Update on *Actinobacillus pleuropneumoniae* - knowledge, gaps and challenges

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**Abstract**

Porcine pleuropneumonia, caused by the bacterial porcine respiratory tract pathogen *Actinobacillus pleuropneumoniae*, leads to high economic losses in affected swine herds in most countries of the world. Pigs affected by peracute and acute disease suffer from severe respiratory distress with high lethality. The agent was first described in 1957 and, since then, knowledge about the pathogen itself, and its interactions with the host, has increased continuously. This is, in part, due to the fact that experimental infections can be studied in the natural host. However, the fact that most commercial pigs are colonised by this pathogen has hampered the applicability of knowledge gained under experimental conditions. In addition, several factors are involved in development of disease, and these have often been studied individually.

In a DISCONTOOLS initiative, members from science, industry and clinics exchanged their expertise and empirical observations, and identified the major gaps in knowledge. This review sums up published results and expert opinions, within the fields of pathogenesis, epidemiology, transmission, immune response to infection, as well as the main means of prevention, detection and control. The
gaps that still remain to be filled are highlighted, and present as well as future challenges in the control of this disease are addressed.

Introduction

*Actinobacillus pleuropneumoniae* (App) is a small, Gram-negative, encapsulated rod with typical coccobacillary morphology. The bacterium is the etiological agent of porcine pleuropneumonia, a contagious respiratory disease that affects pigs and causes economic losses in the swine industry worldwide (Gottschalk, 2012). The organism was first isolated in 1957 in Great Britain (Pattison et al., 1957) and originally named *Haemophilus pleuropneumoniae* (Shope, 1964). App was later assigned to the *Pasteurellaceae* family and *Actinobacillus* genus (Pohl et al., 1983). Acute disease, characterised by fibrino-haemorrhagic and necrotizing pleuropneumonia, is often fatal. Asymptomatic carriers of the bacterium, either those having survived acute disease or those that were subclinically infected, may harbour App in nasal cavities, tonsillar crypts, and chronic lung lesions, thus becoming a source of infection for naïve subpopulations (Chiers et al., 2002a; Tobias et al., 2014b). The estimation of the economic burden of this disease is mainly based on the occurrence of acute outbreaks characterised by high mortality, loss in production, and high medical costs. Results from different studies about the impact of App infections on production parameters vary greatly. Few studies have confirmed the broadly accepted perception that average daily weight gain and feed conversion rate are negatively affected by the disease, mainly in pigs with chronic lung alterations (Holmgren et al., 1999; Hoflack et al., 2001; Straw et al., 1990). A reduction in daily weight gain by 33.6% and decrease in feed efficiency ranging from 0.77% to 25.5% have been reported (Holmgren et al., 1999). Positive serological findings alone were not correlated with a decrease in average daily weight gain (Andreasen et al., 2001).

Severity of disease can be influenced not only by differences in virulence potential of different App isolates, but also by differences in susceptibility of the host. The latter can be affected by intrinsic factors such as presence/absence of
specific or cross-reactive antibodies (Devenish et al., 1990b; Chiers et al., 2002a), as well as stress or co-infections with other pathogens (van Dixhoorn et al., 2016; Marois et al., 2009), and extrinsic factors such as poor husbandry and insufficient biosecurity (Beskow et al., 1998; Maes et al., 2001; Rosendal and Mitchell, 1983).

Although considerable research has been done on App disease pathogenesis and virulence mechanisms, as well as host immune mechanisms, there is still a lack of solutions for dealing with the disease under field conditions. Gaps in knowledge exist regarding: factors triggering disease outbreaks in subclinically infected herds; appropriate diagnostic tools and procedures to differentiate between subclinically infected pigs and those which do not carry the pathogen; genetic markers for naturally occurring disease resistance in pig breeding lines; how to prevent App persistence; and how to achieve optimal immunity in endemically infected herds by vaccination.

This review summarises the work of the ‘Actinobacillus pleuropneumoniae DISCONTOOLS expert group’ (www.discontools.eu, swine A. pleuropneumonia), who exchanged their knowledge regarding different aspects of the bacterium and the disease, and highlighted gaps and challenges that should be addressed in future research (Table 1).

Description of disease

Natural infection and co-infection

Only animals belonging to the Sus scrofa species, namely wild boar, domestic and feral pigs, are infected by App, likely due to requirement for some host specific factors such as cell surface ligands and iron source (Schryvers and Gonzalez 1990; Jacques and Paradis 1998; Hamer-Barrera et al., 2004). More than 50% of the wild boar population in Slovenia was found to be seropositive (Vengust et al., 2006), while in Germany 35.8% of hunted wild boars were PCR positive for App (Reiner et al., 2010), thus raising awareness of the possible role of wild boars as a source of infection. In Canada, wild boars were found to be seropositive for
serovar 14, which has never been described in domestic pigs in America (McGregor et al., 2015). Two possible ways wild boars may become infected are: contact with infected domestic swine, which would require close contact between the two species, or contact with infected wild boars from other countries. The high prevalence of serovar 14, which is present in Europe (Nielsen et al., 1997), but has never been isolated in North America in domestic pigs, supports the second theory. European wild boars were introduced in the 1900s, and have remained a part of the wildlife population in North America since then (Mayer and Brisbin, 1991). The actual risk of transmission of App from this wild reservoir to conventional pig farms should be further assessed.

Porcine contagious pleuropneumonia can occur in different clinical forms. The peracute form is characterised by a high mortality rate and sudden death. Clinical signs resemble those of a systemic shock: cardiovascular failure, high fever (41°C), dyspnoea with mouth breathing, subsequent drop in the rectal temperature and apathy. A typical anamnesis is the finding of dead animals without any premonitory signs, and with typical bloody and foamy nasal discharge.

In acute disease outbreaks, morbidity can range from 10-100%, and mortality of 1-10% has been described in the literature (Klinkenberg et al., 2014; Fenwick and Henry, 1994). Anorexia, fever (40.5-41°C; 104-105.8°F), and severe respiratory distress are predominant signs. Acute lung damage developing in the first days after infection is decisive for the further course of disease (Hoeltig et al., 2009). Pigs with subacute disease show milder symptoms with lower fatality. Frequently, pigs that overcome acute disease remain chronically infected, showing no clinical signs, but harbouring chronic lung alterations such as fibroblastic pleurisy and lung tissue sequesters surrounded by fibrotic tissue (Liggett et al., 1987; Merialdi et al., 2012). Chronic infections without previous acute stages of disease also occur. Factors affecting the variability of the course of disease, especially the development of subclinical carrier status (such as host age and/or breed, environmental, and bacterial strain-related virulence determinants), should be further analysed. In a recent study, the beneficial effect
of environmentally and socially enriched pens on the outcome of co-infection with mild virulent App and Porcine Reproductive and Respiratory Virus (PRRSV) strains was shown, and supported the hypothesis, that the hypothalamic-pituitary-adrenal (HPA) axis and mood of pigs can impact on susceptibility to disease (van Dixhoorn et al., 2016).

Primary infections with other respiratory pathogens like Aujeszky disease virus and/or Mycoplasma hyopneumoniae can worsen the course of the disease (Sakano et al., 1993; Marois et al., 2009). Experimental dual infections with swine influenza virus (SIV) and App resulted in increased severity of SIV-like lesions, and enhanced viral replication in the lung and nasal SIV shedding (Pomorska-Mól et al., 2017). Additionally, the association of App/PRRSV has been studied (Pol et al., 1997; van Dixhoorn et al., 2016). Although many practitioners in Europe believe that the co-existence of these two pathogens in pigs may have a significant impact on the health status of a herd, experimental studies showed that a previous PRRSV infection did not always enhance the severity of the disease caused by a secondary App infection (Pol et al., 1997). Even though the recovery of both pathogens from the same pig was associated with pleuritic lesions (Fablet et al., 2012), the mechanism of interaction between App and PRRSV still needs to be clarified. More recent co-infection studies with a mild virulent PRRSV strain followed by a mild virulent App strain resulted in histological lung lesions, while this was not the case in mono-infected pigs (van Dixhoorn et al., 2016). On the other hand, in an in vitro model, App appeared to induce cell cycle arrest in the G2/M-phase and inhibit PRRSV replication (Ferreira Barbosa et al., 2015) resulting in an antiviral activity (Lévesque et al., 2014). It cannot be excluded that the order and interval between both infections is decisive for the final outcome of co-infection.

