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Reappearance of Salmonella serovar Choleraesuis var. Kunzendorf in Danish pig herds

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ABSTRACT

Salmonella enterica serovar Choleraesuis is a porcine adapted serovar which may cause serious outbreaks in pigs. Here we describe outbreaks of salmonellosis due to *S. Choleraesuis* in four Danish pig farms in 2012–2013 by clinic, serology, and microbiology and compare the isolates to those of a previous outbreak in 1999–2000. The infection was in some herds associated with high mortality and a moderate to high sero-prevalence was found. In 2012–2013 the disease contributed to increased mortality but occurred concomitant with other disease problems in the herds, which likely delayed the diagnosis by up to several months. Nine isolates from the four farms in 2012–2013 and 14 isolates obtained from the outbreak in Denmark in 1999–2000 were subjected to typing using pulsed-field gel electrophoresis (PFGE). Seven isolates were selected for whole genome sequencing (WGS). The PFGE results of 23 isolates displayed five different profiles. The isolates from 2012 to 2013 revealed two distinct profiles, both different from the isolates recovered in 1999–2000. Two of the 2012–2013 farms shared PFGE profiles and had also transported pigs between them. The profile found in the two other 2012–2013 farms was indistinguishable but no epidemiological connection between these farms was found. Analysis of the number of single nucleotide polymorphisms (SNPs) from the WGS data indicated that the isolates from the farms in 2012–2013 were more closely related to each other than to isolates from the outbreak in 1999. It was therefore concluded that the infection was a new introduction and not a persistent infection since the outbreak in 1999. It may further be suggested that there were two or three independent rather than a single introduction. The re-introduction of *S. Choleraesuis* in Denmark emphasizes the importance of strict hygiene measures in the herds. Further investigations using WGS are now in progress on a larger collection of isolates to study clonality at European level and trace the origin of the infections.

1. Introduction

Pork is one of the most important sources of human foodborne salmonellosis in the EU (EFSA, 2013) and the USA (Gould et al., 2013). Pigs can be colonized with a

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variety of *Salmonella* serotypes (EFSA, 2008, 2009) but mostly, pigs are asymptomatic carriers. In Denmark, the most common *Salmonella* serovars in pigs are *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (including monophasic *S.* 4,[5],12:i:-), *S.* Derby and *S.* Infantis (Argüello et al., 2013, 2014). These serovars may also cause clinical salmonellosis in pigs, but the extent of clinical salmonellosis in pigs in Denmark is uncertain.

S. Choleraesuis is a serovar, which is host-adapted to pigs, and may cause serious outbreaks of salmonellosis and paratyphoid (Griffith et al., 2006). The majority of the *S.* Choleraesuis outbreaks in pigs are caused by var. Kunzendorf (Fedorka-Cray et al., 2000). In the USA, *S.* Choleraesuis was by far the most frequently found serovar in pigs until the mid-1990s. In 1986, 71% of the isolates from pigs were *S.* Choleraesuis, but thereafter the prevalence of this serovar declined while other serovars increased, and from 1995 and onwards, *S.* Typhimurium and *S.* Derby have been most prevalent (Foley et al., 2008). Yet, in 2005 *S.* Choleraesuis still constituted 9% of all clinical *Salmonella* isolates from pigs in the USA (Foley et al., 2008). In Europe, *S.* Choleraesuis is a relatively rare serovar, both in slaughter pigs and in breeding herds but it has been reported with low frequency in a number of countries (EFSA, 2008, 2009). Out of 42,417 isolates from pigs and pork in 2011, 695 were *S.* Choleraesuis (EFSA, 2013), but its significance as source of clinical salmonellosis – human or in pigs – is not known.

In the USA, the disease is typically a porcine post weaning disease with septicaemia, enterocolitis and pneumonia and it has been reported to occur most often in farms where pigs of different ages and litters are mixed (Anderson et al., 2000). *S.* Choleraesuis seems more often to be isolated from non-gastrointestinal organs than other serovars, most notably from the lungs (Gray et al., 1996).

