JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2009, p. 951–958 Vol. 47, No. 4
0095-1137/09/$08.00+0 doi:10.1128/JCM.02029-08

Isolation, Antimicrobial Resistance, and Virulence Genes of *Pasteurella multocida* Strains from Swine in China▽

Xibiao Tang,[1] Zhanqin Zhao,[1,2] Junyong Hu,[1] Bin Wu,[1]* Xuwang Cai,[1] Qigai He,[1] and Huanchun Chen[1]

State Key Laboratory of Agricultural Microbiology, Laboratory of Animal Infectious Diseases, College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan, China,[1] and Laboratory of Animal Infectious Diseases and Microbiology, College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China[2]

Received 19 October 2008/Returned for modification 5 January 2009/Accepted 12 January 2009

A total of 233 isolates of *Pasteurella multocida* were obtained from 2,912 cases of clinical respiratory disease in pigs in China, giving an isolation rate of 8.0%. Serogroup A *P. multocida* isolates were isolated from 92 cases (39.5%), and serogroup D isolates were isolated from 128 cases (54.9%); 12 isolates (5.2%) were untypeable. *P. multocida* was the fourth most frequent pathogenic bacterium recovered from the respiratory tract, after *Streptococcus suis*, *Haemophilus parasuis*, and *Escherichia coli*. All isolates were characterized for their susceptibilities to 20 antibiotics and the presence of 19 genes for virulence factors (VFs). The frequency of antimicrobial resistance among *P. multocida* isolates from swine in China was higher than that reported among *P. multocida* isolates from swine in from other countries, and 93.1% of the isolates showed multiple-drug resistance. There was a progressive increase in the rate of multiresistance to more than seven antibiotics, from 16.2% in 2003 to 62.8% in 2007. The resistance profiles suggested that cephalosporins, florfenicol, and fluoroquinolones were the drugs most likely to be active against *P. multocida*. Use of PCR showed that colonization factors (*ptfA*, *fimA*, and *hsf-2*), iron acquisition factors, sialidases (*nanH*), and outer membrane proteins occurred in most porcine strains. The VFs *pfhA*, *tadD*, *toxA*, and *pmHAS* were each present in <50% of strains. The various VFs exhibited distinctive associations with serogroups: concentrated in serogroup A, concentrated in serogroup D, or occurring jointly in serogroups A and D. These findings provide novel insights into the epidemiological characteristics of porcine *P. multocida* isolates and suggest that the potential threat of such multiresistant bacteria in food-producing animals should not be neglected.

Pasteurella multocida is an important cause of pneumonia and atrophic rhinitis in pigs and is responsible for significant losses on large farms worldwide (11, 16, 30). Strains of *P. multocida* are grouped into five capsular serogroups (serogroups A, B, D, E, and F) and are further classified into 16 somatic serotypes (serotypes 1 to 16), which are primarily based on lipopolysaccharide antigens (22, 31, 34). To date, only serogroups A, B, and D have been recovered from swine (11, 33). Together with *Bordetella bronchiseptica*, toxigenic strains of *P. multocida* serogroups A and D can cause atrophic rhinitis (4, 11). Both toxigenic and nontoxigenic strains of serogroups A and D can cause pneumonic pasteurellosis (15, 30), whereas isolates of serogroup B cause hemorrhagic septicemia and are less frequently associated with pigs (16, 33). Since pasteurellosis was identified as one of the most important zoonoses in 1959 (3), *P. multocida* has been reported to be the cause of a series of outbreaks, especially in Australia, Vietnam, Canada, and the United States (4, 15, 30, 33). In addition, this organism is typically associated with subacute or chronic pleuritis. Both vertical and horizontal transmissions occur, with the most common route of transmission being nose-to-nose contact. Interspecies transmission of *P. multocida* is also thought to occur under certain conditions (12).

Although antimicrobial therapy is a widely available tool for the prevention and control of clinical infections (5, 25, 26), antibiotic resistance in pathogenic bacteria from food-producing animals and environmental sources is recognized as a global problem for public health (6, 37). Over the past decade, the high degree of resistance to common antibiotics and the worldwide emergence of multidrug resistant phenotypes have become of increasing concern (8, 36, 37). Previous studies have reported that the imprudent use of antimicrobials bears a high risk for the selection of resistant bacteria and promotes the spread of resistance genes located on plasmids, integrons, and transposons (23, 25). This has resulted in a reduction in the efficacies of the antimicrobial agents that are currently available for the treatment of infections in food-producing animals (25). Indeed, the antimicrobial classes that are commonly used for the treatment of infections in humans may be misused in animals either for therapy or for the prevention of disease, which has a large potential impact on public health (8, 36).

The pathogenicity of *P. multocida* is associated with various virulence factors (VFs) (18, 20, 24). The key factors that have been identified to date include the capsule and lipopolysaccharide (10, 21). The recognized VFs of this organism also include diverse adhesins (e.g., filamentous hemagglutinin, type 4 fimbriae, and Flp pilin), toxins (dermonecrotic toxin), siderophores (e.g., iron acquisition proteins), sialidases (which may enhance bacterial virulence by unmasking key host receptors and/or reducing the effectiveness of host defenses), and outer

* Corresponding author. Mailing address: State Key Laboratory of Agricultural Microbiology, Laboratory of Animal Infectious Diseases, College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Shizishan Street No. 1, Wuhan, Hubei 430070, China. Phone: 86-27-87286974. Fax: 86-27-87282608. E-mail: wub@mail.hzau.edu.cn.

