 kb, 115 kb and 18 kb were shared by pig *S. hyicus* and *S. chromogenes* strains.

DISCUSSION

S. hyicus is the causative agent of porcine EE [36]. The organism occurs frequently on the skin and in the nares or tonsils of healthy pigs [7, 22, 34, 37]. The factors which enable it to survive on the skin and occasionally produce EE are still poorly understood. An epidermolytic toxin (exfoliative toxin) [1, 30, 42] has been implicated in the pathogenesis of disease due to *S. hyicus*. In the epidemiological studies (source, transmission, and spread of causative agent) of EE in pigs, there is a problem in discriminating pathogenic (virulent) *S. hyicus* strains from apparently non-pathogenic (avirulent) *S. hyicus* strains in clinical material. Hunter *et al.* [17] and Amtsberg *et al.* [2] reported that there are pathogenic and non-pathogenic porcine strains of *S. hyicus* and that these strains can be distinguished by an agglutination test using absorbed antisera. On the other hand, Devriese *et al.* [10] and Takeuchi *et al.* [37] reported that isolates from diseased and healthy pigs resembled each other in most of their biochemical properties. Takeuchi *et al.* [38] also examined the extracellular proteases of *S. hyicus* with a zymogram method and could not differentiate between isolates from diseased and healthy pigs. In the present study, we were interested in whether or not there was a difference in the PFGE patterns between the isolates of diseased pigs and those from healthy carriers. Isolates from the skin, nares or tonsils of healthy pigs showed some deviation from the EE strains with respect to their PFGE patterns. With regard to the isolates from pigs with EE, PFGE patterns were different according to the country. However, variation within a country was not adequately assessed. The outbreaks of EE occurring on four separate pig farms in Japan involved *S. hyicus* with different PFGE patterns, and two or more kinds of PFGE patterns were present in isolates from pigs with EE on a single farm. Kawano *et al.* [20] using phage typing of *S. hyicus* demonstrated that two or more kinds of phage patterns were present in isolates from pigs with EE on the same farm. These results suggest that *S. hyicus* strains from