Experimental infection of pigs and infection of other hosts

Experimental App infection in swine is widely used to elucidate host-pathogen interactions, to test vaccine candidates and the efficacy of new therapeutic approaches. Also, in naïve pigs, outcome of infection depends on the virulence of the strain, dose, and the route of infection (Baarsch et al., 2000).
Jacobsen et al. (1996) showed little difference in virulence between biovar 1 serovars 2, 5b and 6 strains, whereas a biovar 2 untypable strain was less virulent, in an aerosol model of infection. Aerosol infection with $10^2$ colony forming units (CFU)/litre (inhaled for ten minutes), of App serovar 2 (Menzel et al., 2014), serovar 7 (Hoeltig et al., 2009), and serovar 9 (Maas et al., 2006a) strains, induced acute disease. Doses of $10^{3-10}$ CFU of the same App serovar 2 strain were given intratracheally to fattening pigs, and a dose of $10^3$ CFU found to be adequate for the development of clinical signs and lung alterations, but not death (Hennig-Pauka et al., 2008). The adequate dose of the same strain for intranasal infection was $4 \times 10^4$ CFU (Sassu et al., 2017b).

A standardised dose-response challenge experiment by endobronchial infection was documented by van Leengoed et al. (1989). While intratracheal infection is most appropriate to guarantee infection of the lung with a defined number of bacteria, nasal infection leads to milder symptoms due to loss of the inoculum by coughing and swallowing, with a chance to produce chronic infection (Tobias et al., 2013).

Other than the natural host, the most widely used infection model to assess virulence of App is the mouse, but rats and guinea pigs have also been used (Idris et al., 1993; Montaraz et al., 1994; Perfumo et al., 1999). Dependent on strain, high doses ($10^{8-12}$ CFU) of bacteria are typically given to mice (5-10 per group) by intraperitoneal injection, and the numbers of surviving/dead animals at specific time points recorded. The results obtained may reflect App toxin and/or lipopolysaccharide (LPS) shock. A correlation between virulence potential in the pig and mouse, e.g. attenuation of the znuA mutant, has been reported (Yuan et al., 2014).

Infection of the larva of *Galleria mellonella* (the Wax Moth), has also been used to assess the comparative virulence of App wild-type and mutants (Pereira et al., 2015). As it has been shown in the pig (Subashchandrabose et al., 2013), an App *hfq* mutant was attenuated for virulence in *G. mellonella* (Pereira et al., 2015). These results suggest that the App-*G. mellonella* model of infection has promise to
identify App mutants attenuated for virulence. However, further work with different App serovars and types of mutants (e.g. metabolic, LPS, Apx toxin), is required to determine the full extent of correlation of virulence in *G. mellonella* with that obtained in the pig.

Reports about naturally occurring disease due to App in hosts other than swine are rare. Recently, a bacterium with a 16S rDNA gene showing 99% homology with that of App serovars 3 and 7 was recovered from layer hens with upper respiratory infection (Pérez Márquez et al., 2014). While experimental infection of chicken embryos with this isolate resulted in 100% mortality, challenge experiments in specific-pathogen-free (SPF) chickens were inconclusive (Pérez Márquez et al., 2014). In addition, only one report of human 'infection' is known, which describes a local gangrenous necrosis of the thumb provoked by an accidental injection of a live attenuated App strain (Rycroft et al., 2011). However, App is not considered a zoonotic agent.

**Disease pathogenesis**

Pathobiology of App infection has been intensively studied [see reviews (Bossé et al., 2002; Chiers et al., 2010)]. The incubation period preceding appearance of clinical signs of App infection can be extremely variable. It can be as short as 12 hours, especially under the influence of stress factors such as mixing, moving or weaning, and the first cases of death can be observed as early as 24 hours after infection (Gottschalk, 2012).

A variety of virulence factors have been described for App, and can be allocated to the categories of adhesion, acquisition of nutrients, induction of lung lesions, evasion of immune system and persistence (Bossé et al., 2002; Chiers et al., 2010). Iron metabolism is of high importance for the pathogen to survive and multiply in the host. It has been shown recently that catecholamine binding to App facilitates iron uptake, although iron availability is highly decreased during acute infection as a physiological acute reaction during inflammation (Humann-Ziehank et al., 2014; Li et al., 2015). The fact that more than 50 App genes are involved in
iron uptake and metabolism (Xu et al., 2008), some of which are differentially expressed during infection (Deslandes et al., 2010; Klitgaard et al., 2012), supports the major importance of this bacterial adaptation strategy for disease pathogenesis (Bossé et al., 2002). Genetically defined differences in disease susceptibility between breeding lines were, in part, due to differences in the iron-transport protein transferrin, because different protein variants might be bound with variable intensity by the transferrin binding receptors of App (Daniłowicz et al., 2010; Reiner et al., 2014a).

Of major importance, in regard to virulence, are the Apx toxins, with different degrees of cytotoxicity, haemolytic activity and distribution among serovars (Frey, 1995; Schaller et al., 2000; Yang et al., 2011; Sárközi et al., 2015). ApxI is strongly haemolytic and strongly cytotoxic, and is produced by serovars 1, 5a, 5b, 9, 10, 11, 14 and 16; ApxII is weakly haemolytic and moderately cytotoxic, and occurs in all serovars except for 10 and 14; ApxIII is non-haemolytic, strongly cytotoxic, and is expressed by serovars 2, 3, 4, 6, 8 and 15. A fourth RTX toxin, ApxIV, has not been characterised with regards to haemolytic or cytotoxic capacity. It is produced by all serovars in vivo and is therefore widely used for diagnostics (Dreyfus et al., 2004), although some isolates not producing ApxIV have been reported (Tegetmeyer et al., 2008).

High levels of hyaluronidase (an enzyme involved in the degradation of the interstitial barrier), that may lead to enhanced infiltration of the pathogen in the lung, were detected in bronchoalveolar lavage fluid (BALF) of pigs experimentally infected with App (Kahlisch et al., 2009).

**Epidemiology of App strains**

So far, 16 serovars of App are known, which differ in their capsular polysaccharide composition, with serovar 16 identified only recently (Bossé et al., 2017b; Sárközi et al., 2015). Depending on their requirement for nicotinamide adenine dinucleotide (NAD) to grow, App strains can be further classified as biovar I (also called “typical”) that are NAD-dependent, or biovar II (or “atypical”)
that are NAD-independent. Normally, serovars 1-12 and 15-16 are biovar 1, and serovars 13 and 14 are biovar 2. However, some biovar 2 isolates belonging to serovars other than 13 and 14 (Dom and Haesebrouck, 1992; Beck et al., 1994; Maldonado et al., 2009), as well as a biovar 1 serovar 13 isolate (Perry et al., 2012), have been identified. Additionally, some untypable isolates have been reported (Kokotovic and Angen, 2007; Ito et al., 2016). Different serovars/biovars are predominant in different countries, however, temporal changes in geographic distribution of serovars have been reported (Blackall et al., 2002; Gottschalk and Lacouture, 2014; Gottschalk, 2015). Assessment of the virulence of strains and characterization of their antigenic profile is a diagnostic challenge, especially when multiple serovars can be present on a farm and also within one individual pig (Broes et al., 2007).

