Isolation of *Actinobacillus Pleuropneumoniae* from Pigs in Israel

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ABSTRACT

In a farrow to finish holding of 1,200 sows, mortality in growing and fattening pigs increased up to 8% with several cases of sudden mortality due to respiratory diseases. Lesions at necropsy were suggestive of *Actinobacillus pleuropneumoniae* (App) infection. Lungs from necropsies were submitted for laboratory investigation for the purpose of isolation, typing and sensitivity testing. Blood samples from sows, growers and fatteners were submitted for serological investigation for different antigens responsible for respiratory diseases in pigs. Cultures typing from isolates and serological tests confirmed App involvement in the outbreak. Sensitivity tests allowed for the organization of herd treatment which reduced mortality in the affected holding. To date there are no vaccines registered in Israel against App.

INTRODUCTION

App is a Gram negative, non-sporigenic, immobile, capsulated bacteria, responsible for septicemia and pleuro-pneumonia which may result in mortality or chronic – subclinical disease (1, 2) with reduced growth performances, and increased medication costs. Furthermore App is primarily recognized as being responsible for pleurisy and pleuro-parietal adhesions in pigs at abattoirs (2, 3).

Two bio-types, I and II, are recognized in relation to their different laboratory growth requirements: Bio-type I shows NAD - dependence and presence of "fostering" *Staphylococcus aureus* or *epidermitis* streaks. Bio-type II does not need the presence of *Staphylococcus aureus* or *epidermitis* and the bacterial biotype grows easily on blood-agar plates (1, 2). According to capsular polysaccharides (CPS) and cell-wall lipopolysaccharides (LPS) at least 15 sero-types of App are recognized, 13 of which belong to bio-type I and two of which belong to bio-type II. Furthermore, cross reactions among serotypes are known due to similarity in LPS structure. Hemolytic activity is one of the main characteristics of App growth on blood-agar plate; this is the consequence of the release of toxins, classified as Apx I, Apx II, Apx III, belonging to repeats in the structural toxin *(RTX)* family with strong hemolytic or cytolytic properties (1, 2).

App has high specificity for pigs and infects the respiratory tract and in the course of disease it may be isolated from lung lesions and nasal discharges. Infection is generally perpetuated at herd level by breeders (sows) which infect piglets during the nursing period. Infected sows also transfer, through colostrum, high level of antibodies to piglets. These piglets rarely develop disease, due to the neutralizing effect of passive antibodies, until weaning (4 weeks of age) and even later (6 to 10 weeks of age). There is probably a strong association between infection without clinical signs and presence of colostral antibodies (2). Piglets are later responsible for App diffusion by direct contact or by droplet infection but only over a short distance. Typical clinical signs of App pneumonia are observed beginning at 10 weeks of age (around 25 to 30 kg body weight) until the finishing period (100 kg body weight and more). App adheres first to tonsils and then, after overcoming the clearing function of mucociliary system, localizes on the alveolar epithelium. If phagocytized by macrophages, App can survive for a certain period: CPS and LPS provide resistance to complement activity and Apx toxins are

Table 1: Toxins productions and complement resistance according to serotypes (1 modified; 2)

Toxins	Serotypes	Complement resistance (*responsible structure*)
Apx I	1, 5, 9, 10, 11, 14	serotype 1 (LPS)
Apx II	1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 15	serotype 5 (CPS)
Apx III	2, 3, 4, 6, 8, 15	

the responsible for impairing macrophages and neutrophils phagocytic function.

When multiplying at alveolar level, App resists phagocytosis through the function of its thick capsule (1) and releases toxins which exacerbate the inflammatory response at lung level. Toxins have hemolytic, cytotoxic, cytolytic and edema-inducing activities.

Toxin production differs according to different App biotypes and serotypes, as schematized in Table 1.

Results of active multiplication of App in the lungs together with toxins released include severe edema, hemorrhage, and necrosis in peracute to acute cases (4), and alveolar and interlobular edema, intravascular fibrinous thrombosis and necrotizing pleuropneumonia in acute-subacute cases (5). Correlation with high virulence is associated with the productions of two toxins (6) or differences in virulence due to capsular or LPS composition (7, 8, 9).

