

Full Length Research Paper

Prevalence and antimicrobial susceptibility of bacterial pathogens isolates from diseased swine in southwest, China

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The purpose of this study was to investigate the prevalence of antimicrobial resistance in the clinical bacterial isolates from diseased swine in southwest, China during 2009-2010. A total of 504 bacterial isolates (19 species) were collected from the 364 clinical samples. The activity of 6-14 antibiotics to each bacterial species was examined. The sensitivity was tested by the disk diffusion method and performed according to CLSI guidelines in Mueller-Hinton agar. The most common pathogens were *Escherichia coli* (n=154; 30.56%), *Staphylococcus* spp. (n=110; 21.83%), *Enterococcus faecalis* (n=58; 11.51%), *Klebsiella pneumoniae* (n=44; 8.73%), *Proteus mirabilis* (n=43; 8.53%) and *Streptococcus suis* (n=30; 5.93%). All isolates revealed high level of resistance to ampicillin (47.6-100%), amoxicillin (52.6-100%), cephalothin (29-100%), norfloxacin (52.6-83.3%), gentamicin (45.1-83.3%) and terramycin (61.9-100%). Moreover, 93% of the isolates exhibited multiple drug resistance (MDR; resistance \geq 3 antimicrobials). Only ticarcillin/clavulanate exhibited very high activity against *E. coli* (98.1%), *Staphylococcus* spp. (91.9%), *K. pneumoniae* (92.3%) and *P. mirabilis* (97.2%), respectively. These findings suggest that antimicrobial resistance of bacterial pathogens isolates is commonly present among diseased swine in Southwest, China, and they also suggest the need for more prudent use of antibiotics by farmers and veterinarians.

Key words: Identification, antimicrobial susceptibility, pathogen, swine.

INTRODUCTION

As acute infections and outbreaks of infectious diseases in groups or herds become more common, use of an effective antimicrobial treatment as early as possible is critically important. The empirical treatment is generally based on knowledge of the resistance patterns of the different bacterial pathogens toward antimicrobial agents used in the particular animal species. Uncontrolled usage of antimicrobial agents is recognized as the most important factor that favors the development and spread of resistant microorganisms (Van den Bogaard and Stobberingh, 1999; White, 2002). The digestive tracts of

pigs can harbor antimicrobial-resistant bacteria among the commensal flora, which contain a reservoir of antibiotic-resistance genes potentially transmissible to humans through the food chain and environment (Caprioli et al., 2000). In addition to the human health concerns, antimicrobial-resistant pathogens also pose a severe and costly health problem in that they may prolong illness and decrease productivity through higher morbidity and mortality (Yang, 2004). However, data on the prevalence of antimicrobial-resistant veterinary pathogens are sparse, particularly in Southwest, where animal husbandry was more developed than other area.

Therefore, the purpose of this study was to investigate the prevalence of bacterial infection and resistance to antimicrobial agents in the clinical isolates obtained

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from diseased swine in Southwest, China.

MATERIALS AND METHODS

Clinical samples

364 Samples, including lungs, lymph nodes, livers, hearts, spleens, kidneys, and blood (obtained between May 2009 and July 2010) were collected from 72 pig farms in southwestern China. All the samples were obtained from diseased pigs which had at least one of the following symptoms: septicemia, arthritis, enteritis, meningitis, pneumonia, pleuritis, diarrhea, edema, fever, pneumonia, endocarditis, and dysentery. Before sampling, all samples surface were being aseptically by burning of ethanol. Aseptically collected samples were appropriately processed and seeded in chocolate agar and blood agar. All of the samples were transported to the laboratory of Sichuan Animal Science Academy for bacterial counts and isolation within 6 h.

Bacteria isolation

The strains were incubated at 37°C for 18-24 h. One loopful from each enrichment were streaked on tryptic soy agar, blood agar (5% fresh rabbit blood), MacConkey agar (Tianhe Microorganism Reagent Co., Ltd.) and Salmonella-Shigella agar respectively. All plates were incubated at 37°C in air for 24 h and purified by standard methods (Murray et al., 2003). Single colony was obtained from the isolates and stored in Luria-Bertani containing 20% glycerol, at -80°C until use. No replicate isolates from the same samples were used. All media agar were purchased from Tianhe Microorganism Reagent Co., Ltd.