It has been shown that virulence of App strains can be serovar- or biovar-related, which is dependent, to some extent on, the respective production of Apx toxins (Dom and Haesebrouck, 1992; Beck et al., 1994; Frey, 1995; Jacobsen et al., 1996). Reported differences in virulence of certain isolates of the same serovar may be due, at least in part, to lack of production of one of the Apx toxins through deletion, point mutation, or insertion of a transposon such as ISApl1. For example, serovar 2 strains from Europe are highly virulent, while North American isolates of the same serovars are almost non-virulent (Gottschalk, 2015). The low virulence North American biovar 1 serovar 2 isolates lack ApxIII, which is expressed by most European biovar 1 serovar 2 isolates. One UK biovar 1 serovar 2 isolate was reported to lack ApxIII, as did two biovar 2 serovar 2 isolates from Switzerland; whereas a biovar 1 serovar 2 isolate from the Netherlands was found to be lacking ApxII (Beck et al., 1994). Beck et al. also reported isolates of serovar 6 lacking ApxII (Denmark), serovar 10 lacking ApxI (Norway), and serovar 11 lacking ApxII (Switzerland), though the relative virulence of these isolates was not investigated (Beck et al., 1994). Atypical serovar App isolates that only have genes for ApxIII and ApxIV have been recovered from the lungs of a few pigs in Germany and Switzerland (Kuhnert et al., 2011). The Swiss isolates were confirmed as serovar 3 by PCR, whereas the German isolates did not produce a specific amplicon in diagnostic mPCR for any of serovars 3, 6 or 8, and no further
investigation was made to determine the serovar for these isolates. There has been a report of a presumably less virulent serovar 1 isolate in Canada, lacking ApxI, recovered from the tonsils of clinically healthy pigs (Broes et al., 2007). Moreover, Kamp et al. reported the existence of a serovar 2 and a serovar 9 isolate from Australia, both of which produced ApxII only, and a serovar 7 isolate from the Netherlands that produced ApxII and ApxIII instead of only ApxII (Kamp et al., 1994). Although in most cases, the mechanism of toxin loss has not been investigated, some serovar 7 isolates were reported to spontaneously delete the genes encoding ApxII, through homologous recombination between direct repeats flanking the toxin genes (Anderson et al., 1991). In some cases, reported differences in virulence of certain serovars from different countries may be due to serological errors in typing. For example, serovar 3 isolates are normally considered less virulent, however it was considered to be the most prevalent disease-causing serovar in the UK until molecular typing by PCR revealed that most clinical isolates were, in fact, serovar 8 (O’Neill et al., 2010).

The awareness that virulence of App serovars can differ, illustrates the need for establishment of an epidemiological database summarising on-going serovar prevalence (indicating method of typing) over time at the country, region and herd level. Additional information regarding presence/absence of virulence factors such as the Apx toxins would also be beneficial. Factors other than Apx toxins may also contribute to serovar-specific differences in virulence. However, other than capsule and LPS, which, by their nature contribute to differentiating serovars, little is known regarding the distribution of other various putative virulence factors amongst the different serovars of App. A genomic comparison has indicated potential virulence-associated genes conserved amongst the more virulent serovars (Xu et al., 2010), however this study was based on genome sequences from single isolates, and not all serovars were represented. In most cases, genomes are available for serovar reference strains rather than clinical isolates. Most are draft sequences, and complete genomes are only available for four serovars (Genomes OnLine Database: https://gold.jgi.doe.gov, last access on 15.02.2017). More whole genome sequences (representing multiple clinical
isolates of each serovar), along with relevant metadata, are required to allow meaningful interrogation of virulence-related traits.

Transmission

With regard to the occurrence of clinical signs, a mathematical simulation study provided evidence that, in most outbreaks, disease is more likely caused by a trigger in already colonised pigs (Klinkenberg et al., 2014). Therefore, knowledge on the transmission between colonised animals is highly relevant for design of preventive measures. Transmission from pig to pig occurs mainly by direct oral or nasal contact or by droplets of aerosol spread over short distances of 1-2 metres (Lechtenberg et al., 1994; Velthuis et al., 2003; Kristensen et al., 2004b; Tobias et al., 2014a). Transmission of App by aerosols generated during coughing, sneezing and by fluids from the respiratory tract was shown nearly 50 years ago (Nicolet et al., 1969). Highest infectivity was proven for pathogen-containing droplets 0.5-3 µm in diameter, which are inhaled deeply into the lung alveoli (Nicolet et al., 1969). Subclinically infected pigs are of major importance as the source of infection for distribution of the bacterium (Fenwick and Henry, 1994; Torremorell et al., 1997). An experimental transmission study indicated that direct contact was required for spread of App from infected to uninfected animals; no indirect transmission was observed to uninfected pigs housed in separate pens in the same room as infected pigs (Tobias et al., 2013). Under field conditions, indirect transmission was found to be ten times less efficient than direct contact (Tobias et al., 2014a). Nevertheless, there exist some epidemiological empirical reports about air or indirect transmission of App over longer distances up to kilometres (Desrosiers and Moore, 1998). For direct transmission in clinically affected pigs, the severity of clinical signs was inversely related to efficient transmission (Tobias et al., 2013).

Early transmission from infected sows to their offspring was confirmed from the tenth day of life (Vigre et al., 2002). Although it is assumed that, in infected herds, most sows carry the pathogen, transmission to suckling piglets does not take place in all litters (Tobias et al., 2014b). For this reason, litter-wise
grouping of piglets after weaning can help to reduce transmission within a herd (Tobias et al., 2014a). Longer suckling periods - although improving welfare of piglets - might have negative effects on dynamics of infection, because they result in more colonised piglets at weaning if the sow is the source (Vigre et al., 2002; Tobias et al., 2014b). The impact of maternal antibodies on colonisation is uncertain (Tobias et al., 2014a). High levels of maternal antibodies induced by a specific serovar may prevent homologous colonisation. However, a different serovar may result in low levels of maternal antibodies, so that transmission to and colonisation of piglets will be more prevalent (Broes et al., 2007).

Disease in suckling piglets is a rare event, but is especially prevented by proper levels of protective maternal antibodies, which can be induced either by infection or vaccination (Krejci et al., 2005). The time until maternal antibodies in the suckling period decrease below detection limits is reported to range between two weeks after birth (Vigre et al., 2003) to the twelfth week of age, depending on the level of antibodies in sows and the serological test used, leaving piglets highly susceptible to infection (Gardner et al., 1991; Chiers et al., 2002a; Cruijsen et al., 1995). It is assumed that the decline in maternal antibodies is associated with replication of bacteria in the tonsils, and with a higher risk of disease development from the third month of life (Chiers et al., 2002a; Cruijsen et al., 1995; Sjölund et al., 2011b).

Experimental and field observations defined the transmission rates for App, with direct transmission among weaned pigs shown to be $\beta=0.06–0.1$ per day, and indirect transmission occurring with $\beta=0.006$ per day (Tobias et al., 2014a; Velthuis et al., 2002). Transmission of the agent among pigs mostly occurs without the development of any clinical symptoms. In an experimental setup, severely affected pigs shed more bacteria, but they transmitted App less efficiently than mildly affected pigs. A possible explanation put forward was that behavioural changes resulted in reduced contact rates between diseased and contact pigs (Tobias et al., 2013).
Infection dynamics of App within herds and recommended diagnostic methods are summarised from literature reports in Figure 1 (Gottschalk, 2015; Vigre et al., 2002; 2003).

**App in the environment and biofilms**

Regarding indirect transmission, App is not known to survive for long in the environment, especially in warm and dry conditions. It can survive for longer periods if embedded in organic material, and up to 30 days if in water at 4 °C (Assavacheep and Rycroft, 2013). There has been one report that App may use biofilm formation as a strategy to survive in drinking water on swine farms (Loera-Muro et al., 2013).