▽ Published ahead of print on 21 January 2009.

membrane proteins (e.g., OmpA, OmpH, Oma87, and PlpB) (2, 17, 18, 23, 35). These VFs facilitate the colonization and invasion of the host, the avoidance or disruption of host defense mechanisms, injury to host tissues, and/or stimulation of a noxious host inflammatory response (20, 23). Thus, the informed selection of the VFs to be targeted for the prevention of *P. multocida* infections requires knowledge of which VFs are prevalent in specific clinical syndromes, as may be revealed by epidemiological studies. Furthermore, it was reported that there is an obvious correlation between some VFs and capsular serogroups, with the filamentous hemagglutinin gene *pfhA* being associated with serogroups A, B, E, and F; the iron acquisition gene *tbpA* being associated with serogroups A and B; and the dermonecrotoxin *toxA* gene being associated with serogroup D (17). Because pathogenic behavior is predicted both by the VF repertoire and by the serogroup (20), the clonal associations of VFs must be evaluated.

The current investigation is the first large study in China of the prevalence of *P. multocida* in clinical samples collected from 16 provinces between 2003 and 2007. To obtain more information about the epidemiology of porcine *P. multocida* infection and to characterize clinical isolates, we investigated a total of 233 isolates of *P. multocida* that were associated with clinical disease in swine for the distributions of the capsular serogroups, the phenotypic antimicrobial resistance profiles, and the presence of 19 virulence genes.

MATERIALS AND METHODS

Clinical specimens, culture, and *P. multocida* screening. Over more than 4 years (from June 2003 to September 2007), 2,912 clinical samples from pigs with clinical respiratory infections which were collected by the Clinical Microbiology Laboratory of the College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, for routine pathogen identification were screened for *P. multocida*. The samples were plated on tryptic soy agar (Difco, Detroit, MI) containing 10 μg/ml NAD (Sigma, St. Louis, MO) and 5% bovine serum, MacConkey agar, and blood agar (5% fresh sheep blood). All plates were incubated at 37°C in air for a minimum of 48 h. After this isolation stage, the isolates were purified and cultured by standard methods for the identification of strains of bacteria, including *Haemophilus parasuis*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Escherichia coli*, and *Staphylococcus aureus* (7, 28). Presumptive isolates of *P. multocida* were confirmed by a PCR assay with primers specific for the amplification of the KMT1 gene (34).

All isolates of *P. multocida* were subsequently characterized biochemically by using a MicroStation system (Biolog Inc.) and their capsules were serotyped. For samples in which all isolates were identical with respect to their capsular serotype, only one colony was selected. When one sample yielded colonies with different capsular serotypes, one colony of each serotype was selected for further characterization. All isolates were freeze-dried and kept at −80°C.

Capsule typing. The capsular types of the isolates were determined by multiplex capsule PCR typing with the capsule-specific primer pairs (primers specific for *capA*, *capB*, *capD*, *capE*, and *capF*) described by Townsend et al. (34). All oligonucleotides were synthesized with a DNA synthesizer (with finishing done by Sangon Biological Engineering Technology Inc., Shanghai, China). The primer sequences used in the multiplex capsule PCR typing assay for *P. multocida* are listed in Table 1.

Antimicrobial susceptibility testing. All determinations of antimicrobial MICs for the *P. multocida* strains were performed by the broth microdilution methods recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) (29). The antimicrobial agents tested included amoxicillin, cefazolin, ceftiofur, spectinomycin, kanamycin, neomycin, gentamicin, amikacin, chloramphenicol, florfenicol, erythromycin, tilmicosin, lincomycin, clindamycin, chlortetracycline, tetracycline, ciprofloxacin, sulfamethazine (sulfadimidine), trimethoprim-sulfamethoxazole, and polymyxin B. They were supplied by the National Institute for the Control of Pharmaceutical and Biological Products in Beijing, China. Determination of the MICs and their evaluation were done by use of the interpretive criteria of the CLSI (29). The MIC was defined as the lowest concentration that prevented visible growth. Ranges of susceptibility, along with the MIC_{50}s and the MIC_{90}s of the isolates, were recorded. The breakpoint used for ciprofloxacin was that previously used by Aarestrup et al. (1) for Danish strains of *H. parasuis*. For the other antimicrobials, the breakpoint values were taken from the CLSI guidelines (29). Reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control organisms in all antimicrobial susceptibility tests. Isolates that were nonsusceptible to at least three different antibiotic classes were classified as multidrug resistant.

Detection of virulence genes by PCR. Nineteen pairs of oligonucleotide primers to be used for the detection of VFs were designed by using the software program Prime (version 5.0; Premier Biosoft International, Palo Alto, CA) and were synthesized commercially (with finishing done by Sangon Biological Engineering Technology Inc.). The base sequences and the predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. The bacterial lysates used as templates for the PCR were prepared as follows. A loopful of bacteria from a fresh overnight culture on a tryptic soy agar plate was resuspended homogeneously in 200 μl of sterile water, and the mixture was boiled at 100°C for 5 min to release the DNA and centrifuged. A 4-μl volume of the supernatant was used as a template for each 25-μl PCR mixture. The appropriate positive and negative controls for amplification were generated from clinical isolates of *P. multocida* by PCR carried out with a GeneAmp PCR system 9700 instrument (Applied Biosystems, Foster City, CA) and were confirmed by sequencing. The amplified products were analyzed in 0.8% agarose gels by electrophoresis, and the results were recorded with a gel documentation system. All tests were repeated three times in parallel with the relevant positive and negative controls. Discrepant results for each VF were investigated further, and samples were sequenced for gene verification.

Clinical data and statistical analysis. Clinical data were collected by retrospective analysis of the protocols. Statistical testing was performed with SPSS software (version 12.0; SPSS Inc., Chicago, IL). Comparisons of proportions were made by two-tailed Fisher's exact test or the χ^2 test. Comparisons of the prevalence of different traits within the same population were made by McNemar's test. Aggregate VF scores were compared by the Mann-Whitney U test. *P* values of <0.05 were considered statistically significant.

RESULTS

Prevalence of *P. multocida* in porcine clinical samples. We analyzed clinical specimens from diseased pigs with pneumonia or atrophic rhinitis from 16 provinces in China for the presence of *P. multocida*. Strains of *P. multocida* were detected in 233 (8.0%) of the 2,912 cases investigated, and the isolation rate at different time points (years) ranged from 6.4 to 10.2% (Table 2). Isolates of *P. multocida* of capsular type A were obtained from 92 cases (39.5%), capsular type D strains were isolated from 128 cases (54.9%), and 1 isolate was identified as capsular type B, whereas 12 isolates were untypeable. Capsular types E and F were not detected in the population sampled. All these isolates gave positive results by the PCR assays with primers specific for *P. multocida* (34).