Bacteria identification

Identification was based on colony type and morphology, Gram staining characteristics, and standard biochemical tests. All isolates were identified at species level by VITEK (Vitek System, bioMérieux). They were also confirmed using primers 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' TAC GGC TAC CTT GTT ACG ACT T 3') by the polymerase chain reaction (PCR) assay (Wilson, 1990). The template DNA was prepared by suspending an overnight cultured of bacteria in 400 µL Milli-Q water. The suspensions were heated at 95°C for 5 min and centrifuged at 13,000 rpm for 5 min. Each 50 µL of PCR mixture consisted of 4 µL of template, 5 µL 10xPCR Buffer, 1.5 mM MgCl₂, 200 µM dNTP, 0.4 µM each of the seven primers and 2.5 U Taq DNA polymerase. PCR was performed in a DNA thermal cycler (Bio-Rad, Hercules, CA) using the following program: an initial denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min, followed by a final elongation at 72°C for 10 min. The amplified PCR products were analyzed on 0.8% (w/v) agarose gels. Subsequently, the PCR product was sequenced by Shanghai Sangon Bioengineering Co., Ltd. The nucleotide sequences were analyzed with software (BLAST) available from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Antimicrobial susceptibility

Susceptibility to 15 antimicrobial agents, ampicillin (AMP; 10 µg), ampicillin/sulbactam (SAM; 10/10 µg), ticarcillin (TIC; 75 µg), amoxicillin (AMO; 10 µg), amoxicillin/clavulanate (AMC; 20/10 µg), ticarcillin/clavulanate (TIM; 75/10 µg), cephalothin (KF; 30 µg), ceftiofur (EFT; 30 µg), gentamicin (CN; 10 µg), amikacin (AMK; 30

µg), terramycin (OT; 30 µg), erythromycin (E; 15 µg), clindamycin (DA; 2 µg), norfloxacin (NOR; 10 µg) and ciprofloxacin (CIP; 5 µg), were tested by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2009). The control strains used for all susceptibility tests were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

Detection of bla_{TEM} resistance genes

The template DNA was prepared as described above. The forward primer ATGAGTATTCAACATTTCGTG and the reverse primer TTACCA ATGCTTA ATCAGTGAG were used to amplify the bla_{TEM} gene by following procedure: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 0.8% (w/v) agarose gels. The PCR product was sequenced and analyzed as above.

RESULTS

Bacterial isolates

504 bacterial isolates (which belong to 19 difference species) were collected from the 364 clinical samples. The isolates were all obtained between May 2009 and July 2010 from diseased pigs, including suckling pigs, nursery pigs, grower pigs and grower-finisher pigs. The results revealed that *E. coli* (n=154; 30.56%) was the most widespread bacterial isolates, followed by *Staphylococcus* spp. (n=110; 21.83%), *E. faecalis* (n=58; 11.51%), *K. pneumoniae* (n=44; 8.73%), *P. mirabilis* (n=43; 8.53%) and *S. suis* (n=30; 5.93%). Only 7 *H. parasuis* (1.39%) and 6 *P. multocida* (1.19%) isolates were obtained from all clinical samples.

Antimicrobial susceptibility

The majority of enterobacteria, including *E. coli*, *K. pneumoniae* and *P. mirabilis*, were resistant to ampicillin (88.4 to 100%), amoxicillin (92.3 to 100%), ticarcillin (50 to 76.9%), norfloxacin (61.5 to 83.3%), ciprofloxacin (67.3 to 83.3%) and terramycin (98 to 100%), and they were susceptible to ticarcillin/clavulanate, ampicillin/sulbactam and amoxicillin/clavulanate. The Gram-positive bacteria showed resistance to erythromycin (57.1 to 71.4%), clindamycin (79.6 to 85.7%), ampicillin (47.6 to 52.6%), amoxicillin (52.6 to 76.2%) and terramycin (61.9 to 84.2%) and were susceptible to ceftiofur, ticarcillin/clavulanate, ampicillin/sulbactam and amoxicillin/clavulanate (Table 1). In addition, most isolates from the swine were resistant to multiple classes of antimicrobial agents. Four hundred and seventy (93.25%) isolates from diseased swine were resistant to at least 3 of the 15 antimicrobial agents. 50 (9.92%), 83 (16.47%), 133 (26.39%), 144 (30.56%) and 50 (9.92%) isolates were resistant to 3, 4, 5, 6 and 7 antimicrobial agents, respectively.

bla_{TEM} resistance genes detection

Based on the results of the susceptibility tests, 154 *E. coli*, 44 *K. pneumoniae* and 43 *P. mirabilis* were selected to amplify the *bla*_{TEM} genes. The PCR analysis and sequencing showed that 140 (92.1%) *E. coli*, 44 (100%) *K. pneumoniae* and 42 (97.67%) *P. mirabilis* isolates harbored a *bla*_{TEM1} gene.