Anaerobic conditions during early and chronic stage of infection lead to upregulation of genes involved in central metabolism and biofilm formation (Buettner et al., 2009; Deslandes et al., 2010; Klitgaard et al., 2012; Li et al., 2014). Enhanced production of biofilm under anaerobic conditions might reflect bacterial adaptation mechanisms to environmental stress in early infection, in damaged host tissue as well as during colonisation (Klitgaard et al., 2012; Li et al., 2014; Auger et al., 2009). The transcriptional profiles of App during acute infection indicate metabolic adjustment is decisive for virulence (Deslandes et al., 2010; Klitgaard et al., 2012), and global anaerobic regulators (ArcA and HlyX) and anaerobic enzymes (AspA, DmsA, FrdABCD) are essential for infection (Jacobsen et al., 2005; Baltes et al., 2003, 2005; Buettner et al., 2008a, 2008b, 2009). Most App field isolates are able to form biofilms in response to stress and under specific growth conditions (Kaplan and Mulks, 2005; Labrie et al., 2010; Bossé et al., 2010), which may contribute to increased resistance of the pathogen to antibiotics as well as immune defense mechanisms (Izano et al., 2007; Fux et al., 2005). Poly-N-acetylglucosamine (PGA) is one major component of biofilm formation and respective PGA synthesis genes are located in the pga operon (Kaplan et al., 2004). Biofilms comprising bacterial aggregates embedded in host material were recently described in naturally infected pigs (Tremblay et al., 2016).
Immune response to infection

Genetically determined innate immune mechanisms are considered to be decisive for naturally occurring resistance against App in different pig breeding lines and are crucial for the later outcome of disease (Hoeltig et al., 2009). It was shown after experimental infection that a clinical respiratory health score was highly correlated to the severity of lung lesions, and that acute lung damage varies between pig breeding lines and was decisive for the course of disease (Hoeltig et al., 2009). Quantitative trait loci (QTL) associated with resistance were found on S. scrofa chromosome 2 (SSC2); candidate genes on SSC2 are IL-9 and CD14, which are involved in lung immunity and LPS recognition, respectively (Reiner et al., 2014a). Further, candidate genes on other chromosomes were members of the signal transducer and activator of transcription (STAT) gene family, which, depending on the type of cytokine receptor that is activated during the infection, can selectively stimulate multiple signal pathways during the inflammatory response (Reiner et al., 2014b). In the past, some hints that multi-trait selective breeding may enhance humoral responses induced by commercial vaccines were reported (Magnusson et al., 1997). Nevertheless, more studies are needed to verify the existence of specific genes that are directly responsible for resistance to App infection.

Innate immune response against App infection

Cytokines appear early in the blood stream after experimental infection, but the high variability in their kinetics has led to differences in interpretation of the significance of the results (Fossum et al., 1998; Huang et al., 1999; Baarsch et al., 1995). Recently, to detect variation in cytokine serum concentrations during the early response, IL-1β, TNF-α and IL-6 were measured by multiplex analysis every two hours for 18 hours post App infection. Mean peak serum concentrations of TNF-α and IL-6 appeared at 12 and 10 hours post infection, respectively (Wyns et al., 2015). Among pro-inflammatory cytokines, IL-6 was found to be reliable both for monitoring bacterial infections in pigs (Fossum et al., 1998), and for evaluating the efficacy of antibiotic treatments (Lauritzen et al., 2003).
Additionally, an induction of IL-17, as indicated by both transcriptional and protein expression levels, has been observed during App infection (Brogaard et al., 2015; Sassu et al., 2017b).

Host transcriptional studies of App affected lungs (Cho et al., 2005; Podolska et al., 2012; Zuo et al., 2013; Brogaard et al., 2015), lymph nodes (Yu et al., 2013) and liver (Skovgaard et al., 2010) identified several differentially expressed genes. Differential gene expression of these organs during App infection has also been demonstrated by using cDNA microarrays (Hedegaard et al., 2007). In the liver, 51 genes were found to be differentially expressed in App infected animals; among them genes encoding acute phase proteins and cytokines such as IL-1, TNF-α, IL-6 and IL-8 were up-regulated, while those encoding α₁-acid glycoprotein and surfactant proteins were down-regulated (Skovgaard et al., 2010). Pig α₁-acid glycoprotein has been described to act as a negative acute phase protein in App infected pigs (Heegaard et al., 2013).

Acute phase proteins such as haptoglobin, C-reactive protein, major acute phase protein (MAP) and serum amyloid A have been designated as putative biomarkers to evaluate pig health status (Heegaard et al., 1998; Hulten et al., 2003; Heegaard et al., 2011), and their increase upon infection was observed in serum (Skovgaard et al., 2009; Gomez-Laguna et al., 2014), saliva and meat juice (Soler et al., 2013; Gomez-Laguna et al., 2010). Higher levels of fetuin A, as well as lower levels of haptoglobin and surfactant protein D, were suggested as biomarkers of the innate immune system for a higher resistance of pigs against App infection, mainly due to preventing excessive immune reactions detrimental to the host (Kahlisch et al., 2009).

Neutrophils, monocytes and macrophages are the first line of defence after infection (Liggett et al., 1987; Ondrackova et al., 2013). The antibacterial peptide PR-39, which is mainly produced by neutrophils but also other cell types (Gabner et al., 2017), is excreted but does not seem to be especially effective in killing App (Hennig-Pauka et al., 2006; 2012). In vitro, no growth inhibition of App was found with an approximate ten-fold concentration of PR-39 compared to that estimated
in the lung epithelial lining fluid (0.5 µM). However, resistance to PR-39 killing has
been described in App and mutation studies have indicated that, at least in part,
this mechanism is mediated by the SapA protein (Xie et al., 2017). It is
hypothesised that, within disease pathogenesis, this multifunctional peptide plays
a role in fibrotic tissue repair processes rather than displaying high-level
antibacterial activity against App. Other major soluble components of the innate
immune system, such as defensin and lactoferrin, were found to be more effective
than PR-39 against App assessed in artificial test systems (Luna-Castro et al.,
2014; Yang et al., 2015). Transgenic pigs expressing porcine beta-defensin 2 in
different organs showed a better protection against App infection than non-
transgenic pigs (Yang et al., 2015). Furthermore, ficolins, collagenous lectins that
bind bacteria in a N-acetylglucosamine (GlcNAc) dependent manner, are able to
bind App and can be distinguished in two forms: ficolin alpha mainly found in
porcine plasma (Brooks et al., 2003a) and ficolin beta expressed by activated
porcine neutrophils (Brooks et al., 2003b).

**Humoral immune response against App infection**

After a primary exposure to the pathogen in the lung, antibody titres may
be detected as early as 7-14 days, and increasing up to 4-6 weeks, after infection,
and can persist for several months (Gardner et al., 1991; Bossé et al., 1992).
Maternal antibodies are detectable up to an age of 2 to 12 weeks (Gardner et al.,
1991; Vigre et al., 2003). Piglets born to sows with high levels of antibodies against
App are able to mount a higher antibody response than pigs derived from sows
with low antibody levels (Sjölund et al., 2011b). Antibodies can neutralise the Apx
toxins and enhance the phagocytosis via opsonisation, thus reducing the severity
of clinical symptoms (Devenish et al., 1990a; Cruijsten et al., 1995). Nevertheless,
antibodies are not efficient in clearing App, especially when opsonisation and
complement-mediated killing is inhibited by the presence of a thick capsule
(Inzana, 1990; Rycroft and Cullen, 1990), and they cannot prevent the
development of carrier status (Haesebrouck et al., 1997; Dubreuil et al., 2000).
Infection of the tonsils may occur without induction of an immune response,
especially against toxins (Chiers et al., 2002b), thus impairing the detection of
subclinically infected animals (Gottschalk, 2015). Recently, a host-pathogen study reported low immunological responsiveness in tonsils and showed, in parallel, that App isolates from this host compartment undertake distinct metabolic adaptations to the upper respiratory tract as early as 8 hours after App intratracheal infection (Sassu et al., 2017a).