Lung lesions may be appreciated as early as 3 hours after experimental infections or after an incubation period of 8-24 hours in field infections. Edges of damaged areas are well demarcated from surrounding non-affected lung tissue; chronic cases result in necrotic residual areas with intense fibrosis, pleural adherences of various extensions which persist until slaughter (3). Toxins induce immune response and circulating toxins-neutralizing and anti-Outer Membrane Protein (OMP) antibodies can be detected after 10-14 days (2), persisting several months. In non-vaccinated populations, detection of antibody titers, either anti-Toxins, anti-OMP, anti-CPS or anti-LPS, is of help in determining the epidemiology of the App infection.

Past experiences in Israel for the spread of serotypes 2 and 9 in pigs populations, revealed a sero-prevalence of 55,8% and 54,2% respectively among examined animals and in 88% of examined herds (10). However until now, App has never been isolated and identified from specific lung lesions. The purpose of this paper is to present the first isolation of an App strain from the Israel pig population.

MATERIALS AND METHODS

Farm and animals

Samples were obtained at beginning of 2010 from a farrow to finish unit consisting of about 1,200 sows, located in the Northern Region of Israel. The owner complained of a mortality increase in the fattening – finishing phase, with several cases of sudden death and/or acute respiratory cases which rapidly proved fatal. Mortality reached an average of 8% on a monthly basis with respect to the fattening-finishing pigs population.

Dying pigs presented cyanosis seen on the ears, snout, ventral part of the neck and abdomen; with sero-hemorrhagic froth leaking from snout. (Figure 1) Necropsies at the farm revealed a number of cases of intense pleuro-pneumonia, often unilateral, mainly at ventral – caudal lung lobes, with necrotic foci, hard at touch, in some cases extending to the size of an egg (Figure 2). Trachea and main bronchi contained the same sero-hemorrhagic froth observed at snout level. In some cases intense and extended pleuro-parietal adherences were observed, leading to lung tissue lacerations during the extraction of the lungs from thorax. (Figure 3). Severity and fatality of the disease, the age of affected animals, macroscopic lesions at necropsies pointed towards a tentative diagnosis of an App outbreak.

Samples

Samples from 4 lungs collected in two different episodes were sent to the laboratory for isolation and antibiotic sensitivity tests. From 30 pigs in total (10 sows; 10 growers; 10 fattening pigs), after immobilization with hog-snare, a blood sam-