In humans, *S.* Choleraesuis tends to be more invasive and cause less gastrointestinal manifestations than most other serotypes and thus, it is a serious infection with a significant mortality (Cohen et al., 1987). Yet, this organism is not a common human pathogen in EU (EFSA, 2013) or in the USA in spite of its relatively high prevalence in American pigs (CDC, 2008). In Denmark, the latest case of human infection with *S.* Choleraesuis was a var. Decatur case in June 2012 and before that a var. Kunzendorf case in December 2011, both travel related (Dr. Eva Møller Nielsen, Statens Serum Institut, Copenhagen, personal communication). However, in Asian countries, such as Thailand and Taiwan, this serovar continues to be important for human illness (Chiu et al., 2004; Hendriksen, 2010), although the incidences seem to be declining (Su et al., 2014).

In Denmark, *S.* Choleraesuis was last found in pigs at an outbreak in 1999 (Baggesen et al., 2000), but in 2012 and 2013 it reappeared with outbreaks of severe salmonellosis in four farms. It has neither in relation to the outbreak in 1999 (Baggesen et al., 2000) nor to the outbreaks in 2012 and 2013 been possible to identify the primary introduction of infection to the Danish pig herds. This may have been due to limitations in the epidemiological information available but also by an insufficient resolution of isolates by the epidemiological typing methods applied.

In the present study, we describe the reappearance of *S.* Choleraesuis in Danish pig farms during 2012 and 2013. We investigated the clonality of those isolates by the application of pulsed-field gel electrophoresis (PFGE), antimicrobial susceptibility testing (MIC), and whole genome sequencing (WGS), and compared to isolates from the previous Danish outbreak in 1999.

2. Material and methods

2.1. Farm data Salmonella isolates for epidemiological investigations

Farm data was retrieved from observations made by the Danish Pig Research Center and registrations via the *Salmonella* control programme (https://www.retsinformation.dk/Forms/R0710.aspx?id=141725). Data from the serological meat juice surveillance for Salmonella was extracted from the Danish Zoonosis Register. The serological test includes LPS antigens from *Salmonella* serovars *S.* Typhimurium and *S.* Choleraesuis and covers the O factors O1, O4, O5, O6, O7 and O12 (Nielsen et al., 1995). The herds were assigned to one of three infection levels on the basis of serological examination of meat juice samples collected at the slaughterhouse and action was taken for herds reaching levels two or three (Alban et al., 2012). Serological results for the four farms were extracted for the period 2010–2014 in order to analyse the time before, during, and after the diagnosis was made in the farms. Other farm data was retrieved from the Central Herd Register (https://chr.fvst.dk). Twenty-three S. Choleraesuis isolates from an outbreak on four pig farms in 2012–2013 ($n = 9$) and an outbreak in 1999–2000 ($n = 14$) were included in the study and subjected to PFGE analysis. On the basis of the PFGE results, seven isolates were further analysed using WGS and MIC determination, including three from the outbreak in 1999–2000 and one from each of the outbreaks on four different farms in 2012–2013.

2.2. Serotyping and biotyping

Serotyping was performed by slide agglutination with polyclonal antisera (Statens Serum Institut, Copenhagen, Denmark) according to the White–Kauffmann–Le Minor scheme (Grimont and Weill, 2007) and distinction between *S.* Paratyphi C, *S.* Typhisuis and the biovars of *S.* Choleraesuis, var. Kunzendorf and var. Decatur, was performed by biochemical tests (Grimont and Weill, 2007).

2.3. Pulsed-field gel electrophoresis

PFGE was carried out according to the PulseNet protocol as previously described (Ribot et al., 2006) using *Xba*I (Fermentas, Lifesciences) as restriction enzyme and electrophoresis carried out in a Chef-DR® -III (Bio-Rad®). Banding patterns were analysed in BioNumerics® version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) with a position tolerance of 1.5% and optimization of 1.5%. Results were compared using the Dice coefficient for similarity and unweighted pair group method with arithmetic averages (UPMGA) for clustering.