After *Streptococcus suis*, *Haemophilus parasuis*, and *Escherichia coli*, *P. multocida* was the bacterial pathogen that was the fourth most frequently isolated from clinical porcine specimens in this study (Table 2). *Streptococcus suis* was isolated from 24.2% of the study samples, *Haemophilus parasuis* was isolated from 17.1%, *Escherichia coli* was isolated from 12.6%, *Bordetella bronchiseptica* was isolated from 7.2%, *Staphylococcus aureus* was isolated from 3.2%, and *Actinobacillus pleuropneumoniae* was isolated from 0.9%. The simultaneous detection of *P. multocida* and other species of bacteria pathogenic for pigs occurred in 125 specimens. *Haemophilus parasuis* (53.6%), *Streptococcus suis* (48.8%), *Escherichia coli* (27.2%) and *Bordetella bronchiseptica* (18.4%) were the agents that were the most frequently found in coinfections with *P. multocida.* Eleven toxigenic strains of *P. multocida* of serogroup D

TABLE 1. Primers used for the detection of virulence-associated genes in strains of *P. multocida*

Gene function and gene	Description	Direction[a]	Primer sequence (5′–3′)	Amplicon size (bp)
Adhesins				
ptfA	Type 4 fimbriae	s	TGTGGAATTCAGCATTTTAGTGTGTC	488
		a	TCATGAATTCTTATGCGCAAAATCCT GCTGG	
fimA	Fimbriae (from Pm70)	s	CCATCGGATCTAAACGACCTA	866
		a	AGTATTAGTTCCTGCGGGTG	
hsf-1	Autotransporter adhesion (from Pm70)	s	TTGAGTCGGCTGTAGAGTTCG	654
		a	ACTCTTTAGCAGTGGGGACAACCTC	
hsf-2	Autotransporter adhesion (from Pm70)	s	ACCGCAACCATGCTCTTAC	433
		a	TGACTGACATCGGCGGTAC	
pfhA	Filamentous hemagglutinin	s	TTCAGAGGGATCAATCTTCG	286
		a	AACTCCAGT TGGTTTGTCG	
tadD	Putative nonspecific tight adherence protein D	s	TCTACCCATTCTCAGCAAGGC	416
		a	ATCATTTCGGGCATTCACC	
Toxins				
toxA	Dermonecrotic toxin	s	CTTAGATGAGCGACAAGG	864
		a	GAATGCCACACCTCTATAG	
Iron acquisition				
exbB	Accessory protein Ton-dependent transport of iron compounds	s	TTGGCTTGTGATTGAACGC	283
		a	TGCAGGAATGGCGACTAA A	
exbD	Accessory protein Ton-dependent transport of iron compound	s	CGTTCTGATTACAGCCTCTT	247
		a	AACGAAATCTTGGAAACTGG	
tonB	Iron transporters, transport ferric-siderophore complexes	s	CGACGGTGAAACCTGAGCCA	261
		a	CCGAGCGATAAGCATTGACT	
hgbA	A hemoglobin-binding protein	s	TCAACGGCAGATAATCAGGG	267
		a	GCGGGAATGCTGAAGATAAG	
fur	Ferric uptake regulation protein	s	GTTTACCGTGTATTAGACCA	244
		a	CATTACTACATTTGCCATAC	
Sialidases				
nanB	Outer membrane-associated proteins, an autotransporter protein	s	CATTGCACCTAACACCTCT	555
		a	GGACACTGATTGCCCTGAA	
nanH	Outer membrane-associated proteins, small sialidases	s	GTGGGAACGGGAATTGTGA	287
		a	ACATGCCAAGTTTGCCCTA	
Hyaluronidase				
pmHAS	Hyaluronan synthase	s	TCAATGTTTGCGATAGTCCGTTAG	430
		a	TGGCGAATGATCGGTGATAGA	
Protectins				
ompA	Outer membrane protein A	s	CGCATAGCACTCAAGTTTCTCC	201
		a	CATAAACAGATTGACCGAAACG	
ompH	Outer membrane protein H	s	CGCGTATGAAGGTTTAGGT	438
		a	TTTAGATTGTGCGTAGTCAAC	
oma87	Outer membrane protein 87	s	GGCAGCGAGCAACAGATAACG	838
		a	TGTTCGTCAAATGTCGGGTGA	
plpB	Lipoprotein B	s	TTTGGTGGTGCGTATGTCTTCT	282
		a	AGTCACTTTAGATTGTGCGTAG	
Capsule serotypes				
KMT1	Identification of all *P. multocida* isolates	s	ATCCGCTATTTACCCAGTGG	460
		a	GCTGTAAACGAACTCGCCAC	
hyaD-hyaC	Serogroup A *cap* gene	s	GATGCCAAAATCGCAGTCAG	1048
		a	TGTTGCCATCATTGTCAGTG	
bcbD	Serogroup B *cap* gene	s	CATTTATCCAAGCTCCACC	758
		a	GCCCGAGAGTTTCAATCC	
dcbF	Serogroup D *cap* gene	s	TTACAAAAGAAAGACTAGGAGCCC	647
		a	CATCTACCCACTCAACCATATCAG	
ecbJ	Serogroup E *cap* gene	s	TCCGCAGAAAATTATTGACTC	512
		a	GCTTGCTGCTTGATTTTGTC	
fcbD	Serogroup F *cap* gene	s	AATCGGAGAACGCAGAAATCAG	852
		a	TTCCGCCGTCAATTACTCTG	

[a] s, sense; a, antisense.

