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Eur J Wildl Res (2010) 56:493–502
DOI 10.1007/s10344-009-0339-3

ORIGINAL PAPER

Salmonella enterica subspecies *enterica* serovar Choleraesuis in a wild boar population in Germany

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Received: 9 July 2009 / Revised: 13 October 2009 / Accepted: 15 October 2009 / Published online: 4 November 2009

Abstract *Salmonella* (*S.*) *enterica* subspecies *enterica* serovar Choleraesuis, the swine-adapted serovar is found rarely in Western European countries including Germany. However, the regional laboratory of the federal state Thuringia in Germany examined diseased wild boars routinely also for the occurrence of *Salmonella* organisms. Between 2006 and 2008, only the serovar *S.* Choleraesuis was islolated from 24 animals, three strains isolated from domestic pigs were included. In order to detect a possible epidemiological context, the strains of *S.* Choleraesuis were characterised by macrorestriction and plasmid analysis, repetitive sequence PCR, antimicrobial testing and determining the biochemical profile. A combination of all methods enabled the identification of five epidemiological groups. Two groups were detected in the same territory but three other discriminative groups were predominant in different regions. *S.* Choleraesuis strains of the different epidemiological groups circulate in wild boar populations in the corresponding regions. However, it could be concluded that both natural barriers like mountains and artificial barriers like arterial roads may cause the separation of wild boar populations and as a result also the respective *S.* Choleraesuis organisms. The occurrence of the identical epidemiological groups in wild boars and domestic pigs indicates the possible mutual exposure of the pathogen. To avoid risks for human and domestic pig health regular inspection of meat from wildlife by official veterinarians and advice of hunters and persons who prepare and consume wild boar meat should be enhanced.

Keywords Wild boar · *Salmonella* Choleraesuis · Typing · Epidemiology

Communicated by F.-J. Kaup

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Introduction

Salmonella (*S.*) Choleraesuis is a host-adapted, facultative intracellular pathogen that causes swine paratyphoid with clinical manifestations of enterocolitis and septicaemia (Reed et al. 1986). As it is rarely isolated from non-porcine reservoirs, the source of *S.* Choleraesuis seems to be limited to carrier pigs and facilities previously contaminated with this serovar (Gray et al. 1996). Despite its swine-adapted character, *S.* Choleraesuis may also cause human infections. Although *S.* Choleraesuis is not often detected from human sources in the USA (Centers for Disease Control and Prevention 2000) and the European Union (EFSA 2007), it is an important serovar in several Asian countries. In Thailand (Bangtrakulnonth et al. 2004) and Taiwan (Chang et al. 2005), *S.* Choleraesuis is not only frequently isolated in humans but identified as the main cause of salmonellosis. During 1950s and 1960s, *S.* Choleraesuis, including variant Kunzendorf, was the predominant serovar isolated from pigs worldwide. At the present time, this serovar is still highly prevalent in North America and Asia, but is detected rarely in Australia and Western European countries (Fedorka-Cray et al. 2000). A baseline survey on the prevalence of *Salmonella* in

slaughter pigs in the EU from 2006–2007 revealed that *S.* Choleraesuis and *S.* Choleraesuis var. Kunzendorf were detected in four out of the 25 participating member states in altogether only 10 out of 2,600 lymph nodes examined (EFSA 2008). The reasons for the differences between North America and Europe are not known but may be related to husbandry practises (Fedorka-Cray et al. 2000). In Germany, the National Reference Laboratory for *Salmonella* received from 2001 to 2008 no single strain of *S.* Choleraesuis isolated from domestic pigs for further typing (Schroeter, personal communication) indicating that the swine-adapted serovar is not resident in pigs in Germany. However, in the regional laboratory of the federal state Thuringia in Germany diseased wild boars of different ages originating from several regions were examined routinely also for the occurrence of *Salmonella* organisms. Between 2006 and 2008 only the serovar *S.* Choleraesuis was detected in 24 out of 118 animals examined. Reports on the occurrence of *S.* Choleraesuis in wild boars in Germany are few and concern mainly single cases (Plötner et al. 1979; Weber et al. 1990; Müller et al. 2004). The aim of the present study was to characterise German wild boar strains of *S.* Choleraesuis by phenotypical and genotypical methods in order to detect a possible epidemiological context.