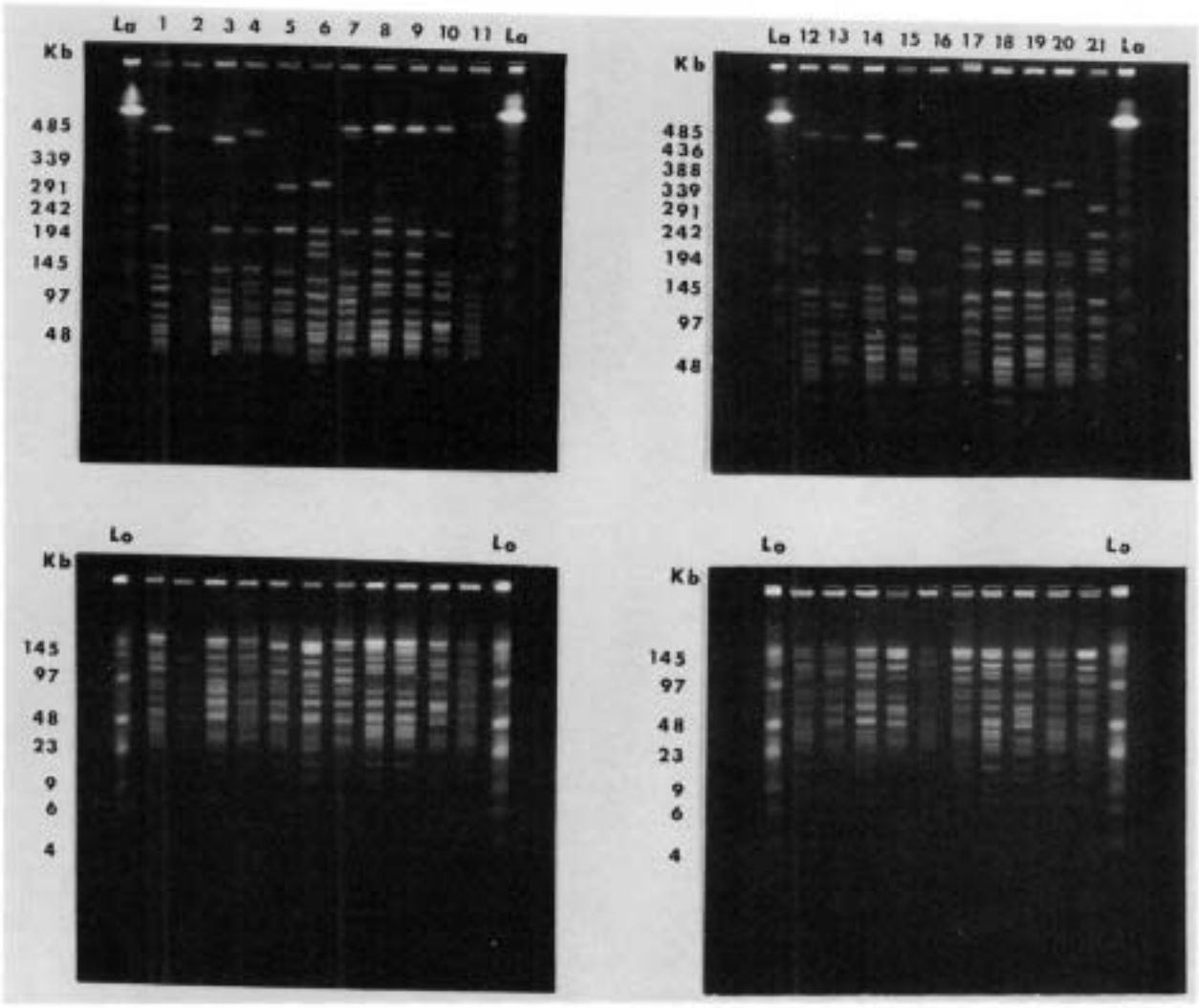

Fig. 3. PFGE of *Sma*I-digested, large (top) and small (bottom) genomic fragments of *S. chromogenes* isolated from pigs and cows. The number of isolates belonging to each pattern is listed in Table 3. Lanes 1–5, pig normal skin; lane 6, pig, Type strain CBCC 1462^T; lane 7, cow aborted fetus; lanes 8–21, cow mastitic milk. See Fig. 1 for key. To identify the uppermost band in lanes 7–12, a separate gel was prepared and run with a pulse time of 15 to 55 sec at 200 V for 22 hr.

porcine EE do not belong to a single genotype or subpopulation.

S. hyicus is a common inhabitant of the skin, nares and tonsils of cows [22, 33, 35, 37, 39], and it has also been frequently isolated from the skin or nares of chickens [22, 35, 37, 39]. One purpose of the present study was to determine whether PFGE analysis could be utilized for the differentiation of pig, cow and chicken strains. There are no remarkable differences in biological characteristics among strains of *S. hyicus* isolated from pigs, cows and chickens [37, 39], though compared to cow and chicken strains the frequency of fibrinolysin-positive strains is very high in pig strains [10]. Shimizu *et al.* [32] using phage typing of *S. hyicus* reported that all of the chicken strains and most of the bovine strains were susceptible to the phage CH11 (from a lysogenic strain of *S. hyicus* of chicken origin), while most of the pig strains were resistant to the phage. Takeuchi *et al.* [38] reported that the isolates from pigs could be distinguished from those isolated from chickens and cows by the caseinolytic and gelatinolytic zymograms. However, phage typing and zymotying could not discriminate between isolates from chickens and cows. In this study, strains of *S. hyicus* isolated from pigs showed some deviation from the chicken, cow or goat strains with respect to their PFGE patterns. Furthermore, the chicken, cow and goat strains had host-specific fragments of approximately 415 kb or 425 kb, 370 kb or 475 kb, and 1,125 kb, respectively. These fragments were not found in the pig strains. Interestingly, the PFGE could differentiate between chicken and cow strains that were previously indistinguishable by phage typing and zymotyping. The results suggest that PFGE analysis might be a useful marker for distinguishing ecovars within *S. hyicus*, though the number of strains from cows, and goats examined in this study was too small to draw definite conclusions.

Table 4. Fragments conserved among species

Fragment (s)[a] conserved among indicated percentage of strains												
S. hyicus									*S. chromogenes*			
EE[b] pig (n=40)[c]		Healthy pig (n=48)		Chicken (n=45)		Cow (n=3)	Goat (n=2)		Pig (n=6)		Cow (n=15)	
100%	90–98%	100%	88–92%	100%	91–98%	100%	100%		100%	83%	100%	93%
210	115	23[d]	31	415 or 425	163	370 or 475	1125		210[f]	145	115[f]	210[f]
63	103	4	18	23[d]	145	88[e]	145		115[f]	40	86[f]	145
31	42	2	10	13	128	23[d]	128		86[f]		68[f]	26[f]
23[d]	7	< 1		8	88[e]	< 1	88[e]		68[f]		51[f]	
10				5	55		23[d]		51[f]		35	
4				< 1	31		7		45		18[f]	
2					18		< 1		26[f]		8[f]	
< 1					10				18[f]		< 1	
									11			
									8[f]			
									< 1			

a) Fragments designated according to size in kilobases.
b) Exudative epidermitis.
c) No. of strains.
d) Fragment shared by pig, chicken, cow and goat *S. hyicus* strains.
e) Fragment shared by chicken, cow, and goat *S. hyicus* strains.
f) Fragment shared by pig and cow *S. chromogenes* strains.

Recently, coagulase-negative staphylococci (CNS) have been attracting interest as potential human and animal pathogens. Animal habitant species *S. chromogenes* and *S. hyicus* frequently colonize the bovine teat skin, teat canals, milk and interior of the mammary gland [8, 41]. *S. chromogenes* is the most frequently isolated CNS from cows with subclinical or clinical mastitis [4, 16, 19, 25] and IMI [40]. The presence of *S. chromogenes* in the milk of cows suffering from mastitis may suggest a pathogenic role for this species. Compared with *S. hyicus*, the ecology and epidemiology of *S. chromogenes* are not well understood, because of the lack of good discriminatory typing methods. In this study, we applied PFGE procedure to the strains of *S. chromogenes* isolated from cows with mastitis in a herd. The PFGE pattern of the strains was highly conserved, but they are different from each other in fragments of size ranging from about 242 kb to 545 kb (Fig. 3). The PFGE appears to be a useful molecular epidemiologic tool in the study of *S. chromogenes*.

The differentiation of *S. hyicus* and *S. chromogenes* species has been based on extensive phenotypic character analyses and DNA-DNA hybridization studies [10, 15]. In this study, the PFGE analysis of *S. hyicus* and *S. chromogenes* DNA showed distinct species *Sma*I digest fragment patterns. *S. chromogenes* strains could be distinguished from *S. hyicus* strains by PFGE analysis, especially fragments in the range of about 305 kb to 545 kb.

We conclude that genomic DNA fingerprinting by PFGE is a useful tool not only for species or strain identification but also for ecological or epidemiological studies of *S. hyicus* and *S. chromogenes.*

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