TABLE 2. Isolation of bacterial species and the prevalence of *P. multocida* in clinical samples from China from June 2003 to September 2007

Yr	No. of samples analyzed	No. of samples from which the following bacterial species were isolated[a]:							
		P. multocida	*S. suis*	*H. parasuis*	*E. coli*	*B. bronchiseptica*	*S. aureus*	*A. pleuropneumoniae*	Others[b]	None[c]
2007	594	49 (33)	167 (15)	132 (15)	87 (11)	42 (5)	26 (2)	2	112 (9)	61
2006	726	74 (46)	214 (22)	156 (23)	95 (11)	69 (5)	31 (1)	2	137 (6)	86
2005	493	35 (17)	126 (9)	91 (13)	68 (3)	35 (3)	17	5 (3)	83 (7)	55
2004	502	32 (14)	97 (7)	64 (8)	55 (5)	25 (4)	9	11 (3)	59 (3)	48
2003	597	43 (15)	101 (8)	55 (8)	62 (4)	39 (6)	10 (1)	6 (3)	64 (3)	52
Total no. (%) of samples from which bacterial species were isolated	2,912	233 (8.0)	705 (24.2)	498 (17.1)	367 (12.6)	210 (7.2)	93 (3.2)	26 (0.9)	455 (15.6)	302 (10.3)
Total no. (%) of samples containing other pathogenic bacteria as well as *P. multocida*		125	61 (48.8)	67 (53.6)	34 (27.2)	23 (18.4)	4 (3.2)	9 (7.2)	28 (22.4)	

[a] Values in parentheses in the rows for the years 2003 to 2007 indicate the number of samples containing other pathogenic bacteria among 125 samples coinfected with *P. multocida*.
[b] Others include, for example, *Salmonella enterica*, *P. aeruginosa*, *Staphylococcus epidermidis*, *Actinobaculum suis*, *Streptococcus equinus*, and *Erysipelothrix rhusiopathiae*.
[c] None, no bacteria were isolated from the clinical samples.

were isolated from 37 samples of nasal swabs and lungs lesions from pigs with typical clinical signs of atrophic rhinitis. Six strains of *Bordetella bronchiseptica* and five strains of *Pseudomonas aeruginosa* were also cultured from the same samples.

All infections with *P. multocida* detected in this study were from territorial outbreaks on pig farms, and the sources of infection were not identified in most cases. Strains of *P. multocida* were isolated throughout the year without seasonal variation, and 62.2% of the organisms were detected from growing pigs 60 to 110 days old (145 cases). Eleven toxigenic strains of *P. multocida* were collected from growing pigs 80 to 100 days old from June to August.

Antimicrobial susceptibility. Two hundred thirty-three isolates of *P. multocida* recovered from diseased swine were tested for resistance to 20 antibiotics (Table 3). The most prevalent phenotypes detected were resistance to lincomycin (96.6%), sulfamethazine (85.4%), amoxicillin (80.3%), clindamycin (80.3%), trimethoprim-sulfamethoxazole (74.2%), chlortetracycline (65.2%), and tetracycline (58.0%), followed by tilmicosin (28.3%), amikacin (14.2%), gentamicin (13.7%), kanamycin (12.8%), and spectinomycin (12.0%). Less than 10% of the isolates were resistant to erythromycin or chloramphenicol (6.0 and 2.6%, respectively). No resistance to cefazolin, ceftiofur, florfenicol, or ciprofloxacin could be detected. The MIC_{90}s of neomycin and polymyxin B for the *P. multocida* isolates tested were 32 μg/ml and 4 μg/ml, respectively. The proportion of *P. multocida* isolates categorized as resistant could not be evaluated in this study because the breakpoints of neomycin and polymyxin B for veterinary use have not been determined according to the CLSI criteria (29). In addition, it was observed that 98.6% of the isolates were resistant to at least one antibiotic and 93.1% were multiresistant (resistant to from 3 to 10 antibiotics). Multiresistance was predominant in isolates of serogroup D, including toxigenic *P. multocida* strains. Resistance to amoxicillin, chlortetracycline and tetracycline, lincomycin and clindamycin, and sulfamethazine and trimethoprim-sulfamethoxazole was the common feature of these multiresistant isolates. The percentage of isolates with resistant to at least three antimicrobials was equally high in all years from 2003 to 2007 (Fig. 1). Isolates resistant to more than five antimicrobials became more frequent over time. The prevalence increased from 47.8% in 2003, 54.1% in 2004, and 57.6% in 2005 to 81.6% in 2006 and 97.1% in 2007. It is important to note that the proportion of isolates resistant to more than seven antimicrobials increased approximately fourfold between 2003 and 2007, from 16.2% to 62.8% ($P < 0.05$).

Distribution of virulence genes. Among the 233 porcine *P. multocida* isolates, the 19 virulence gene regions ranged in prevalence from 4.7% (*toxA*) to 100% (*ompA*). Multiple adhesins (including *ptfA*, *fimA*, and *hsf-2*), all iron acquisition factors (*exbB*, *exbD*, *tonB*, *hgbA*, and *fur*), *nanH*, and various outer membrane proteins (*ompA*, *ompH*, *oma87*, and *plpB*) were each found to occur in over 90% of the strains (Table 4). This shows that these virulence genes are highly prevalent in porcine isolates of *P. multocida*. Of the adhesin-encoding genes studied, *hsf-2* (99.1%) was more prevalent than *hsf-1* (67.0%; $P < 0.001$, McNemar's test), and *tadD* was more prevalent than *pfhA* ($P < 0.001$, McNemar's test). However, there was no statistically significant difference in the prevalence of *hsf-1* and *tadD* (67.0% and 43.3%, respectively; $P > 0.05$,