Materials and methods

Bacterial strains

In total, 24 strains of *S.* Choleraesuis were isolated from a total of 118 sickened and shot wild boars of different age (shoats, juveniles and adult animals) in several regions in Thuringia, a federal state in Germany, between 2006 and 2008 (Fig. 1). Additionally, three isolates of *S.* Choleraesuis from domestic pigs detected during that time were included. All isolates were typed according to the Kauffmann–White scheme (Grimont and Weill 2007) and to differentiate between the biovars *S.* Choleraesuis sensu stricto or var. Choleraesuis and *S.* Choleraesuis var. Kunzendorf (Le Minor et al. 1985) the biochemical profile was determined using the identification system API 20 E (bioMerieux, France). Furthermore, pathomorphological changes of the wild boars were recorded.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities to ampicillin (AMP), amoxicillin/clavulonsäure (AUG2), chloramphenicol (CHL), ceftiofur (XNL), ciprofloxacin (CIP), colistin (COL), florfenicol (FFN), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), neomycin (NEO), spectinomycin (SPE), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET), trimethoprim (TMP) and trimethoprim/sulfamethoxazole (SXT) were assessed by determining the minimum inhibitory concentration (MIC) using the NCCLS (NCCLS 2000) broth microdilution method (Trek Diagnostic Systems, UK). Breakpoints (μg/ml) given from the NCCLS (2001) and from DANMAP 98 (1999) were used.

Plasmid analysis

Plasmid DNA was isolated by using the alkaline denaturation method as described before (Kado and Liu 1981). Samples were analysed by electrophoresis in 1×TBE buffer (90 mM Tris; 90 mM boric acid; and 2.5 mM Na_2-EDTA, pH 8.3) at 20 V for 20 min and at 54 V for 4 to 5 h on 1.0% agarose gels. Extracted DNA of four plasmid-containing strains of *Escherichia coli* (R27, R1, RP4 and Col E1) were mixed and used as size markers (three parts of strain R27 and one part of strain R1, RP4 and Col E1 each). The molecular weights of the plasmids of the *E. coli* strains are 112 MDa (R27), 62 MDa (R1), 36 MDa (RP4), and 4.2 MDa (ColE1). Additionally, a supercoiled DNA ladder (Invitrogen, Karlsruhe, Germany) was used as size marker. The molecular weights of the plasmids were calculated by comparison with the markers.

Repetitive sequence polymerase chain reaction

Salmonella strains were grown overnight in Luria-Bertani broth (SIFIN, Berlin, Germany). Template DNA was purified using a commercial kit (QIAmp DNeasy Tissue kit, Qiagen, Hilden, Germany) according the instruction manual of the manufacturer. DNA of each isolate was amplified by polymerase chain reaction (PCR) using five primers for repetitive DNA sequences. For BOX element (BOXA)-PCR the primer BOXA 1R, for Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR the primer pair ERIC 1R, ERIC 2 and for Repetitive Extragenic Palindromic (REP)-PCR the primer pair REP 1R-I, REP 2-I were used. PCR was performed using primer sequences by Versalovic et al. (1991) and Weigel et al. (2004): BOXA 1R (5′CTACGGCAAGGCGACGCTGACG-3′); ERIC 1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′); ERIC 2 (5′-AAG TAAGTGACTGGGGTGAGCG-3′); REP 1R-I (5′-IIIIC GICGICATCIGGC-3′); REP 2-I (5′-ICGICTTATCIGGCC TAC-3′). Primers were used at a final concentration of 50 pmol using a thermal cycler (Uno Thermoblock, Biometra, Göttingen, Germany). Amplification reactions were performed in a final volume of 25 μl with 1.0 μl (approximately 25 ng) *Salmonella* DNA and 12.5 μl Mastermix (HotStarTaq, Qiagen). The reactions were incubated for 15 min at 95°C, followed by 32 cycles at 94°C for 1 min, 52°C (ERIC- and BOXA-PCR) or 40°C (REP-PCR) for 1 min and 72°C for 1 min and a final

Fig. 1 Location of the federal state Thuringia in Germany. *x* indicates the place of origin of *Salmonella* Choleraesuis isolates from wild boars or *o* from domestic pigs

extension step at 72°C for 10 min. High-purity water was used as negative control and DNA from *Samonella Typhimurium* strain LT2 as positive control. Aliquots of the products (10 µl) were resolved in 1.5% agarose gel with a length of 25 cm (Agarose Lm, BioRad) containing 0.5× Tris–acetate–EDTA buffer (0.4 M Tris, 0.2 M acetic acid and 0.01 M EDTA; pH 8.5). Two DNA ladders (DNA Ladder High Range and DNA Ladder Low Range, Fermentas, St. Leon-Rot, Germany) were used as size markers. After electrophoresis at 70 V for 20 h at 4°C the DNA was stained with ethidium bromide and photographed on a UV-transiluminator (Biostep, Jahnsdorf, Germany).