*Cell-mediated immune response against App infection*

Reports on cell-mediated immune responses against App are rare. However, Furesz et al. showed that cell-mediated delayed-hypersensitivity to App infection was positively associated with antibody responses (Furesz et al., 1997). Moreover, a difference in variation of CD4:CD8 ratio was reported in pigs immunised with low or high doses of App (Appleyard et al., 2002) and CD8α-γδ T cells were found to be increased in BALF after contact with bacterial antigen (Faldyna et al., 2005). Recently, the frequency of Th17 cells in blood and lungs of pigs chronically infected with App was shown to positively correlate with the presence of lung lesions and antibody titres (Sassu et al., 2017b). Whether the presence of specific T cells initiates the immune evasion of App, leading to its retreat to tissue sites that are less accessible to immune cells, needs to be further investigated. The function of specific T cell subpopulations and their role during the chronic stage of infection is not yet known. Studies that aim at characterising cell-mediated immune responses against App infection are of high relevance for disease prevention, since antigen-specific lymphocytes can be transferred via colostrum to suckling piglets (Nechvatalova et al., 2011).

**Diagnostic methods**

*Bacteriological and molecular biological examination*

Bacteriological examination of affected lung tissue obtained during necropsy is most adequate for diagnosis of respiratory disease caused by App (Runge et al., 1996; Hennig et al., 1998). Alternatively, in order to increase the number of animals tested, oral fluids and BALF can be sampled from living pigs.
Further options for living pigs are nasal swabbing or tonsillar scraping to collect representative samples from the upper respiratory tract. Since infected animals do not necessarily seroconvert (Chiers et al., 2002b; Cheong et al., 2016; Costa et al., 2011), tonsillar brush or scrape sampling with subsequent PCR diagnostics is the most sensitive method to confirm or refute negative serological test results in certified negative herds, although it is invasive and not easy to perform (Costa et al., 2011; Tobias et al., 2012). Bacteriological examination of tonsils is problematic because App resides deep in the tonsillar crypts, and commensal tonsillar bacteria tend to overgrow App in culture (Costa et al., 2011; Gottschalk, 2015). Improved diagnostic methods using enrichment of App by immunomagnetic isolation (Gagné et al., 1998; Angen et al., 2001), PCR (Fittipaldi et al., 2003) or by loop-mediated isothermal amplification (LAMP) (Yang et al., 2009) have been published for several serovars. The immunomagnetic separation assay has a good sensitivity but is time-consuming and expensive.

Under experimental infection conditions, PCR detection rates for App from oral fluid harvested from a cotton rope provided for eight pigs, for 20-30 minutes, differed between the serovars tested, and was only successful until day 7 after infection (Costa et al., 2011), indicating that this is not an appropriate approach for routine diagnosis, especially for subclinically or chronically affected pigs. The sensitivity of sampling living pigs can be increased by parallel PCR testing of nasal, tonsillar and oropharyngeal swabs. This approach was successful in detecting more than 30% of healthy carrier sows in a positive herd (Fablet et al., 2010). Although the direct detection of App from nasal swabs or tonsillar samples by PCR is more frequently used, the successful isolation of App strains is, in most routine laboratories, still the pre-requisite for determination of its serovar by PCR (Gottschalk, 2015).

Hitherto, the classification of the different serovars has been based on the recognition of capsular polysaccharides (CPS) and LPS antigens. Ito et al. described the nucleotide sequence analysis of the CPS loci as a major tool for determining non-typeable App isolates (Ito et al., 2016). The results showed that insertion element ISApl1 of App can interfere with both serologic and molecular
typing methods, and that nucleotide sequence analysis across the capsular gene clusters was an excellent tool for revealing the cause of serological non-typeability (Ito et al., 2016). A database with all the detected isolates needs to be available in order to detect new serovars or variants with mixed CPS and LPS loci.

Serological tests

The elaboration of effective prevention strategies in herds is based on specific and sensitive diagnostic tools, because not all App serovars have the same impact on the health status of the pigs.

The number of samples, the frequency of testing, the animals to be sampled and the test to be used depend, in part, on farm characteristics and the related risk of introduction of carriers (Dohoo et al., 2009). Moreover, it depends on the accepted probability of missing an introduction (false negative results), as well as the accepted probability of false positive results. Monitoring of App-free herds should be based on a combination of syndromic surveillance (absence of clinical signs), absence of suspected slaughter lesions, fallen stock monitoring and serological or, with lower sensitivity, bacterial monitoring with PCR based diagnostic tools. There is an urgent need for internationally accepted standards, as the international trade of pigs often ignores the infection status for App. Up to now, no widely accepted monitoring guidelines with respect to sampling size and frequency are available, which would depend on expected prevalence (Martin et al., 1992; Dohoo et al., 2009). Although there is no universal consensus on how a herd must be monitored to be classified as "negative", it may be recommended that at least 30 serum samples obtained from 6 month-old pigs should be tested between 2 to 4 times/year (unpublished data). In the Danish SPF system, for farms to be declared free from App, it is recommended that 20 animals be tested once a month over a time period of 24-36 months (www.gruppoveterinario suinicolomantovano.it/documenti/Szancer.pdf; accessed 31.05.2017). Some German associations recommend serological examination of 15 samples 4 times/year (unpublished data). For the interpretation of serological data, knowledge about prevalence of serovars isolated from diseased animals in
respective countries should be available, because clinically relevant serovars differ between countries, continents and also over time (Gottschalk, 2015), as discussed above.

Serological tests are available to allow the detection of antibodies against the bacterial species App, as well as serovar/serogroup-specific antibodies (Gottschalk, 2015). In Germany, approximately 80% of herds are serologically positive for a broad spectrum of serovars, mostly with no obvious clinical impact (Brackmann et al., 2015). An ApxIV-based ELISA is widely used for serological screening diagnostics, but positive results do not reveal any information on virulence or serovar (Gottschalk, 2015). In addition, the sensitivity of this method can be impaired, when a genetic insertion element (ISApII) inhibits the expression of ApxIV in specific App strains (Tegetmeyer et al., 2008). An LPS-based ELISA, with good sensitivity and specificity against predominant serovars/serogroups involved in clinical cases, can be used as an alternative test (Opriessnig et al., 2013; Gottschalk, 2015). Serological tests based on LPS antigens were found to be serogroup-specific under experimental and field conditions (Gottschalk, 2015). Tests based on ApxII are considered to be of lower specificity due to serological cross-reactions with ApxII toxins of other Actinobacillus species, such as A. suis or “A. porcitonsillarum”, that have almost identical biochemical and phenotypic profiles (Kuhnert et al., 2005). In swine herds which have been continuously confirmed to be negative for App, monitoring on a regular basis can be performed by species-specific tests of relatively low sensitivity but high specificity, because it can be expected that introduction of App in a naïve herd will lead to a significant seroconversion, followed by a high seroprevalence (Gottschalk, 2015). This might not be the case if only colonisation of the tonsils takes place (Chiers et al., 2002b; Tobias et al., 2013; Velthuis et al., 2002).

It is not possible to differentiate between maternal and acquired antibodies in piglets, and infected piglets will not seroconvert in the presence of maternal antibodies (Vige et al., 2002; 2003). If piglets are infected, it will take approximately 2-4 weeks until active seroconversion. A negative serological result can occur despite colonization of piglets’ tonsils. To perform a diagnosis in
this specific age group, the sows should be tested for antibodies to evaluate the risk for infection of the piglets (Gottschalk, 2015).