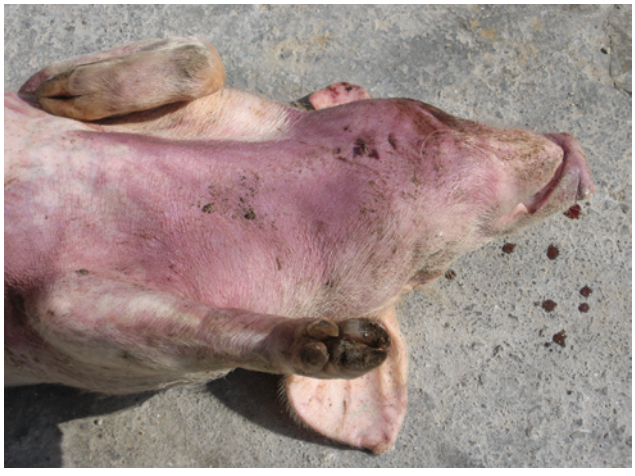

Figure 1: Intense cyanosis; hemorrhagic leak from snout

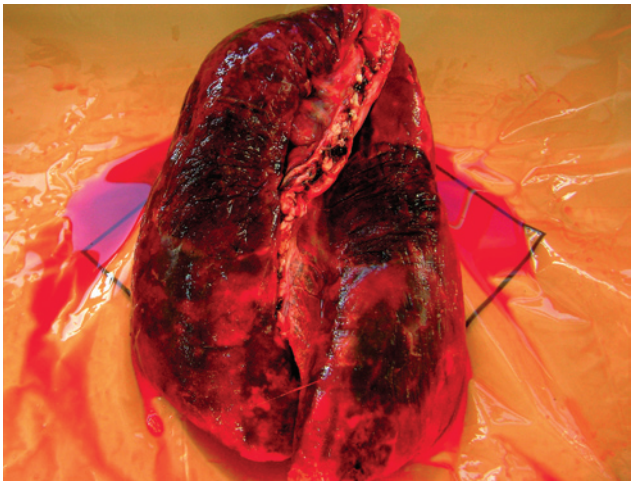

Figure 2: Severe pneumonia and pleurisy

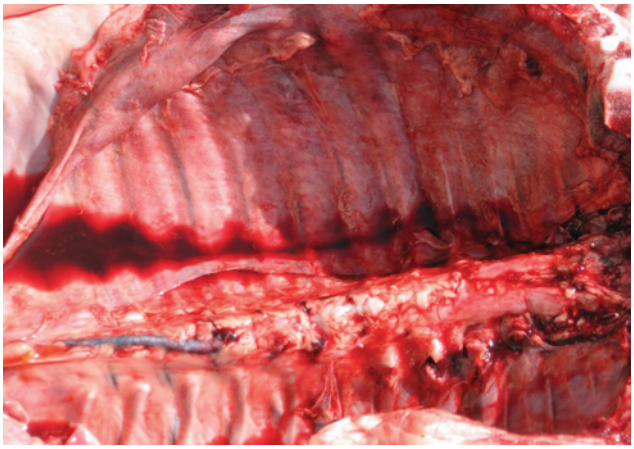

Figure 3: Severe pleurisy on parietal pleura

ple was collected from jugular vein, using vacutainer without anti-coagulant and a new needle for every pig. Blood samples were centrifuged and the serum separated, frozen and then sent to Intervet International Laboratory, Boxmeer (The Netherlands) for serological investigations (10, 11). The sampling size is adequate to detect at least one positive sample in a population of 3,000 pigs at 10% expected prevalence with 95% confidence level (12).

Culture method

An inoculum was prepared on MacConkey agar, 5% sheep blood trypticase agar, and on Chocolate agar (containing both X and V factors). The isolates were cultivated after 24 hours of incubation at 37°C in aerobic conditions from the Chocolate agar plates only.

The colonies cultivated from the Chocolate agar were then inoculated onto 5% sheep blood trypticase agar with a streak of *Staphyloccocus aureus* (*S. aureus*) inoculum and incubated for 24 hours at 37°C in aerobic conditions.

Being the first time App isolated in Israel, the isolation was required to be validated by a reference Laboratory. For this purpose, colonies were streaked on Chocolate agar supplemented with 1% blood–agar and incubated 24 hours at 37° C. The plates were sealed and three replicates of isolate were forwarded to Animal Health Institute "IZS-LER", Brescia, Italy, for confirmation and validation of isolate. Presumptive biochemical identification and PCR tests to confirm the identity of App strains were carried out.

Typing

Three replicates from the isolated strain were submitted to the Ring Precipitation Test (RPT). The RPT test is based on specific rabbit antisera – antigen reaction in comparison with known positive and negative control sera run simultaneously (14). Specific post culture PCR tests were carried out for typing of the isolated strains. Tests were used to obtain the genetic profile and to identify difference between App serotypes (17).

Serological testing

Sera were inactivated at 56°C for 4 minutes; then submitted to an "in house" ELISA test against antibodies to apx I, apx II, apx III, OMP antigens. In the test, microtiter plates were coated with purified antigens and then incubated with positive and negative standard and/or reference sera and the unknown Israel samples. 1:2 dilutions are made before next reaction. Antibodies present in the standard and/or reference sera and in unknown samples are quantified using a goat anti-swine peroxidase conjugate and TMB substrate. Results are expressed as reciprocal of last dilution at which a reaction occurred.

Statistical testing

Average antibody titers for each group of animals, Standard Deviation (SD) within each group and towards each antigen were calculated and evaluated. Antibody titers of each group and for each antigen were compared using Student's t-test. A value of $p<0.05$ was considered significant.

RESULTS

Culture

There was no growth on the MacConkey or Blood agar plates. The colonies on the chocolate agar plates were small,

grey, translucent and smooth. (Figure 3). Stained organisms appeared as Gram negative coccobacilli. Organisms showed satellitism to *S. aureus*. (Figure 4 and 5). The colonies on blood agar showed beta haemolysis (13, 14). Satellitism to *S. aureus* is indicative of App bio-variant 1, NAD dependent. Biochemical tests and CAMP phenomenon confirmed the identity of three strains to App.

Typing

RPT testing on the submitted isolates confirmed that all the three isolates belonged to the same App sero-type 13. Genomic profile obtained from PCR panel of each of the three strains was identical confirming the sero-type 13.