TABLE 3. MICs for 20 antimicrobial agents against 233 strains of *P. multocida*

Antimicrobial[a]	No. of isolates with MIC of (μg/ml):																Breakpoint MIC (μg/ml)[b]	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistance[c]
	≤0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	≥1,024				
AMX						4	1	2	1	38	49	62	54	12	7	3	32	64	128	80.3
FAM	1	1	8	101	96	18	6	1	1								32	0.5	0.5	0
XLN	165	58	6	1	3												8	≤0.03	0.06	0
SPT									14	36	71	84	15	7	4	2	128	32	128	12.0
KAN						17	34	66	58	21	7	18	10	2			64	4	64	12.8
NEO						14	18	62	48	45	26	9	11				ND[c]	8	32	ND
GEN				2	11	30	62	56	40	23	7	2					16	4	16	13.7
AMK						2	2	23	76	49	48	17	14	2			64	16	64	14.2
CHL			13	99	85	12	9	3	2	4	5	1					32	0.5	2	2.6
FFC		8	48	107	54	9	3	4									8	0.25	0.5	0
ERY			16	39	18	36	54	56	12	1		1					8	2	4	6.0
TYL			2	19	17	10	33	36	28	22	24	17	12	9	4		32	4	128	28.3
LIN						1	7	22	102	71	25	5					4	8	32	96.6
CLI				1	1	12	32	61	61	42	18	5					4	4	16	80.3
CTET						5	14	28	34	36	34	33	25	20	2	2	16	16	128	65.2
TET					1	13	25	41	18	22	36	32	27	8	10		16	16	128	58.0
CIP	91	84	31	22	3	1	1										4	0.06	0.25	0
SDM											2	9	12	11	67	132	512	≥1,024	≥1,024	85.4
SXT									1	2	5	7	24	21	43	130	512	≥1,024	≥1,024	74.2
PB	2	5	12	26	39	51	37	39	16	3	3						ND	1	4	ND

[a] AMX, amoxicillin; FAM, cefazolin; XLN, ceftiofur; SPT, spectinomycin; KAN, kanamycin; NEO, neomycin; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; FFC, florfenicol; ERY, erythromycin; TYL, tilmicosin; LIN, lincomycin; CLI, clindamycin; CTET, chlortetracycline; TET, tetracycline; CIP, ciprofloxacin; SDM, sulfamethazine; SXT, trimethoprim-sulfamethoxazole; PB, polymyxin B.

[b] The values, with the exception of those for ciprofloxacin, are based on CLSI standards.

[c] ND, not determined.

McNemar's test). Of the sialidase-encoding genes studied, *nanH* (97.0%) was more prevalent than *nanB* (81.5%; $P < 0.001$, McNemar's test). Of note was the substantial prevalence of the gene for hyaluronan synthase (*pmHAS*) and the low prevalence of the virulence genes *toxA* (4.7%) and *pfhA* (15%). The *tbpA* determinant was not detected in any of the 233 clinical strains studied.

The distribution of the virulence-associated genes among capsular serogroups, which was compared by Fisher's exact test or the χ^2 test, is presented in Table 4. As anticipated, the great majority of VFs, including *ptfA*, *fimA*, *hsf-2*, *exbB*, *exbD*, *tonB*, *nanH*, *ompA*, *ompH*, *oma87*, and *plpB*, were equally distributed in each capsular serogroup. However, when each serogroup was compared with all other capsular serogroups combined, *tadD* was significantly associated with serogroup A, *hsf-1* and *nanB* were significantly associated with serogroup D, and *hgbA* and *fur* were significantly associated with serogroups A and D. The *pfhA* and *pmHAS* genes were less common in isolates of serogroup D than in isolates of the other serogroups. It was noted that the *toxA* gene, which is involved in the pathogenesis of progressive atrophic rhinitis in pigs, was found in only 11 strains, and it was strictly restricted to strains belonging to capsular serogroup D. The different capsular serogroups exhibited disparate median aggregate VF scores: serogroup A, 15.6 (range, 12 to 18); serogroup D, 15.0 (range, 9 to 17); serogroup B, 14.0 (only one strain); and the nonaligned strains (nonserogroup strains), 14.1 (range, 11 to 16). These results did not differ significantly (for all comparisons, $P > 0.05$, Mann-Whitney U test).

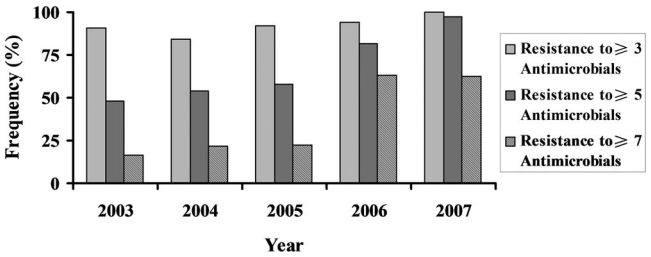

FIG. 1. Distribution by year of *P. multocida* isolates that showed phenotypes of multidrug resistance to the antimicrobial agents tested. All determinations of antimicrobial MICs with the *P. multocida* isolates were performed by broth microdilution methods recommended by the CLSI (29). The breakpoint used for ciprofloxacin was those previously used by Aarestrup et al. (1) for Danish strains of *H. parasuis*. For the other antimicrobials tested, breakpoint values were taken from the CLSI guidelines (29). Isolates that were nonsusceptible to at least three different antibiotic classes were classified as multidrug resistant. The percentage of isolates resistant to at least three antimicrobials was equally high between 2003 and 2007. The proportion of isolates resistant to more than five antimicrobials became more frequent, with a progressive increase from 47.8% in 2003 to 97.1% in 2007. The proportion of isolates resistant to more than seven antimicrobials increased approximately fourfold between the years 2003 and 2007, from 16.2% to 62.8% ($P < 0.05$).