Recent serological studies in Germany revealed frequent detection of serogroup 3/6/8 (Seitz, 2014), while serovar 2 was most frequently isolated from pigs in this country (Dubreuil et al., 2000). This supports the hypothesis that low virulence serovars, occurring independently of disease, might boost specific immune reactions over time, and may superimpose serological findings for more virulent serovars.

**Prevention, control and treatment**

*Prevention and control*

Strategies to prevent porcine pleuropneumonia are mainly based on external biosecurity measures to avoid introduction of new serovars/strains by carrier pigs, as well as internal biosecurity measures to interrupt infection chains, e.g. by age-segregated rearing (Cleveland-Nielsen et al., 2002) and litter segregated rearing (Tobias et al., 2014a). In addition, identification and elimination of the so-called “triggers for disease” are decisive for disease control in infected herds (Klinkenberg, 2014). Several empirical reports confirm the influence of trigger and herd factors on the respiratory health status of pigs (Beskow et al., 1998; Maes et al., 2001; Rosendal and Mitchell, 1983). It can be hypothesised that all circumstances causing acute stress, such as mixing or transport, increase the risk that already colonised animals become clinically affected. In addition, all factors that impact mucosal immune defence mechanisms, such as dust or aerial ammonia, are likely to increase susceptibility to respiratory infectious disease (Seedorf, 2013). The mechanisms by which the various stress factors influence colonisation, pathogenesis and clinical outcome need to be further investigated, as suggested previously (Klinkenberg et al., 2014). Of high importance for future preventive approaches is the understanding of the complex interface between immune and nervous systems and how it can be modulated in farm animals.
The effect of stress hormones such as catecholamine on virulence factor expression in App has been shown at a molecular level (Li et al., 2015). In addition, a status of well-being of pigs in an enriched housing system had an influence on immune parameters and led to a reduction of lung alterations after experimental infection (van Dixhoorn et al., 2016). Moreover, delineating the interplay of App with the oral and nasal mucosa, as well as the microbiome, might be of importance in understanding the basis and kinetics of immunogenicity and the formulation of measures to prevent colonisation/carrier status (Alverdy and Luo, 2017).

To our knowledge, there exists no published report comparing different housing systems with respect to the prevalence of either App or App-related respiratory disease. The effect of husbandry and management risk factors on the prevalence of specific App serovars, independent of the type of housing system, has been evaluated (Maes et al., 2001). It was shown that the number of origins of purchased pigs and poor biosecurity measures were risk factors for prevalence of serovar 2, and the latter also for serovar 9, while a higher risk for prevalence of serovar 3 was found for climate factors (Maes et al., 2001). These results indicate that risk factors may vary depending on serovar. In out-door systems, potential abiotic risk factors for disease, e.g. ammonia or dust, might be negligible, whereas the influence of sub-optimal temperature regimes could also be a crucial factor in determining herd infection (Beskow et al., 1998). Furthermore, in areas where there are wild boars, which can have a high prevalence of App (Reiner et al., 2010), external biosecurity may be important to lower the risk of introduction of App into domestic herds in outdoor housing systems.

Control of pleuropneumonia and associated economic losses during an outbreak is based on: i) early detection of diseased pigs; ii) treatment of diseased and exposed pigs; iii) identification and removal of respective trigger factors. Meticulous daily clinical inspections are necessary to detect early signs of disease and to facilitate timely interventions. Comparing cleaning, vaccination and medication strategies (and combinations thereof) for mitigation of pleuropneumonia, it was found that the choice of protocol with regards to
economic value depended on prevalence and severity of disease as well as efficiency of the protocol used (Stygar et al., 2016). In peracute disease, pigs often die without showing any typical clinical signs (Gottschalk, 2012). A reduced feed intake in finishers can be an early indicator of forthcoming clinical disease. After the onset of first clinical symptoms, early treatment with effective antimicrobials is recommended. Metaphylactic treatment of exposed but not yet diseased pigs is necessary to minimise losses during an outbreak. Establishing the risk of exposure of pigs depends on identification of herd specific risk factors for dissemination of the bacterium and potential trigger factors, as well as the measures available to reduce or eliminate these risks.

An ambitious prevention strategy for the future is the development of genetic markers for selection of disease resistant pigs for breeding strategies. QTL on SSCs 2, 6, 12, 13, 16, 17 and 18, associated with resistance/susceptibility to App, were identified in controlled infection experiments in cross-bred Hampshire/Landrace pigs, and explained 6-22% of variance in disease severity (Reiner et al., 2014a, b). Another group found six QTL, also including SSCs 2, 12, 13 and 18, associated with dorso-caudal chronic pleuritis (Gregersen et al., 2010). Combining chromosomal position with functional data could support the identification of functional candidate genes involved in App resistance or susceptibility.

Treatment

Nowadays, relatively few acute outbreaks are present in Canada and the USA, while they still remain a problem in Asia, Latin America and some European countries (Gottschalk, 2012). In general, vaccination and use of antibiotics are able to reduce the severity of clinical symptoms and mortality. A wide range of antimicrobials is effective against the pathogen, although an increase of resistance to non-critical antimicrobials such as tetracyclines, penicillins and trimethoprim-sulphonamides has been observed (Vanni et al., 2012; Bossé et al., 2017a).
Minimal inhibitory concentrations (MICs) against App have been reported by various authors. Thirty years ago, a relatively high prevalence of Canadian App isolates were found to be resistant to common antibiotics such as penicillins, chloramphenicol, spectinomycin, lincomycin and spiramycin. Sixty-eight percent of the isolates were resistant to tetracycline, but most were susceptible to trimethoprim, erythromycin and gentamicin (Nadeau et al., 1988). In a more recent study, most Canadian isolates were reported to be susceptible to the majority of tested antimicrobials, but still a high level of resistance to chlortetracycline (88.4%) and oxytetracycline (90.7%) was observed (Archambault et al., 2012). It has to be noted that serovars involved in clinical cases have significantly changed with time in Canada (Gottschalk and Lacouture, 2015).

In a Spanish retrospective study from 1994 to 2009 a high or an increasing trend for resistance against beta-lactam antibiotics, tetracyclines and tilmicosin was recorded, while most isolates were susceptible to amphenicols, fluoroquinolones and ceftiofur (Vanni et al., 2012). In a recent study only 33% of UK App isolates were negative for resistance genes, while 57% of the isolates were resistant (as adjudged by MICs) to tetracycline, 48% to sulfisoxazole, 20% to ampicillin, 17% to trimethoprim and 6% to enrofloxacin (Bossé et al., 2017a).

Recently, plasmids conferring resistance to florfenicol and chloramphenicol were isolated from App clinical isolates from Greece and Brazil (Bossé et al, 2015; Cunha da Silva et al., 2017), and enrofloxacin resistant strains have been reported in Taiwan and the UK (Wang et al., 2010; Bossé et al. 2017a). It has been demonstrated that whole genome sequencing can be used as predictor for App resistance to antimicrobial substances (Bossé et al., 2017a). In general, variation in levels of antimicrobial resistance of App isolates within the same herd can be high (Dayao et al., 2015), and there is not always an association between in vitro test results and success after treatment of disease.

Clinical breakpoints are already available for App in broth micro dilution assays, with MICs (µg/ml) for the following antimicrobial agents: ampicillin
(sensitive <0.5, resistant >2.0), ceftiofur (sensitive <2, resistant >8.0),
tulathromycin (sensitive <64), tilmicosin (sensitive <16, resistant >32),
tildipirosin (sensitive <16), florfenicol (sensitive <2, resistant >8.0), tiamulin
(sensitive <16, resistant >32), tetracycline (sensitive <0.5, resistant >2) (CLSI,
2015).