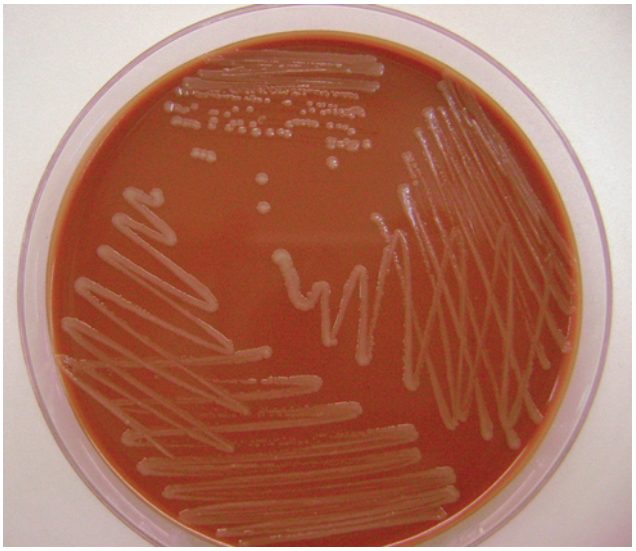

Figure 4: App strips on agar-chocolate

Figure 5: App "satellism": intense growth along *S. aureus* streak (upper streak). App needs V factor for growth. Because App cannot synthesize it from the blood agar, it tends to grow beside the Staphylococcus streaks. Colonies are very small and translucent. Beside the lower streak there are fewer colonies because this strain of App prefers satellism to *S. aureus* then to *S. epidermidis*

Table 2: Average antibody titers and standard deviation (SD) against App toxin antigens in sows, growers and fatteners

	apx I	apx II	apx III	OMP
SOWS	13.6	13.8	10.2	9.1
SD	*0.58*	*0.28*	*0.65*	*0.91*
GROWERS	10.4	12.3	9.0	8.7
SD	*1.73*	*2.22*	*1.16*	*1.14*
FATTENERS	10.4	13.6	9.3	8.2
SD	*0.90*	*0.39*	*1.09*	*0.74*

Serological tests

All the 30 sampled pigs were seropositive for the four App antigens tested and results are presented in Table 2. Serological tests confirmed diffused positive results to *Mycoplasma hyopneumoniae* (Mhyo), Porcine Circovirus type 2 (PCV2), and negative results to Swine Influenza Virus (SIV) sub-types H1N1; H3N2; H2N2, typical of Israeli swine farms as previously demonstrated (10, 11, 16).

Sensitivity testing

Isolates of App were sensitive to Tetracycline; Florfenicol; Cefthriazone; Enrofloxacin; Cefquinone and resistant to Amoxycillin; Ampicillin; Trimethoprim/sulfamethoxazole.

DISCUSSION AND CONCLUSIONS

Laboratory investigations confirmed the diagnosis of App infections anticipated on the basis of the clinical respiratory symptoms and necropsy findings.

The serology profile pattern to App showed higher standard deviation values for all the four antigens in the growers. This is probably due to the ongoing sero-conversion processes in this category of pigs compared to sows and fatteners, which had already seroconverted earlier on in time.

There was no statistical differences ($P > 0.05$) between antibody titers against all the four App antigens between growers and fatteners, while differences were statistically significant between sows and growers-fatteners (grouped together) to antigens apx I ($P < 0.005$); apx III ($P < 0.005$); OMP ($P < 0.05$) and not significant relative to antigen apx II ($P = 0.082$). Serotype 13 is recognized as more active (but not exclusively) in apx II – toxins release (1, 2) so that a high and more uniform immune response to this toxin can be speculated, with less or no significant differences in anti-

body titer increases, at least in animals a long time after exposure and seroconversion, as in this case (P = 0.082). In fact standard deviation (SD) values for apx II within sows and within fatteners appear lower than the standard deviation for growers and the standard deviations for other the three App antigens in all ages.

Evolution of the outbreak

Despite registered in several countries, there are no App vaccines registered in Israel, so that control measures were mainly concentrated on antibiotic prophylaxis and therapy.

Results relative to sensitivity tests allowed to abandon tentative therapy and to organize a specific treatment: Tetracycline was added as feed medication at 1,400 ppm during weaning period; at the beginning of growing phase and, later on, at beginning of fattening phase. A five day medication cycle was used for each group of pigs. Alternatively Cefquinone 2mg/kg once a day for 3 -5 days or Florfenicol 15 mg/kg every 48 hours, were used as individual therapy in affected animals. Mortality was progressively reduced to 2.6 – 2.5 % in about one month.

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