Macrorestriction analysis using pulsed-field gel electrophoresis

S. Choleraesuis strains were grown overnight at 37°C in 5 ml of Luria-Bertani broth and adjusted to optical density ($OD_{580\ nm}$) of 0.3 by dilution with sterile Luria-Bertani broth. Genomic DNA was prepared as follows: *Salmonella* cells were embedded after centrifugation and washed with cold PET IV buffer (1 M NaCl, 10 mM Tris, pH 8.0 and 10 mM Na_2-EDTA) in agarose (pulsed-field certified, BioRad, Munich, Germany). Agar plugs were prepared by adding 500 µl of bacterial suspension in PET IV buffer to 500 µl of 1.2% hot agarose (56°C), mixing thoroughly and filling approximately 100 µl into each plug mould. The solidified agarose plugs were added into TE buffer (10 mM Tris and 1 mM Na_2-EDTA; pH 8.0) and stored at 4°C until lysis. For lysis agarose plugs were incubated in lysis buffer (1 M NaCl, 10 mM Tris, pH 8.0, 10 mM Na_2-EDTA, 5% *N*-lauroylsarcosine sodium salt [Sigma, Deisenhofen, Germany], 0.2% [wt/vol] deoxycholic acid sodium salt [Sigma], 2 µg/ml [wt/vol] RNase [Roth, Karlsruhe, Germany] and 1 mg/ml [wt/vol] lysozym [Roth]) for 2 h at 37°C. After removing the lysis buffer agarose plugs were incubated overnight at 56°C in 1 ml ES buffer (0.5 M Na_2-EDTA, pH 9.0 and 1% *N*-laurylsarcosine sodium salt) with proteinase K (Merck, Darmstadt, Germany) at a final concentration of 650 µg/ml. The ES buffer was replaced with fresh ES buffer-proteinase K solution, followed by a second overnight incubation at 56°C. To prepare samples for restriction endonuclease digestion, the plugs were washed four times extensively in 1 ml Tris–EDTA buffer (10 mM Tris and 1 mM Na_2-EDTA; pH 8.0) for 30 min at 4°C by shaking and cut into two pieces of equal size. The plug pieces were equilibrated with 50 µl of the appropriate digestion buffer at room temperature for 15 min. After

removing the samples were incubated in 50 µl digestion buffer containing 20 U of the restriction endonuclease *Xba*I (Fermentas, St. Leon-Rot, Germany) or 10 U of restriction endonuclease *Spe*I or *Bln*I, respectively, (Roche Diagnostics, Mannheim, Germany) for 4 h at 37°C. Electrophoretic separation of the DNA fragments in 1.0% (wt/vol) agarose gels was performed using a contour-clamped homogeneous electric field apparatus (CHEF DR III, BioRad, Munich, Germany) under the following conditions: 6 V cm^{-1}, pulse times ramping from 5 to 50 s for *Xba*I and *Spe*I, and 20 to 80 s for *Bln*I, electrode angle of 120° and a temperature of 10°C for 24 (*Xba*I, *Spe*I) and 25 h (*Bln*I), respectively. The running buffer contained 0.5× Tris–borate–EDTA (45 mM Tris, 45 mM boric acid and 2.5 mM EDTA; pH 8.0). Finally, DNA fragments were stained with ethidium bromide, viewed under UV light and documented using a gel imaging system (Biostep, Jahnsdorf, Germany). Similarities between profiles, based on band positions, were calculated with Gelcompar II software, version 4.011 (Applied Maths, Austin, USA). Dice similarity coefficients were calculated based on pairwise comparison of the patterns. For the determination of the genetic relationship among the isolates the matrix of the calculated coefficients was used to generate dendrograms based on the UPGMA method (unweighted pair group method using arithmetic averages) with a tolerance of 2.0% and optimisation of 1.0% for all enzymes. Patterns were normalised by interpolation to reference lanes. A difference of at least one restriction fragment larger than 48.5 kb in the patterns was the criterion for discriminating between different clones or strains (Tenover et al. 1995). Bands with lower molecular weights were not included in the cluster analysis with Gelcompar. As molecular weight standard, concatemers of lambda DNA (molecular size from 48.5 to 727.5 kb; New England BioLabs, Ipswich, USA) were run on three lanes (both sides and the middle) of each gel.

Calculation of the discriminatory indices

The discriminatory value for each endonuclease used in macrorestriction analysis using pulsed-field gel electrophoresis (PFGE) was calculated as an index of discrimination (D) according to Hunter and Gaston (1988). It is based on the probability that two unrelated strains randomly sampled from the test population will be assigned to different typing groups.

Results

Pathological findings in wild boars

Nearly all diseased and shot wild boars (especially shoats and juveniles) examined showed clinical signs of septicaemia. Pneumonia and hepatitis were detected regularly but enterocolitis only in single cases. Some wild boars were suffering from severe osteomyelitis, *S.* Choleraesuis could be isolated from the affected tissue. In most cases it was possible to cultivate pure colonies of the agent from different organs directly without pre-enrichment in buffered peptone water.