DISCUSSION

To investigate the prevalence of bacterial infections in swine in southwest, China, a total of 504 bacterial isolates (19 species) were collected and identified from the 364 clinical samples. The results revealed that *E. coli* and the *Staphylococcus* spp. were the most widespread bacterial isolates. In swine, *E. coli* is an important pathogenic bacteria including enterotoxigenic (ETEC) and extracellular pathogenic *E. coli* (ExPEC) strains which are common causes of a variety of clinical syndromes, including urinary tract infections, abdominal infections, pneumonia, neonatal meningitis, sepsis, neonatal and post weaning diarrhea and edema (Yang, 2004; Wada et al., 2004; Nazareth et al., 2007; Boerlin et al., 2005).

E. faecalis (n=58; 11.51%), *K. pneumoniae* (n=44; 8.73%), *P. mirabilis* (n=43; 8.53%) and *S. suis* (n=30; 5.93%) were the another four frequent isolates. *E. faecalis* is intrinsic not as virulent as other Gram-positive organisms, such as *S. aureus*, *S. pneumoniae* and *S. suis* Type 2 (Bittencourt, 2004; Gaspar et al., 2009). It emerges as an opportunistic pathogen, nevertheless, it is known to cause serious infections such as bacteraemia, septicaemia, urinary tract infections, wound infections, meningitis and endocarditis (Giacometti et al., 2000; Hershberger et al., 2005; Hällgren et al., 2003; Hébert et al., 2007). *K. pneumoniae* is also an opportunistic pathogen that responsible for a wide range of infection in humans and animals, such as urinary tract infections, pneumonia, wound infections and septicemia (Podschn and Ullmann, 1998; Brisse and Duijkeren, 2005). *P. mirabilis* is also often found in human as opportunistic pathogens (Zych et al., 2001). *S. suis*, especially the serotype 2, is an important swine pathogen causing meningitis, septicemia, endocarditis, and arthritis (Marie et al., 2002; Lun et al., 2007; Domínguez-Punaro et al., 2007; Ma et al., 2009). In 2005, an Streptococcal Toxic Shock Syndrome (STSS) human outbreak caused by *S. suis* serotype 2 was found in Sichuan province with 38 human deaths and over 200 human infections, and more than 640 pigs were found to be severely infected (Yu et al., 2006). Although other zoonotic bacterial such as *Salmonella* spp., *H. parasuis*, *P. multocida* and *Actinobacillus* spp. were lesser, they showed higher mortality and morbidity. The traditional zoonotic *E. coli*, *Staphylococcus* spp., *S. suis* and opportunistic *E.*

faecalis, *K. pneumoniae*, *P. mirabilis* were the main pathogens in swine in Southwest, China.

The enterobacteria were resistant to β -lactams, tetracyclines and aminoglycosides as previously described in China (Chang et al., 2002; Yang et al., 2004; Liu et al., 2007; Tian et al., 2009). The *E. coli* isolates assessed in this study displayed similar levels of resistance to tetracyclines, ampicillin, gentamicin and fluoroquinolones as were previously reported for *E. coli* strains isolated from diseased swine in China by Tian et al. (2009). High levels of resistance to tetracycline (~60-95%) have also been detected in *E. coli* isolates recovered from apparently healthy swine on-farm or at slaughter in other countries (Kozak et al., 2003; Kijima-Tanaka et al., 2003; Teshager et al., 2000; Blake et al., 2003). Most of *E. coli* isolates (67.3%) from swine were resistant to fluoroquinolones (e.g. norfloxacin and ciprofloxacin). Somewhat similar findings have been reported in a recent study of clinical *E. coli* isolates from swine by Wang et al., 2010. Resistance to amoxicillin-clavulanic acid does not occur frequently in *E. coli* isolates from diseased swine in China before 2004 (Yang et al., 2004), but 21.1% of our swine isolates were resistant to this antibiotic-inhibitor combination. In the present study, amikacin exhibited moderate activity against all strains tested.

Interestingly, fewer reports of antimicrobial resistance in *K. pneumoniae* isolated from swine have been published in China. In the present study, *K. pneumoniae* was the sixth most frequently encountered pathogen in swine and showed high resistance to antimicrobial. *K. pneumoniae* isolates may be naturally resistant to ampicillin, amoxicillin, carbenicillin, and ticarcillin, but not to extended-spectrum β -lactam antibiotics due to a constitutively expressed chromosomal class A β -lactamase (Haeggman et al., 2004). In the present study, the degree of resistance to cephalothin and ceftiofur was 100 and 48.2%, respectively. The antibiotic-inhibitor combination revealed actively to *K. pneumoniae*. This could be due to the wide use of these classes of cephalosporins in husbandry activities and the considerable increase in prevalence of ESBL-producing and multiple-antimicrobial-resistant isolates from pig farms (Yang et al., 2004; Tian et al., 2009).