DISCUSSION

Pasteurellosis is one of the most common diseases of grower and finisher pigs worldwide. It is widely accepted that specific serotypes and pathotypes of *P. multocida* strains are responsible for most respiratory disease syndromes in pigs that are associated with pneumonia, atrophic rhinitis, and/or mycoplasma infection (4, 11, 30). However, the distribution and prevalence of serotypes and pathotypes can vary considerably from region to region and over time in a given region. This is the first study in China of a large collection of isolates of *P. multocida* obtained from pigs with clinical signs of respiratory infection. Our findings suggest that strains of *P. multocida* are widely prevalent on pig farms, and we have confirmed that on the Chinese mainland, infections caused by *P. multocida* strains of serogroup D are more common than those caused by strains of serogroup A ($P < 0.01$). Similar results were reported by Ewers et al. (17) in Germany (58.1% versus 34.9%) and Chan-

TABLE 4. Distribution of VFs according to capsule serotypes among 233 porcine isolates of *P. multocida*

Associated VF gene[a]	Total no. (% of 233) with trait	No. (%) of VFs within the following capsule serotypes:			*P* value
		capA (*n* = 92)	*capD* (*n* = 128)	Other (*n* = 13)	
ptfA	218 (93.6)	82 (89.1)	123 (96.1)	13 (100)	
fimA	231 (99.1)	90 (97.8)	128 (100)	13 (100)	
hsf-1	156 (67.0)	35 (38.0)	119 (93.0)[d]	2 (15.4)	<0.001
hsf-2	231 (99.1)	91 (98.9)	127 (99.2)	13 (100)	
pfhA	35 (15.0)	23 (25.0)[d]	4 (3.1)	8 (61.5)[d]	<0.001
tadD	101 (43.3)	85 (92.4)[d]	13 (10.2)	3 (23)	<0.001
toxA	11 (4.7)	0 (0)	11 (8.9)[d]	0 (0)	0.009
exbB	231 (99.1)	91 (98.9)	127 (99.2)	13 (100)	
exbD	231 (99.1)	91 (98.9)	127 (99.2)	13 (100)	
tonB	228 (97.9)	87 (94.6)	128 (100)	13 (100)	
hgbA	225 (96.6)	90 (97.8)	125 (97.7)	10 (76.9)[b]	<0.001
fur	216 (92.7)	89 (96.7)	118 (92.2)	9 (69.2)[c]	0.002
nanB	190 (81.5)	60 (65.2)	122 (95.3)[d]	8 (61.5)	<0.001
nanH	226 (97.0)	91 (98.9)	123 (96.1)	12 (92.3)	
pmHAS	105 (45.1)	77 (83.7)[d]	19 (14.8)	9 (69.2)[d]	<0.001
ompA	233 (100)	92 (100)	128 (100)	13 (100)	
ompH	217 (93.1)	88 (95.7)	121 (94.5)	12 (92.3)	
oma87	220 (94.4)	83 (90.2)	125 (97.7)	12 (92.3)	
plpB	231 (99.1)	91 (98.9)	128 (100)	12 (92.3)	

[a] All genes were detected by PCR.
[b] $P < 0.05$ for the indicated group compared with the results for all other strains (negative association).
[c] $P < 0.01$ for the indicated group compared with the results for all other strains (negative association).
[d] $P < 0.001$ for the indicated group compared with the results for all other strains.

drasekaran and Yeap (9) in Malaysia (45% versus 20%). In contrast, in the United States (30) and England and Wales (11), the prevalence of strains of capsular serogroup D is lower than that of serogroup A. It is interesting that although untypeable strains are generally uncommon in pigs (11), only 12 untypeable isolates associated with pneumonic pigs were isolated during the period of investigation. We do not know whether the presence of untypeable isolates is attributable to a lack of available tests.

Atrophic rhinitis is seldom reported in China; but our investigations have confirmed the presence of atrophic rhinitis in Henan, Shandong, Fujian, Hainan, and Hubei Provinces by the isolation of *P. multocida* from clinical cases and the use of assays for toxigenicity. In agreement with the findings of previous studies (4, 11), toxigenic *P. multocida* strains of serogroup D may play a more important role in atrophic rhinitis in China than toxigenic strains of serogroup A. During the study period, other bacterial species, including *Haemophilus parasuis* and *Streptococcus suis*, were often coisolated with pathogenic *P. multocida* strains from the same sample. Although it is not easy to distinguish whether *P. multocida* is a primary or a secondary pathogen in herds with mixed infections, the fact that various bacterial species may coexist in a given herd should be considered when attempts are made to control disease outbreaks (7).

Treatment for infections with *P. multocida* commonly includes broad-spectrum antimicrobials (5, 25, 26). The findings of our antibiotic susceptibility studies, like the findings of Kehrenberg et al. (25) in France, Salmon et al. (32) in North America, and Yoshimura et al. (38) in Japan, indicated that cephalosporins (cefazolin, ceftiofur), florfenicol, and fluoroquinolones (ciprofloxacin) were the most active drugs. The aminoglycoside antibiotics usually showed poor activity against *P. multocida*, as reported by Gutiérrez Martin and Rodríguez Ferri (19) in Spain and Yoshimura et al. (38) in Japan; however, in the present study, spectinomycin, kanamycin, gentamicin, and amikacin exhibited moderate activity against all strains tested. The average prevalence of resistance to conventional antibiotics, including amoxicillin, lincomycin, clindamycin, chlortetracycline, tetracycline, sulfamethazine, and trimethoprim-sulfamethoxazole, among the *P. multocida* isolates was found to be in excess of 60% for each antibiotic. Therefore, preventive and therapeutic effects on porcine *P. multocida* strains should no longer be expected from these antibiotics. Furthermore, the increased incidence of multidrug-resistant pathogenic bacteria has been widely reported in the last decade (8, 25, 37). This is presumably attributable at least in part to the use of antibiotic additives in animal feed and the extensive use of antimicrobial agents in veterinary medicine. Here we have shown that *P. multocida* exhibited a rapid increase in the rate of resistance to a large number of antimicrobial agents. This revealed that a high prevalence of multiple-drug resistance exists among isolates of *P. multocida* from pigs. If this situation continues, there will be no effective antibiotic therapeutic reserve for some bacterial infections. The implications of a large reservoir of multiresistant organisms, particularly *P. multocida*, which is not host specific, with resistance that is potentially transferable among livestock species are obvious (3, 12, 24). Therefore, the use of antimicrobial agents in food animals in ways that minimize the emergence of resistance not only in target pathogens but also in zoonotic bacteria is warranted in the future for the protection of public health.