Biochemical characterisation of *S.* Choleraesuis

All isolates were typed according to the Kauffmann–White scheme and revealed the complete antigenic formula (6, 7: c: 1, 5) for *S.* Choleraesuis. Three out of the 27 *S.* Choleraesuis strains were H_2S negative and, therefore, could be referred to the biovar Choleraesuis sensu stricto or variato Choleraesuis (vC), respectively. Twenty-four *S.* Choleraesuis strains were, according to their positive H_2S reaction, assigned to biovar Kunzendorf (vK; Table 1). Furthermore, the *S.* Choleraesuis strains revealed different fermentation patterns for sorbitol (SOR), melibiose (MEL) and arabinose (ARA). Altogether, four different "Analytical Profile Indices" of the *S.* Choleraesuis isolates were identified and designated as A, B, C or D: 6 504 150 (=A): H_2S+, SOR-, MEL+, ARA-; 6 504 510 (=B): H_2S+, SOR+, MEL-, ARA-; 6 104 150 (=C): H_2S-, SOR-, MEL+, ARA- and 6 504 110 (=D): H_2S+, SOR-, MEL-, ARA- (Table 1).

Antimicrobial susceptibilities

Only against sulfamethoxazole (SMX) and streptomycin (STR) the *S.* Choleraesuis isolates revealed a different antimicrobial resistance pattern (Table 1). Six strains (826, 828, 829, 830, 933 and 934) showed a MIC against SMX of 64/32 µg/ml (sensitive) and against STR of 32 µg/ml (resistant), whereas all other isolates revealed higher MICs to SMX (>512 µg/ml) and STR (64 µg/ml). The MICs (µg/ml) against CIP (0.06); SPE (32); XNL (1), (8); COL (<4); AMP (2); CHL (4); FFN (4); GEN (<1); KAN (<4); NEO (<2); TMP (<4); SXT (<1); AUG (<2) and TET (4–8) were identical in all *S.* Choleraesuis strains tested.

Repetitive sequence polymerase chain reaction

Amplification with primers for ERIC-PCR, BOXA-PCR and REP-PCR produced for each PCR one single pattern for all *S.* Choleraesuis strains tested (not shown).

Plasmid analysis

In all 27 strains of *Salmonella* Choleraesuis only one plasmid of approximately 52 kbp was identified (Fig. 2, Table 1). Figure 2 shows strains from all epidemiologial groups.

Table 1 Characteristics of the *S.* Choleraesuis isolates

Strain	Biochemical Index (API 20E)	Biovar	MIC (μg/ml) to		Size of plasmids (kbp)	Macrorestriction pattern			Macrorestriction group	Epidemiological group[c]
			SMX	STR		*Xba* I X[a]	*Bln* I B[b]	*Spe* I S[a]		
826	A	vK	64	32	52	2	3	2	I	I A
828	A	vK	64	32	52	2	3	2	I	I A
829 (pig)	A	vK	64	32	52	2	3	2	I	I A
830 (pig)	A	vK	64	32	52	2	3	2	I	I A
930	C	vC	64	32	52	2	3	2	I	I C
933	C	vC	64	32	52	2	3	2	I	I C
934	C	vC	64	32	52	2	3	2	I	I C
861	B	vK	>512	64	52	1	1	1	II	II B
890 (pig)	B	vK	>512	64	52	1	1	1	II	II B
929	B	vK	>512	64	52	1	1	1	II	II B
971	B	vK	>512	64	52	1	1	1	II	II B
973	B	vK	>512	64	52	1	1	1	II	II B
1000	B	vK	>512	64	52	1	1	1	II	II B
966	D	vK	>512	64	52	1	1	1	II	II D
967	D	vK	>512	64	52	1	1	1	II	II D
970	D	vK	>512	64	52	1	1	1	II	II D
994	D	vK	>512	64	52	1	1	1	II	II D
843	B	vK	>512	64	52	1	2	2	III	III B
927	B	vK	>512	64	52	1	2	2	III	III B
928	B	vK	>512	64	52	1	2	2	III	III B
932	B	vK	>512	64	52	1	2	2	III	III B
981	B	vK	>512	64	52	1	2	2	III	III B
984	B	vK	>512	64	52	1	2	2	III	III B
986	B	vK	>512	64	52	1	2	2	III	III B
987	B	vK	>512	64	52	1	2	2	III	III B
995	B	vK	>512	64	52	1	2	2	III	III B
1382	B	vK	>512	64	52	1	2	2	III	III B

A 6 504 150, *B* 6 504 510, *C* 6 104 150, *D* 6 504 110, *vK* biovar Kunzendorf, *vC* biovar Choleraesuis sensu stricto, *MIC* minimum inhibition concentration against SMX (sulphamethoxazole) or STR (streptomycin)