Another important observation in this study is that the ceftiofur resistance has increased. Ceftiofur is the only extended-spectrum cephalosporin approved for veterinary use in many countries (Salmon et al., 1995). Because ceftiofur-resistant organisms are cross resistant to ceftriaxone, the use of this antimicrobial agent in food animals has come under increasing scrutiny as a selective agent potentially responsible for the emergence and dissemination of ceftriaxone resistance in *Salmonella* and other enteric pathogens (Alcaine et al., 2005). The rate of resistance to ceftiofur were (9.6%) *E. coli*, (48.2%) *K. pneumoniae*, (30.1%) *P. mirabilis* and (23.8%) *S. suis* isolates in this study, respectively. These findings are

Table 1. Antibiotic resistance in the clinical bacterial isolates.

Antimicrobial agent	Resistance(%) ^a						
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>Staphylococcus</i>	<i>S. suis</i>	<i>E. faecalis</i>	Others ^d
AMP	88.4	100.0	100.0	51.0	47.6	52.6	67.7
AML	92.3	100.0	100.0	61.2	76.2	52.6	80.6
TIC	73.0	76.9	50.0	-	-	-	61.2
SAM	19.2	15.3	16.6	10.2	-	-	9.6
AMC	21.1	15.3	16.6	10.2	-	-	12.9
TIM	1.9	7.7	2.8	8.1	-	-	6.4
KF	40.3	100.0	35.2	40.8	52.3	-	29.0
EFT ^b	9.6	48.2	30.1	12.2	23.8	-	16.1
E	-	-	-	65.3	57.1	71.4	-
DA	-	-	-	79.6	85.7	-	-
NOR	67.3	61.5	83.3	59.1	-	52.6	54.8
CIP	67.3	69.2	83.3	51.0	-	36.8	45.1
CN	55.7	46.1	83.3	44.9	-	-	45.1
AMK	44.2	46.1	16.6	30.6	-	-	51.6
OT ^c	98.0	100.0	100.0	83.6	61.9	84.2	87.1

^aBreakpoints are those recommended by the Clinical and Laboratory Standards Institute (CLSI); -, no CLSI breakpoints were available, ^bAccording to cefotaxime, ^cAccording to tetracycline, ^dincluding *Shigella* spp., *Salmonella* spp., *H. parasuis*, *A. Iwoffii*, *P. multocida* (Aarestrup, 2004). AMP, ampicillin; AML, amoxicillin; TIC, ticarcillin; TIM, ticarcillin/clavulanate; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanate; KF, cephalothin; EFT, ceftiofur; E, erythromycin; DA, clindamycin; NOR, norfloxacin; CIP, ciprofloxacin; CN, gentamicin; AMK, amikacin; OT, terramycin.

very significant difference to the previous reports which displayed high actively to ceftiofur (Marie et al., 2002; Yang et al., 2004; Morioka et al., 2005; Zhou et al., 2010; Wang et al., 2010). The high incidence of ceftiofur resistance in the *K.pneumoniae*, *P. mirabilis* and *S. suis* isolates tested herein was somewhat unexpected, as this drug was introduced into veterinary clinics for use in China only 2 to 3 years ago.

Of all the 154 *E. coli*, 44 *K. pneumoniae* and 43 *P. mirabilis*, 140 (92.1%), 44 (100%) and 42 (97.67%) isolates harboured a *bla*_{TEM-1} gene. The results of the study indicated that TEM-1 was the most common β -lactamase gene among the *K. pneumoniae* and *E. coli* isolates, in agreement with previous studies that reported a high prevalence of the *bla*_{TEM-1} gene among animal *E. coli* isolates (Liu et al., 2007; Rayamajhi et al., 2008; Li et al., 2007; Chander et al., 2011).

In conclusion, our results confirmed a different prevalence of bacterial infection in swine during 2009-2010, in Southwest, China. The *E. coli*, *Staphylococcus* spp., *S. suis*, *E. faecalis*, *K. pneumoniae* and *P. mirabilis* isolates are commonly present among diseased swine. Here we have shown that bacterial pathogens from diseased swine exhibited high level resistance to a large number of antimicrobial agents. If this situation continues, there will be no effective antibiotic therapeutic reserve for some bacterial infections. In the future, the data from monitoring programs and resistance studies should be taken into account for the usage of antimicrobials in veterinary medicine.

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