Acute clinical pleuropneumonia can be successfully treated by a single
injection of fluoroquinolones in a dosage allowing concentration-dependent
bacterial killing, if App strains are susceptible (Grandemange et al., 2017). In an
infection experiment, the high effectiveness of enrofloxacin against App compared
to a tetracycline and procaine-penicillin was shown (Sjölund et al., 2011a).
However, after a second App challenge, pigs that had been previously treated with
the less effective antibiotic were found to be better protected in the second
challenge, due to better immunity (Sjölund et al., 2011a).

A considerable decrease in feed and water intake may be observed in pigs
affected by App (Pijpers et al., 1991). For this reason it is advised to treat affected
pigs with a parenteral injection. Next to the route of administration also the
bioavailability and the mechanism of action of a chosen antimicrobial substance
also have to be taken into account for appropriate dosing and dosing intervals.
Success of antibiotic treatment in general is determined by sufficient tissue
concentrations at the first exposure to kill bacteria, which can be achieved by
adequate dosing regimen (Martinezet al., 2012). Treatment days and intervals, as
well as dosages, are provided by the companies selling the registered drugs, but
results from pharmacokinetic and pharmacodynamic studies might lead to new
dosing recommendations in the future. In a recent study, pharmacokinetic and
pharmacodynamics modelling for marbofloxacin treatment of 12 week old pigs
infected with App resulted in a single dose of 0.66 mg marbofloxacin/kg body
weight to achieve a 90% bactericidal target attainment rate over 48 hours (Dorey
et al., 2017).

In general, use of antibiotics increases the risk for the emergence of
antimicrobial resistant strains of App (Gutiérrez-Martín et al., 2006). Therefore
the choice of the antimicrobial for treatment has to be taken with careful consideration, especially for antimicrobials considered as critically important, such as fluoroquinolones. More sustainable control measures against porcine pleuropneumonia that do not require use of antimicrobial substances are needed.

Vaccination

A comprehensive review of different approaches to vaccination against App is given by Ramjeet et al. (2008). Commercial vaccines are either killed-whole-cell vaccines (bacterins), subunit vaccines, toxoid vaccines or some combination of these (Van Overbeke et al., 2001).

Most App vaccines successfully decrease clinical symptoms, but cannot protect against infection or transmission (Ramjeet et al., 2008). Limited cross-serovar protection hampers the efficacy of bacterins that are based on specific serovars (Fenwick and Henry, 1994). Some subunit vaccines, which contain Apx toxins and either outer membrane proteins or whole bacterial cells, have been commercialised, and are reported to convey better cross-protection than bacterins (Tumamao et al., 2004; Shao et al., 2010; Thevenon et al., 2014; van den Bosch et al., 2003). The commercially available second-generation subunit vaccines contain four to five recombinant proteins, and confer cross-protection against all serovars to some degree (Meeusen et al., 2007). A commercial subunit vaccine, containing the Apx toxins I-III, an outer membrane protein, and the adjuvant alpha-tocopherol acetate is widely used in practice in Europe. Sow vaccination in the 6th and 3rd week prior to farrowing led to an increase in specific antibody levels in piglets by colostrum uptake (Kristensen et al., 2004a). Empirical reports indicated an interference of maternal antibodies and immune response to vaccination, so that in sows with high maternal antibodies the vaccination of piglets should be postponed to a later time point, e.g. weeks 10 to 14 of life (Jirawattanapong et al., 2008).

Live attenuated vaccines, tested in experimental conditions, are considered to have more potential to protect against homologous and heterologous serovars
(Maas et al., 2006a, b). An ideal live vaccine should adhere to ‘Differentiating Infected from Vaccinated Animals’ (DIVA) principles, and might also be used as a vector for expression of antigens from other pig pathogens. There are currently two different methods of introducing unmarked mutations for generation of attenuated strains in App. One uses suicide vectors (Oswald et al., 1999), and the other exploits the phenomenon of natural transformation (Bossé et al., 2004; 2009) for generation of insertion-deletion mutations in a two-step transformation system in highly transformable App isolates (Bossé et al., 2014).

The development of attenuated live marker vaccines, which can be differentiated from naturally occurring App isolates, should be further supported. The effects of live vaccines on mucosal immunity may provide desirable enhanced protective effects, but this needs to be confirmed in future research. After interaction with the pulmonary epithelial surface, bacteria are phagocytised by antigen presenting cells which subsequently migrate to lymphoid tissue and activate lymphocytes, which migrate back to the mucosal site and mediate a local immune response including antibody production with the main emphasis being secretion of immunoglobulin A (Ramjeet et al., 2008). Antigens considered as potential vaccine candidates can be found by genome-wide screening methods as In Vivo Expression Technology (IVET), Signature Tagged Mutagenesis (STM), Selective Capture of Transcribed Sequences (SCOTS) or DNA microarrays (Ramjeet et al., 2008).

In general, the immune response after vaccination is influenced by the route of administration. In contrast to parenteral vaccination and depending on the antigen characteristics of the vaccine, mucosal vaccination induces both specific, and innate immune response, due to binding of antigen to pattern-recognition receptors expressed by resident airway mucosal dendritic cells (Holt et al., 2008; Makoschey, 2015). Mucosal vaccination in the respiratory tract (e.g. intranasal) leads to antigen uptake by macrophages, myeloid and plasmacytoid dendritic cells. These can migrate to the draining lymph nodes, presenting the antigen to naïve-T cells, leading to their activation, proliferation and migration to lung parenchyma or mucosal sites depending on their homing receptor profile.
(Holt et al., 2008). In addition, luminal antigens are transported through microfold (M) cells within the epithelium to nasopharynx-associated lymphoid tissue (NALT), where dendritic cells initiate the development of specific T- and B- cells, which migrate to the regional lymph nodes and then to the effector sites, resulting in mucosal as well as systemic immunity (Kiyono and Fukuyama, 2004). In contrast, parentally administered antigens (intramuscularly, intradermally, subcutaneously) are processed by local dendritic cells and transported to draining lymph nodes, resulting in stimulation of systemic immune response (Makoschey, 2015). Humoral immune responses have been measured after intramuscular administration of commercially available App vaccines (Jirawattanapong et al., 2008). Mucosal vaccination against App resulted in local and distal mucosal as well as systemic immune responses (Wilson and Obradovic, 2015).

Eradication

The most effective measure for prevention of pleuropneumonia is to obtain/maintain SPF status of the herd with regard to App. Some reports describe procedures to eradicate herds already infected with App, though these were not successful (e.g. Hunneman and Oving, 1991). Conversely, many case reports, based on test and removal of infected animals, combinations of treatments with antimicrobials, or vaccination and changes in pig flow, have been published throughout the years (Lariviere et al., 1990; Gjestvang et al., 2008; Baekbo and Lorenzen, 2017). These reports differ in the methods used for confirmation of successful elimination, and regarding the time frame during which SPF status was maintained.

In principle, eradication can be achieved by total depopulation/repopulation or partial depopulation and medication. The latter can be applied in all-in-all-out multiple-site production systems after isolation of the pathogen and testing for susceptibility to antimicrobials. Although genetic material of breeding stock in this way can be preserved, the low probability of success as well as a high use of often critically important antimicrobials should be considered in the decision process.
Experimentally it has been shown that antimicrobial treatments decrease shedding of the pathogen (Fittipaldi et al., 2005), but effective eradication seems to be not attainable in the long run (Fittipaldi et al., 2005; Angen et al., 2008). Although combinations of medication with removal of high risk populations on farms may explain the success of App eradication on farms in some case reports (e.g. Schafzahl and Lillie‐Jaschniski, 2010), the most reliable method to sanitise a farm is a complete depopulation and restocking with App free animals. Given the increasing farm size, the latter method is a financial and logistical challenge, which stresses the need for effective and validated, but also sustainable, eradication protocols.