[a] Pattern numbers correspond to lane numbers in Fig. 3

[b] Pattern numbers correspond to lane numbers in Fig. 4

[c] Epidemiological group as combination of macrorestriction group and biochemical index

Macrorestriction analysis

Three different restriction endonucleases, *Xba*I, *Bln*I and *Spe*I, were used to cleave whole-cell DNA of *S.* Choleraesuis isolates. The resulting fragments were separated by pulsed-field gel electrophoresis; the patterns are shown in Fig. 3. *Xba*I digestion yielded two different patterns (X1 and X2) with 14 fragments in the range of 15 to 720 kbp (Fig. 3, Table 1). The majority of the isolates exhibited pattern X1 whereas pattern X2 was represented by seven strains. Isolates from pigs showed both X1 (n=1) and X2 (n=2) pattern. *Xba*I macrorestriction analysis had a *D* value of 0.400. *Spe*I digestion resulted in two patterns (S1 and S2) of 21 to 22 fragments in the range of 10 to 340 kbp (Fig. 3, Table 1). Ten *S.* Choleraesuis strains exhibited pattern S1 and 17 strains pattern S2. The *D* value of *Spe*I macrorestriction analysis was calculated to be 0.487. *Bln*I digestion produced three patterns (B1, B2 and B3) composed of 13 to 15 fragments in the range of 20 to 970 kbp (Fig. 4, Table 1). Ten strains represented pattern B1, 10 strains B2 and seven *S.* Choleraesuis isolates exhibited pattern B3. *Bln*I macrorestriction analysis had a *D* value of 0.682.

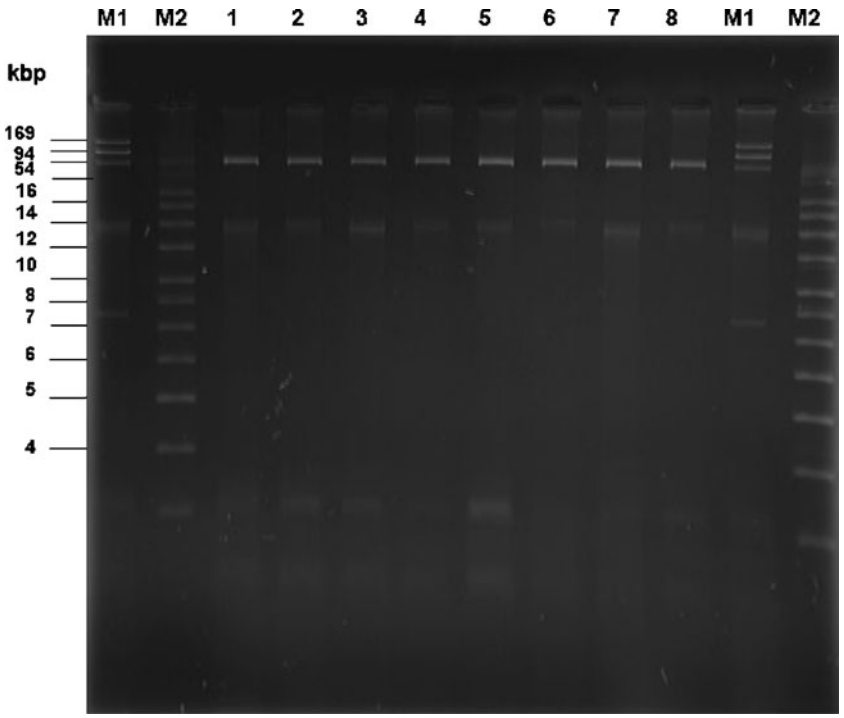

Fig. 2 Agarose gel electrophoretic demonstration of the identical plasmid profile (*lanes 1–8*) observed in the *S.* Choleraesuis isolates from all epidemiological groups