Although the molecular basis of the pathogenicity and host specificity of *P. multocida* is not well understood, several studies have reported that a number of VFs are correlated with the pathogenic mechanisms (20, 23). The present study has provided novel epidemiological information on the prevalence and distribution of the various VFs of porcine strains of *P.*

multocida. Consistent with previous observations (17), the prevalence of 11 of the 19 VF genes examined, which encode colonization factors (*ptfA*, *fimA*, and *hsf-2*), iron acquisition factors, sialidases (*nanH*), and outer membrane proteins, were broadly characteristic of the three categories of isolates (serogroup A, serogroup D, and others [1 serogroup B isolate and 12 untypeable isolates]). These distribution patterns would support some lines of evidence that suggest that factors involved in cross-protection may potentially serve as vaccine candidates that can elicit homologous protective immunity against all serotypes of *P. multocida* (2, 20, 35). However, certain VFs varied significantly among the different serogroups. For example, *hsf-1*, which has been described to be an autotransporter adhesin in a common avian clone, Pm70 (27), was more frequently seen in serogroup D, whereas *tadD*, which has been described as putative nonspecific tight adherence protein D in Pm70 (27), was concentrated significantly in serogroup A. *pfhA*, which governs the adherence of *Bordetella pertussis* to host cells and which plays a role in the virulence of *P. multocida* (18, 20, 27), showed a low prevalence in strains of serogroup D compared with its prevalence in serogroup A or untypeable isolates. Various hyaluronan synthases have been described in the last 5 years (13, 14). Preliminary data from a Southern blot analysis suggested that the *P. multocida* serogroup A hyaluronan synthase PmHAS and the *P. multocida* serogroup D hyaluronan synthase PmHS1 were not similar at the DNA level (13). However, our study showed that PmHAS not only was prevalent in serogroup A strains but also was found in other serogroups of porcine *P. multocida*. It seems probable that different VFs have entered *P. multocida* strains independently at multiple different times in the evolutionary history of the species and at multiple positions within the phylogenetic tree. Moreover, the observed distribution pattern suggests that it is likely that the acquisition of certain VFs has led to divergent patterns of vertical inheritance and horizontal transmission (via pathogenicity-associated islands, plasmids, and transposons) within the *P. multocida* population.

In conclusion, given that it is a pathogenic microorganism that is not host specific, we believe that the occurrence of *P. multocida* in food-producing animals should not be forgotten. In China, as in many other countries, strains of *P. multocida* have frequently been isolated from pigs, and they represent a significant cause of territorial outbreaks of respiratory infections. The high prevalence of multiresistant strains of *P. multocida* in pigs and the association of such strains with serious disease strongly suggest that more attention should be paid to the prudent use of antimicrobials and to vaccination. Nowadays, many key VFs of *P. multocida* are slowly being identified. Further work is required to elucidate the mechanisms of pathogenesis and to determine unequivocally the role of these factors in immunity to pasteurellosis.

ACKNOWLEDGMENTS

We thank Xiangdong Liu (Wuhan, China) for support with the statistical analyses and Feng Liu for help with the capsule typing and biochemical assays.

This study was supported by grants from the National Basic Research Program (973 Program; no. 2006CB504404) and the Science and Technology Brainstorm Project of Hubei Province, China (Emergency Program, 2006–2008).

