Detection of *Mycoplasma hyorhinis* in Porcine Eustachitis

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ABSTRACT. Pathologic study of the ear was performed on 179 young swine, ranging in age from 1 day to 18.6 weeks. Histologically, eustachitis was the most common and its inflammatory reaction appeared to precede otitis. Immunohistochemically, *Mycoplasma hyorhinis* (Mhr) antigens were detected on the luminal surface of the auditory epithelia in 19 of 179 cases (10.6%). All the cases, positive for Mhr antigens, were associated with an acute eustachitis. Ultrastructural examination of two piglets confirmed these immunohistochemical data. The present results indicate that Mhr may be a primary cause of acute eustachitis in young swine.—**KEY WORDS:** eustachitis, *Mycoplasma hyorhinis*, swine.

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Otitis media has been described in swine [12, 14] and other domestic animals [15], including cattle [7], lambs [8], dogs [9], rabbits [4], guinea pigs [2] and mice [5] as well as human beings [6]. We recently reported the prevalence of otitis media in Japanese swine herds [14]. The otitis media occurred from the early stages of life, and first appeared in the auditory tube. *Pasteurella multocida*, *Actinomyces pyogenes* and *Corynebacterium* group E were isolated from the middle ear of swine with otitis media [14], and most of these organisms were common inhabitants of the upper respiratory tracts of normal swine [13]. The auditory tube is lined with the ciliated epithelia similar to those of the upper respiratory tracts, and the mucociliary beat of the epithelial cells accounts for major defence mechanisms of the ear against the bacterial invasions [6]. Therefore, an inefficiency of this defence mechanism appears to be related to the pathogenesis of otitis media of swine. In this report, we indicate that *Mycoplasma hyorhinis* (Mhr) may be a primary agent to cause acute eustachitis in young swine.

Samples for this study were derived from 179 feeder swine ranged in age from 1 day to 18.6 weeks. They were slaughtered between February 1989 and November 1991 due to unfavorable prognosis with clinical signs of weakness, depression and growth retardation. Samples of the right ear were fixed in ethanol (155 cases), and left side were fixed in periodate-lysine paraformaldehyde (PLP) (133 cases). The lungs were fixed in ethanol or PLP. They were embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. Immunohistochemistry was carried out by the labeled streptavidin biotin (LSAB) method using a 1:10,000 dilution of rabbit polyclonal anti-Mhr BTS strain antibody (gift from Dr. K. Kodama of Kyoto-Biken Laboratories), a 1:40,000 dilution of M-61 strain antibody (gift from Dr. T. Yagihashi of Nippon Institute for Biological Science), a 1:10,000 dilution of the rabbit polyclonal anti-*Mycoplasma hyopneumoniae* (Mhp) J strain antibody (gift from Dr. K. Kodama), and a 1:40,000 dilution of MI-3 strain antibody (gift from Dr. T. Yagihashi). These antibodies were raised in rabbits inoculated with whole mycoplasma organisms and were confirmed to be species-specific. The secondary antibody was biotinylated goat anti-rabbit IgG, and the detection system was LSAB kit (DAKO Japan Co.). The chromogen was 3, 3'-diaminobenzidine (DAB). For negative

controls, the rabbit anti-Mhp or anti-Mhr antibodies were either omitted or substituted with normal rabbit serum. Two of 19 auditory tubes which were positively stained for Mhr by the immunohistochemical examination were subjected to electron microscopical examination. After being post-fixed in 1% osmium tetroxide, tissues were dehydrated in graded ethanols and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

Histologically, eustachitis was the most common and its inflammatory reaction appeared to precede otitis (Table 1). Acute eustachitis was characterized by an increased number of goblet cells and mild neutrophilic infiltration, whereas chronic inflammation of the tube consisted of an extensive infiltration of inflammatory cells including lymphocytes, bacterial multiplication and mucosal thickening.