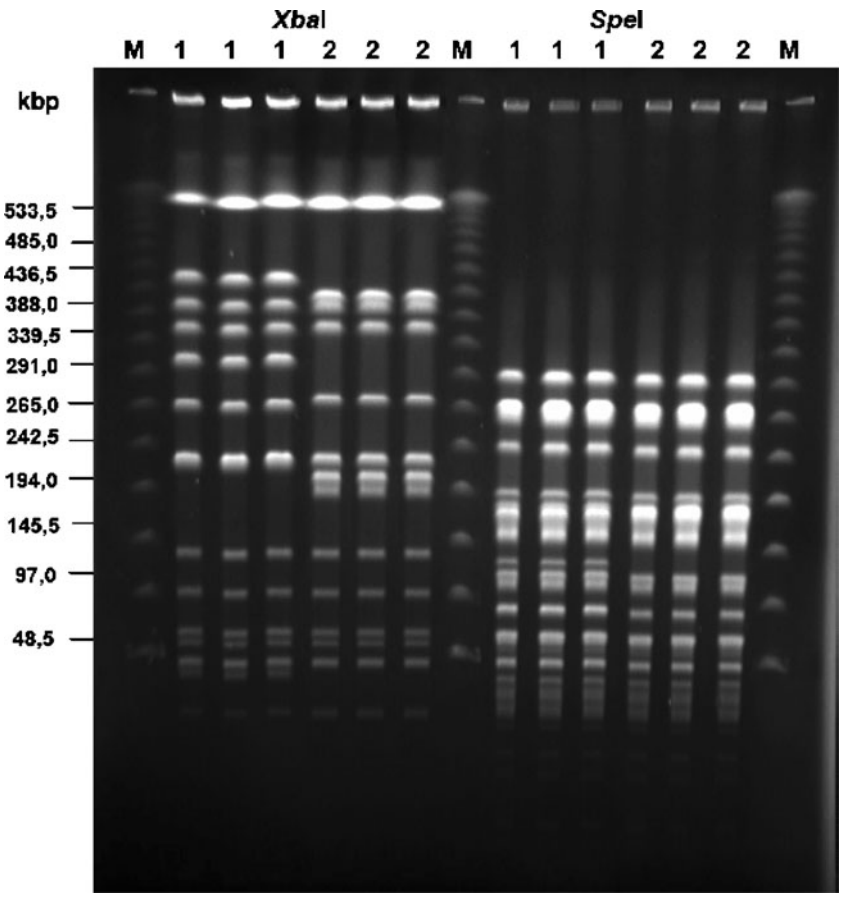

Fig. 3 *Xba*I (X1, X2) and *Spe*I (S1, S2) macrorestriction patterns

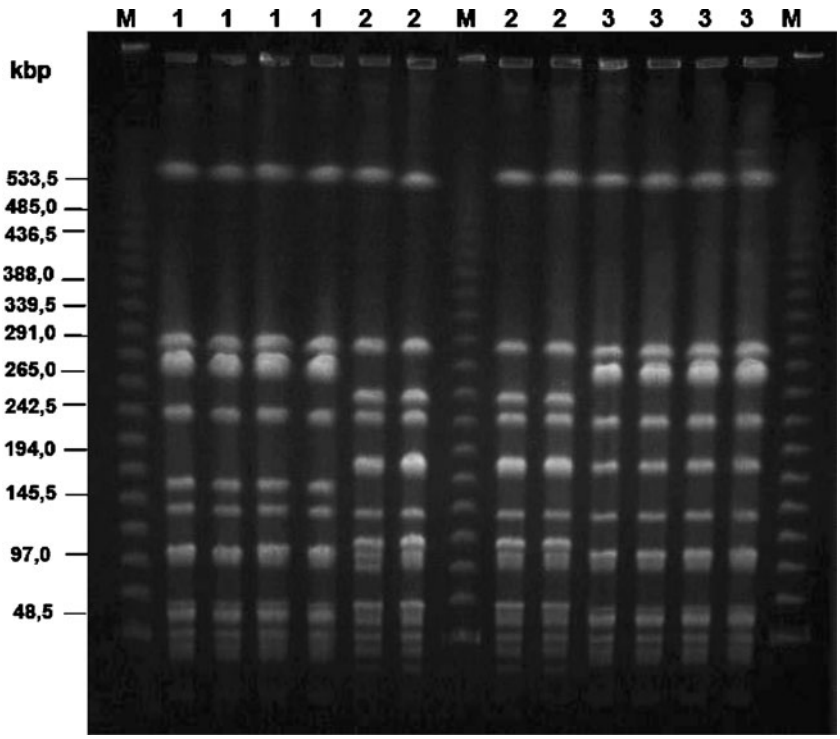

Fig. 4 *Bln*I macrorestriction patterns B1, B2 and B3

Combining of phenotyping and genotyping results

Although plasmid profiling and repetitive sequence PCR did not contribute to the molecular differentiation of the *S.* Choleraesuis isolates, results of macrorestriction analysis allowed the assignment of the 27 strains examined in this study to three macrorestriction groups (Table 1). The different sulfamethoxazole and streptomycin susceptibility patterns of the *S.* Choleraesuis strains did not allow an additional differentiation within these macrorestriction groups but justified this classification as all *S.* Choleraesuis isolates from group I showed a lower MIC against these antimicrobials than the strains from groups II and III. Determination of the biochemical responses revealed four different analytical profile indices for the *S.* Choleraesuis organisms. The assignment of these indices to the macrorestriction groups resulted in a further distinction of the groups, which were termed as epidemiological groups (Table 1). The distribution of the *S.* Choleraesuis strains according to both their affiliation to the different epidemiological groups and their place of origin in the federal state Thuringia in Germany are presented in Fig. 5. It could be shown that the discriminative groups are predominant in different regions. *S.* Choleraesuis strains isolated from both wild boars and pigs belonging to group IA were found only in the north-eastern whereas strains of group IC (var. Choleraesuis) originated only from the south-eastern of Thuringia. Another group, IID, was concentrated in the middle of the western part of this federal state. In the south-western region of Thuringia two genetically different (II and III) but biochemically (B) identical groups of *S.* Choleraesuis strains were dominant. Also in this region the strain isolated from a domestic pig did belong to the same group as the strains from wild boars.