Conclusion

Gaps in knowledge regarding porcine pleuropneumonia are connected to still unsolved problems for swine practitioners and veterinarians. An international database with prevalence data for most frequent and most clinically important App serovars in respective countries should be available to support the interpretation of diagnostic findings. A diagnostic protocol for determination of the subclinical carrier status of pigs with respect to the serovar and its clinical relevance should be established. Scientific consensus is needed regarding the definition of App infection at the herd level, and how to establish and monitor it, in order to facilitate trade between farms and countries, as well as to safeguard animal health.

The influence of strain characteristics as well as host factors on the prevalence of App disease should be analysed in a broader context that also takes into consideration variables that can be introduced after implementation of outside farming, minimal weaning age at 28 days and restrictions on antibiotic usage, all of which may result in new dynamics in App transmission within the herd.

In the opinion of the DISCONTOOLS experts, in relation to good practice in minimising or eliminating App from herds, emphasis should be given to
understanding transmission from sow to piglets, promoting good colostral immunity and high piglet vitality during the suckling period, and reducing or eliminating climatic stressors during weaning and the nursery, and the impact of viral co-infections as PRRSV.

As is evident from this review, while there are still crucial gaps in currently available knowledge, there is already a considerable amount of information available on many aspects of App. Thus, App can be considered as an excellent model organism for the study of many aspects of infectious disease in a natural host such as transmission, virulence mechanisms, host genetics, the interplay between endocrine system and health status of pigs, effect of climate and housing, co-infection with other viral and bacterial species, immune modulation and vaccinology including maternal immunisation.

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**Figure legend**

Figure 1: Dynamic of infection with *Actinobacillus pleuropneumoniae* and diagnostic options (modified according to Vigre et al., 2002; 2003; Gottschalk, 2015).

S: Serological diagnostics: Use ApxIV-based ELISA if herd is negative, and LPS-based ELISA to the known most virulent serovars in the region (or in the region of origin of replacement animals) if herd is positive for low virulence serovars.

TS: PCR diagnostic of tonsillar scrapings. B: Bacteriological examination
Transmission from sow to piglets

Spread of infection + decline of maternal antibodies (low levels at age of ~6-10 weeks)

Transmission within pig group
Variable morbidity and mortality depending on additional trigger factors and virulence of strain

Development of adaptive immunity
Pigs > 16 weeks old: S

Colonization of tonsils
Detection from ~D10 of life

Introduction of pathogen
- Carrier pigs without symptoms
- Inadequate external biosecurity

New pigs in quarantine: S (in case of conflicting results: TS)

Sows: S (in case of conflicting results: TS)

Sows: S (for risk assessment of piglets being carriers; possible false negative serological results, if piglets had been colonised in the presence of maternal antibodies)

Diseased pigs: B

Lung alterations and chronic disease
- Subclinical carrier pigs

Variable morbidity depending on additional trigger factors and virulence of strain (mostly in pigs ~12 weeks and older)
### Table 1: Gaps and challenges in *Actinobacillus pleuropneumoniae* research

<table>
<thead>
<tr>
<th>Known</th>
<th>Required</th>
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<tbody>
<tr>
<td><strong>Disease</strong></td>
<td></td>
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<tr>
<td>Virulence depends, to some extent, on production of Apx toxins which</td>
<td>Epidemiological database with serovar/biovar distribution of isolates</td>
</tr>
<tr>
<td>varies between serovars/biovars. Also the same serovars in different</td>
<td>and their respective production of Apx toxins for most swine-producing</td>
</tr>
<tr>
<td>geographic regions may differ in their Apx toxin production, so that</td>
<td>countries.</td>
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<td>general assumptions of virulence associated with a serovar cannot be</td>
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<tr>
<td>made.</td>
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<tr>
<td>Severe clinical episodes are preceded by infectious (especially co-infection with viral pathogens) or non-infectious triggers.</td>
<td>Checklist of trigger factors for disease outbreaks.</td>
</tr>
<tr>
<td>Association of disease with specific breeding lines.</td>
<td>Genetic markers for natural resistance against App to facilitate strategic breeding.</td>
</tr>
<tr>
<td><strong>Transmission and spread of disease</strong></td>
<td></td>
</tr>
<tr>
<td>App resides in the upper respiratory tract in subclinically infected</td>
<td>Checklist of trigger factors leading to translocation of App from the upper to the lower respiratory tract with the consequence of disease.</td>
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<tr>
<td>or colonised pigs.</td>
<td></td>
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<tr>
<td>Transmission of App from sows to their offspring starts within the first two weeks of life. Rate of transmission is not correlated with clinical signs.</td>
<td>Intervention methods to avoid transmission of App on a farm, and especially from sows to offspring.</td>
</tr>
<tr>
<td>High prevalence of App in wild boars.</td>
<td>Risk assessment for transmission of App from wild boars to domestic pigs.</td>
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<tr>
<td><strong>Diagnostics</strong></td>
<td></td>
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<tr>
<td>Occurrence of untypable App strains or strains of exceptional serovar/biovar and Apx-toxin profile.</td>
<td>Database of analysed atypical App isolates with respect to their virulence and immunity observed in the field.</td>
</tr>
<tr>
<td>Pigs with chronic lung alterations show a decreased growth rate.</td>
<td>Direct or indirect method to detect chronic lung alterations in pigs not showing any clinical signs.</td>
</tr>
<tr>
<td>Mere infection/colonisation of the tonsils, often without induction of an immune response.</td>
<td>Approved standard diagnostic protocols with high sensitivity and specificity for detection of App positive carrier pigs.</td>
</tr>
<tr>
<td>Serological diagnosis is hampered by the high diversity of serovars, as well as other bacteria mimicking App such as <em>A.suis</em>, &quot;A. porcitonsillarum&quot; and <em>A. rossii</em>. Interpretation of serological findings is difficult.</td>
<td>Point of care-based tests for the field, using blood or alternative body fluids (e.g. oral fluids, nasal fluids, meat juice), which are easy to interpret.</td>
</tr>
<tr>
<td>It is not known if serovars and strains vary in their ability to elicit an immune response.</td>
<td>Complementation of PCR diagnosis for isolates with respective immunodiagnostics.</td>
</tr>
<tr>
<td>No conformity in diagnostic procedures between pig breeding companies.</td>
<td>Harmonized definition of the status of an App-free herd based on diagnostic protocols including sampling frequency, sample number, diagnostic tests to be performed and interpretation guidelines.</td>
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<tr>
<td><strong>Prevention</strong></td>
<td></td>
</tr>
<tr>
<td>Exposure of App to endogenous stress hormones promotes expression of virulence factors.</td>
<td>Identification and elimination of situations causing a stress response in farm animals, relevant to the context of infectious diseases.</td>
</tr>
<tr>
<td>Successful eradication efforts are published, but little is known regarding the return on investment or the probability of success of different eradication strategies.</td>
<td>Protocols for eradication including estimation of cost-effectiveness.</td>
</tr>
<tr>
<td>Vaccination does not prevent colonisation, infection or transmission, and shows limited protection.</td>
<td>Effective vaccines (e.g. live marker vaccines) and vaccination strategies improving innate and adaptive immune responses and reducing transmission.</td>
</tr>
<tr>
<td>No diagnostic method available to evaluate and compare protective effects of commercial or autologous vaccines in the field.</td>
<td>Identification of measurable diagnostic correlates of protection.</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Optimal dosing and dosing intervals differ between the antibiotic agents. Antimicrobial treatment can improve clinical disease, but in most cases is not successful in eliminating the pathogen from the host.</td>
<td>Clinical breakpoints for antimicrobial agents used for treatment covering realistic tissue concentrations.</td>
</tr>
<tr>
<td>The interference of antimicrobial treatments with natural immunisation and vaccination is not completely understood.</td>
<td>Recommendation for good clinical practice in treating disease caused by App, minimizing the risk of antimicrobial resistance development, and without negative impact on the immune response.</td>
</tr>
</tbody>
</table>