Immunohistochemically, Mhr (BTS and/or M-61 strains) antigens were demonstrated on the luminal surface of the auditory epithelia in 19 of 179 cases (10.6%) (Fig. 1). Mhp antigens were not detected in the auditory tube. All the cases, positive for Mhr, showed an acute eustachitis. The incidences of Mhr antigens were almost equal between the right (ethanol fixation) and left ears (PLP fixation). In the lungs, Mhp (J and/or MI-3 strains) and Mhr (BTS and/or M-61 strains) antigens were detected in the bronchial epithelia of many cases (Mhp, 44/179; Mhr, 72/179) (Table 2). Of 19 cases, in which Mhr antigens were demonstrated in the auditory tube, 11 had Mhr antigens only in the auditory tube and 8 had in both auditory tube and lungs.

Table 1. Distribution of ear lesions in age groups

Age	Number		Otitis		
	of cases	Eustachitis	media ^{a)}	interna	externa
<1 W ^{b)}	13	7	3	1	1
1 to 4 W	58	44	16	12	9
1 to 2 M	63	56	28	11	13
2 to 3 M	35	32	16	5	5
3 to 4 M	2	1	1	0	0
> 4 M	1	0	1	0	1

a) Inflammation of the tympanic cavity.

b) W=week, M=month.

Ultrastructurally, mycoplasma organisms measuring 0.42 to 0.73 μm in diameter were present adhering to the cilia and the microvilli of the auditory epithelia in the two cases examined. The surface of the organisms was labeled with DAB (Fig. 2).

The primary cause of eustachitis has been reported to be *Mycoplasma pulmonis* in mice [5], and type A influenza virus in both guinea pigs [2] and human beings [6]. In eustachitis of swine, however, there has been no report on the primary cause. The auditory tube connects the middle ear with the nasopharynx and is lined with the ciliated epithelium similar to that in the respiratory tract [6]. Morphologically, an acute eustachitis in the present cases showed an increased number of goblet cells and neutrophilic infiltration. These changes much resembled those

in the early stage of respiratory lesions provoked by experimental inoculation of *Mycoplasma* spp. [1, 3, 13]. Therefore, we postulated that *Mycoplasma* spp. may be a primary pathogen of otitis media in swine.

Table 2. The number of cases with *Mycoplasma hyorhinis* antigen and inflammation in the lungs and auditory tube

	Lung	Auditory tube
Mhr	72	19
Inflammation	131	140

A total of 179 cases were examined.

Mhr: *Mycoplasma hyorhinis* antigen.

Fig. 1. *Mycoplasma hyorhinis* antigen (arrowheads) is shown on the mucosal surface of the auditory tube. Immunohistochemical staining using biotinylated antibody and labeled streptavidin. Light green counterstain. $\times 150$.

Fig. 2. Transmission electron micrograph showing mycoplasma organisms adhering to the cilia and microvilli of the auditory epithelium. The surfaces of the organisms are labeled with diaminobenzidine (arrowheads). Bar = 1.0 μm $\times 18,800$.

Immunohistochemically, we demonstrated Mhr antigens on the cilia of the auditory epithelia in 19 of 179 cases (10.6%). All the cases, positive for Mhr antigen, were associated with an acute eustachitis. Furthermore, ultrastructural examination of two piglets confirmed those immunohistochemical data. Surveys of nasal secretions of weanling piglets in Iowa indicated that 30–40% of them were positive for Mhr [13]. These results suggest that Mhr first evolves an acute eustachitis by ciliostasis and ciliary effacement [10, 11]. The eustachitis injures active mucociliary flow of the auditory tube [6] and contributes to an invasion of bacteria inhabiting at the upper respiratory tract into the tympanic cavity, thus induces otitis media.

Mycoplasma spp. isolated so far from pigs are *M. hyosynoviae*, *M. flocculare*, *M. arginini*, *M. hyopharyngis*, *M. bovis genitalium*, *M. buccale*, *M. gallinarum*, *M. iners*, *M. mycoides*, *M. salivarium* [13], Mhr, and Mhp. In the present study, immunohistochemical examination was performed only for Mhr and Mhp. Therefore, a possible involvement of the other 10 mycoplasmas in the development of otitis media could not be excluded. Furthermore, we used paraffin-embedded sections for immunohistochemistry, and this might have accounted for the low incidence of Mhr antigen at the auditory tube. Immunohistochemistry using frozen sections, isolation of Mhr from the auditory tube, and experimental reproduction of otitis media by inoculation of piglets with Mhr are further needed for the substantiation of the hypothesis that otitis media is a nosologic entity caused primarily by Mhr infection in the auditory tube.

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