Discussion

The pig-adapted serovar *S.* Choleraesuis is still highly prevalent in Asia and North America but is detected rarely in Australia and Western Europe (Fedorka-Cray et al. 2000). During a baseline survey from 2006–2007 on the prevalence of *Salmonella* in slaughtered pigs in the 25 participating member states of the EU *S.* Choleraesuis and *S.* Choleraesuis var. Kunzendorf were detected in Bulgaria [1×], Spain [3×], Poland [3×] and Slovakia [3×] (EFSA 2008). In Germany, since 2001 *S.* Choleraesuis was not isolated from pigs (Hartung 2008) indicating that the swine-adapted serovar is not resident in domestic pig population in this country. However, in the regional laboratory of the federal state Thuringia in Germany diseased wild boars of different ages from several regions were examined routinely also for the occurrence of *Salmonella* organisms. Between 2006 and 2008, only the serovar *S.* Choleraesuis was isolated from 24 animals. Despite few reports on the occurrence of *S.* Choleraesuis in wild boars in Germany (Plötner et al. 1979; Weber et al. 1990; Müller et al. 2004) the occurrence of *S.* Choleraesuis in wild boars in Thuringia

Fig. 5 Distribution of epidemiological groups of *S.* Choleraesuis strains according to their place of origin

might suggest that this serovar is also present but possibly undetected in wild boars in other federal states in Germany. The proved pathomorphological findings, especially septicaemia in the wild boars and the direct detection of *S.* Choleraesuis from affected tissues in this study confirm other results and indicate the invasive character of this pig-adapted serovar (Kingsley and Bäumler 2000; Müller et al. 2004).

To detect a possible epidemiological context between the *S.* Choleraesuis strains originating from different regions in the federal state Thuringia in Germany several methods to discriminate the isolates were applied. PFGE, plasmid analysis, REP-PCR, ERIC-PCR, BOXA-PCR and antimicrobial susceptibility typing are commonly used tools for epidemiological investigations of *Salmonella* (Liebisch and Schwarz 1996; Weide-Botjes et al. 1996; Chmielewski et al. 2002; Chiu et al. 2004). As amplification with primers for ERIC-PCR, BOXA-PCR and REP-PCR each produced only one single pattern for all *S.* Choleraesuis isolates tested, it was obvious that these techniques did not discriminate the strains within this serovar. Similar results were found by Millemann et al. (1996) when analysing *Salmonella* Enteritidis strains. In contrast, Chmielewski et al. (2002) considered REP-PCR and ERIC-PCR as highly discriminatory methods suitable for epidemiological studies of *S.* Enteritidis. However, the capacity of these methods to discriminate between strains may vary with respect to the serovar (Johnson et al. 2001). The results obtained from plasmid analysis corresponded closely to previously published data on plasmids in *S.* Choleraesuis (Montenegro et al. 1991; Weide-Botjes et al. 1996). All isolates from both wild boars and domestic pigs carried the serovar-specific virulence plasmid of approximately 52 kbp but no other larger or smaller plasmids as found in other studies (Weide-Botjes et al. 1996). Therefore, plasmid analysis did not contribute to a further differentiation of the *S.* Choleraesuis isolates.

PFGE is generally accepted as a "gold standard" and the most powerful tool with the best reproducibility for the discrimination of even closely related bacterial isolates (Tenover et al. 1995, Hunter et al. 2005) and most suitable for epidemiological typing of *Salmonella* strains from the same serovar (Olson et al. 1993). Several PFGE analyses of *S.* Choleraesuis isolates have provided reliable information on the discrimination between e.g. field isolates and vaccine strains and also the association between isolates of human and pig origin (Liebisch and Schwarz 1996; Weide-Botjes et al. 1996; Chang et al. 2005; Asai et al. 2008). In the present study the combination of the data obtained from macrorestriction analysis with three different enzymes subdivided the 27 *S.* Choleraesuis isolates examined into three macrorestriction groups. Although the different sulfamethoxazole and streptomycin susceptibility patterns of the *S.* Choleraesuis strains did not enable a further differentiation of these groups they proved to be true the subdivision into the macrorestriction groups as all *S.* Choleraesuis isolates from group I revealed a lower MIC against these antimicrobials than the strains from groups II

and III. In case of *S.* Choleraesuis, biochemical characterisation may also be used as tool for further phenotypical discrimination (Le Minor et al. 1985). Not only the differentiation between the biovar Choleraesuis sensu stricto or variato Choleraesuis (H_2S negative) and the biovar Kunzendorf (H_2S positive) but also the differences in their fermentation patterns for sorbitol, melibiose and arabinose allowed an additional discrimination of the macrorestriction groups in altogether five epidemiological groups.