REFERENCES

1. **Aarestrup, F. M., A. M. Seyfarth, and Ø. Angen.** 2004. Antimicrobial susceptibility of *Haemophilus parasuis* and *Histophilus somni* from pigs and cattle in Denmark. Vet. Microbiol. **101**:143–146.
2. **Adler, B., D. Bulach, J. Chung, S. Doughty, M. Hunt, K. Rajakumar, M. Serrano, A. van Zanden, Y. Zhang, and C. Ruffolo.** 1999. Candidate vaccine antigens and genes in *Pasteurella multocida*. J. Biotechnol. **73:**83–90.
3. **Arashima, Y., and K. Kumasaka.** 2005. Pasteurellosis as zoonosis. Intern. Med. **44:**692–693.
4. **Bäckström, L. R., T. A. Brim, and M. T. Collins.** 1988. Development of turbinate lesions and nasal colonization of *Bordetella bronchiseptica* and *Pasteurella multocida* during long term exposure of healthy pigs affected by atrophic rhinitis. Can. J. Vet. Res. **52:**23–29.
5. **Brogden, K. A., G. Nordholm, and M. Ackermann.** 2007. Antimicrobial activity of cathelicidins BMAP28, SMAP28, SMAP29, and PMAP23 against *Pasteurella multocida* is more broad-spectrum than host species specific. Vet. Microbiol. **119:**76–81.
6. **Bronzwaer, S. L., O. Cars, U. Buchholz, S. Mölstad, W. Goettsch, I. K. Veldhuijzen, J. L. Kool, M. J. Sprenger, and J. E. Degener.** 2002. A European study on the relationship between antimicrobial use and antimicrobial resistance. Emerg. Infect. Dis. **8:**278–282.
7. **Cai, X., H. Chen, P. J. Blackall, Z. Yin, L. Wang, Z. Liu, and M. Jin.** 2005. Serological characterization of *Haemophilus parasuis* isolates from China. Vet. Microbiol. **111:**231–236.
8. **Caprioli, A., L. Busani, J. L. Martel, and R. Helmuth.** 2000. Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. Int. J. Antimicrob. Agents **14:**295–301.
9. **Chandrasekaran, S., and P. C. Yeap.** 1982. *Pasteurella multocida* in pigs: the serotypes and the assessment of their virulence in mice. Br. Vet. J. **138:**332–336.
10. **Chung, J. Y., I. Wilkie, J. D. Boyce, K. M. Townsend, A. J. Frost, M. Ghoddusi, and B. Adler.** 2001. Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. Infect. Immun. **69:**2487–2492.
11. **Davies, R. L., R. MacCorquodale, S. Baillie, and B. Caffrey.** 2003. Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. J. Med. Microbiol. **52:**59–67.
12. **Davies, R. L., R. MacCorquodale, and S. Reilly.** 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. Vet. Microbiol. **99:**145–158.
13. **DeAngelis, P. L., and C. L. White.** 2002. Identification and molecular cloning of a heparosan synthase from *Pasteurella multocida* type D. J. Biol. Chem. **277:**7209–7213.
14. **DeAngelis, P. L., and C. L. White.** 2004. Identification of a distinct, cryptic heparosan synthase from *Pasteurella multocida* types A, D, and F. J. Bacteriol. **186:**8529–8532.
15. **Djordjevic, S. P., G. J. Eamens, H. Ha, M. J. Walker, and J. C. Chin.** 1998. Demonstration that Australian *Pasteurella multocida* isolates from sporadic outbreaks of porcine pneumonia are non-toxigenic (*toxA*$^-$) and display heterogeneous DNA restriction endonuclease profiles compared with toxigenic isolates from herds with progressive atrophic rhinitis. J. Med. Microbiol. **47:**679–688.
16. **Dziva, F., A. P. Muhairwa, M. Bisgaard, and H. Christensen.** 2008. Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*. Vet. Microbiol. **128:**1–22.
17. **Ewers, C., A. Lübke-Becker, A. Bethe, S. Kiebling, M. Filter, and L. H. Wieler.** 2006. Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. Vet. Microbiol. **114:**304–317.
18. **Fuller, T. E., M. J. Kennedy, and D. E. Lowery.** 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb. Pathog. **29:**25–38.
19. **Gutiérrez Martin, C. B., and E. F. Rodríguez Ferri.** 1993. In vitro susceptibility of *Pasteurella multocida* subspecies multocida strains isolated from swine to 42 antimicrobial agents. Zentralbl. Bakteriol. Parasitenkd. Infektkrankh. Hyg. Abt. 1 Orig. **279:**387–393.
20. **Harper, M., J. D. Boyce, and B. Adler.** 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. FEMS Microbiol. Lett. **265:**1–10.
21. **Harper, M., A. Cox, F. St. Michael, H. Parnas, I. Wilkie, P. J. Blackall, B. Adler, and J. D. Boyce.** 2007. Decoration of *Pasteurella multocida* lipopolysaccharide with phosphocholine is important for virulence. J. Bacteriol. **189:**7384–7391.
22. **Heddleston, K. H., J. E. Gallagher, and P. A. Rebers.** 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. Avian. Dis. **16:**925–936.
23. **Hunt, M. L., B. Adler, and K. M. Townsend.** 2000. The molecular biology of *Pasteurella multocida*. Vet. Microbiol. **72:**3–25.
24. **Hunt, M. L., D. J. Boucher, J. D. Boyce, and B. Adler.** 2001. In vivo-expressed genes of *Pasteurella multocida*. Infect. Immun. **69:**3004–3012.
25. **Kehrenberg, C., G. Schulze-Tanzil, J. L. Martel, E. Chaslus-Dancla, and S. Schwarz.** 2001. Antimicrobial resistance in *Pasteurella* and *Mannheimia*: epidemiology and genetic basis. Vet. Res. **32:**323–339.

26. **Lion, C., M. C. Conroy, A. M. Carpentier, and A. Lozniewski.** 2006. Antimicrobial susceptibilities of *Pasteurella* strains isolated from humans. Int. J. Antimicrob. Agents **27:**290–293.
27. **May, B. J., Q. Zhang, L. L. Li, M. L. Paustian, T. S. Whittam, and V. Kapur.** 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc. Natl. Acad. Sci. USA **98:**3460–3465.
28. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken.** 2003. Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, DC.
29. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 2nd ed. NCCLS document M31-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
30. **Pijoan, C., R. B. Morrison, and H. D. Hilley.** 1983. Serotyping of *Pasteurella multocida* isolated from swine lungs collected at slaughter. J. Clin. Microbiol. **17:**1074–1076.
31. **Rhoades, K. R., and R. B. Rimler.** 1987. Capsular groups of *Pasteurella multocida* isolated from avian hosts. Avian Dis. **31:**895–898.
32. **Salmon, S. A., J. L. Watts, C. A. Case, L. J. Hoffman, H. C. Wegener, and R. J. Yancey, Jr.** 1995. Comparison of MICs of ceftiofur and other antimicrobial agents against bacterial pathogens of swine from the United States, Canada, and Denmark. J. Clin. Microbiol. **33**:2435–2444.
33. **Townsend, K. M., D. O'Boyle, T. T. Phan, T. X. Hanh, T. G. Wijewardana, I. Wilkie, N. T. Trung, and A. J. Frost.** 1998. Acute septicaemic pasteurellosis in Vietnamese pigs. Vet. Microbiol. **63:**205–215.
34. **Townsend, K. M., J. D. Boyce, J. Y. Chung, A. J. Frost, and B. Adler.** 2001. Genetic organization of *Pasteurella multocida cap* loci and development of a multiplex capsular PCR typing system. J. Clin. Microbiol. **39:**924–929.
35. **Vasfi Marandi, M., and K. R. Mittal.** 1997. Role of outer membrane protein H (OmpH)- and OmpA-specific monoclonal antibodies from hybridoma tumors in protection of mice against *Pasteurella multocida*. Infect. Immun. **65:**4502–4508.
36. **Walsh, C., and S. Fanning.** 2008. Antimicrobial resistance in foodborne pathogens—a cause for concern? Curr. Drug. Targets **9:**808–815.
37. **White, D. G., S. Zhao, S. Simjee, D. D. Wagner, and P. F. McDermott.** 2002. Antimicrobial resistance of foodborne pathogens. Microbes Infect. **4:**405–412.
38. **Yoshimura, H., M. Ishimaru, Y. S. Endoh, and A. Kojima.** 2001. Antimicrobial susceptibility of *Pasteurella multocida* isolated from cattle and pigs. J. Vet. Med. B **48:**555–560.