As this study describes for the first time the occurrence of *S.* Choleraesuis in a wild boar population, aspects on the epidemiology of the disease can only be concluded from investigations in domestic pigs. The finding that the single epidemiological groups occur almost always in only certain regions of the federal state Thuringia indicates (1) that wild boars live and move only in special parts of Thuringia or (2) that there are barriers between regions which do not allow an exchange of the *Salmonella* organisms between different herds of wild boars, and (3) that the *S.* Choleraesuis strains belonging to the different epidemiological groups persist and circulate in the wild boar populations in the corresponding regions. If there is plenty of food, the boars will stay in a territory of ca. 15 km^2; however, this territory may be considerably enlarged if there is a lack of food. Under these circumstances different wild boar herds may share the same region and possibly exchange not only *S.* Choleraesuis organisms but also other pathogens. This assumption is supported by the occurrence of the two genetically different (II and III) but biochemically (B) identical epidemiological groups in the south-western region of Thuringia. Although it is not known whether both epidemiological groups of *S.* Choleraesuis occur in only one herd of wild boars or each epidemiological group in a different herd, there is a circulation in two herds in the region as the *S.* Choleraesuis strains were regularly isolated in the period from 2006 to 2008.

Since *S.* Choleraesuis is rarely isolated from non-porcine origin (Gray et al. 1996), the reservoir of the serovar seems to be limited to carrier wild boars and the contact with infected faeces is probably the most important source for infecting other animals. Therefore, an exchange of different strains of *S.* Choleraesuis between several herds requires that wild boar herds share the same territory. Presumably more than in domestic pigs the challenge dose for wild boars will vary considerably under natural conditions. It was also indicated that during natural transmission, the minimal infectious dose of *S.* Choleraesuis may be much lower than experimental models have described (Gray et al. 1996). The dose-dependent response of the host after exposure will therefore range from little or no colonisation with *S.* Choleraesuis over few clinical signs of disease to septicaemia and death (Fedorka-Cray et al. 2000). The higher susceptibility of young animals to infection could also be supposed by this study as the majority of wild boars with severe clinical signs and septicaemia were shoats and juvenile animals. As mainly diseased and shot wild boars were examined it might strongly be assumed that the Thuringian wild boar population harbours also an unknown percentage of animals carrying and shedding *S.* Choleraesuis organisms without showing clinical signs. This reservoir might be responsible for the persistence of *S.* Choleraesuis in the whole wild boar population. Faeces from infected wild boars are most probably the permanent existing source for *S.* Choleraesuis infections. This organism can survive for 2 to 4 months in swine faeces in the environment and remains infective to naive pigs from the same or other herds, which may be exposed orally and also by the highly infective intranasal route (Gray et al. 1996; Gray and Fedorka-Cray 2001).

Three other epidemiological groups (IA, IC and IID) of *S.* Choleraesuis identified in this study were separated from each other and also from groups IIB and IIIB, which were found in the same territory over the whole time of the study. The reason is not completely clear but a look on the topographic map shows that motorways divide Thuringia in the north and south or in the east and west, respectively, and this might be largely responsible for the subdivision of the whole wild boar population in this federal state. Therefore, it might be assumed that both natural barriers like mountains or mountain range and artificial barriers like arterial roads or railway lines cause the separation of wild boar but also other wildlife populations and as a result also their according pathogens.

The observation that the epidemiological groups IIIB and IA were detected not only in wild boars but also in domestic pigs strongly indicates that an exchange of *S.* Choleraesuis or rather an exposure of domestic pigs by this organism originating from wild boars may occur. Transmission of *S.* Choleraesuis and perhaps other pathogens like *Brucella suis* or *Trichinella spiralis* (Kruse et al. 2004; Gottstein et al. 2009) might be of special importance in case of keeping domestic pigs in free range or other outdoor farming systems.

Moreover, the finding that wild boars may carry *S.* Choleraesuis (in the present study 20.3% of all wild boars examined) or other serovars of *Salmonella* and also other pathogens are reasons to reinforce attention on game meat inspection in order to improve the safety of pork from wild boars. Therefore, both regular inspection of meat from wildlife by official veterinarians and advice of hunters and persons who prepare and consume wild boar meat are essential measures for reducing risks to human and domestic pig health.

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