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Presence and Seroprevalence of *Actinobacillus pleuropneumoniae* in Pigs in Turkey

K. METINER, S. AK

Department of Microbiology, Veterinary Faculty, University of Istanbul, Avcilar, Istanbul, Turkey

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Abstract

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In this study, the presence and seroprevalence of *Actinobacillus pleuropneumoniae* in pigs raised in various regions of Turkey were investigated. For this purpose, 384 lung, tonsil and blood samples were taken by random sampling during the slaughter. The pigs did not show any signs of clinical respiratory tract disorders at the time of slaughter. Organ samples were used for isolation, and specific antibodies in 384 sera samples (of which 368 belonged to animals used in organ sampling) were investigated by enzyme-linked immunosorbent assay (ELISA). For isolation, the lung and tonsil samples were inoculated onto PPLO agar plates with nicotinamide adenine dinucleotide (NAD), and *A. pleuropneumoniae* (serotype 12) was isolated from only one of the tonsil samples. Of the 384 blood samples, 258 (67.2%) were found to be positive. 209 (54.4%) of the positive samples were determined to be group 1, and 49 (12.8%) were determined to be group 2.

The effect of age and sex of the samples were found to be non-significant by statistical analysis of the ELISA data ($p > 0.05$).

Isolation of *A. pleuropneumoniae* indicates the presence of the infection in Turkey for the first time and high seroprevalence supports this finding.

Actinobacillus pleuropneumoniae, isolation, ELISA, Turkey

Porcine pneumonia caused by *Actinobacillus pleuropneumoniae* causes significant economic losses in pig breeding worldwide. It is highly contagious, lethal in acute cases, and characterized by breathing problems and growth disorders in young pigs (Dubreuil et al. 2000). In acute infections, extensive and fibrinohaemorrhagic lung lesions are observed. In chronic infections, the lesions are localized, necrotizing and associated with pleuritis. The rate of mortality is increased in acute cases (Fenwick and Henry 1994). All age groups are reported to be sensitive to the infection; young pigs in the fast growing period are susceptibile the most. The initiation and severity of infection are reportedly associated with crowded transportation of pigs and adverse weather conditions (Inzana et al. 1988).

Researchers have indicated that the highly contagious pig pneumonia can cause worldwide epidemics with an incubation time of 2 to 5 days by direct transmission from a subclinically infected pig. Indirect transmission may also be observed. The regulation of the course of infection has been reported with introduction of chronic or subclinically infected pigs to farms (Fenwick and Henry 1994). Porcine pneumonia occurs in three forms including hyperacute, acute and chronic (Rycroft and Garside 2000; Fenwick and Henry 1994). Not many infections are reported to progress as fast as the porcine pneumonia, and death is certain within 24 hours following the initial clinical symptoms (Fenwick and Henry 1994). It has been proposed to perform culture, serological tests and molecular diagnostic techniques, and for the isolation, the use of lung, tonsil and nasal swab samples have been recommended (Satran and Nedbalcova 2002; Thielmann 1989). Non-symptomatic pigs have been detected as seropositive, and this seropositivity reflects the degree of the infection (Kume et al. 1984).

The infection, the presence of which was unknown in Turkey so far, has been reported

Address for correspondence:
Kemal Metiner DVM, PhD
Department of Microbiology
Veterinary Faculty, University of Istanbul
Avcilar, 34320, Istanbul, Turkey

Phone: +90 0212 473 70 70
Fax: +90 0212 473 72 41
E-mail: kmetiner@istanbul.edu.tr
http://www.vfu.cz/acta-vet/actavet.htm

worldwide (Blackall et al.2002; Chevallier et al. 1997; Nielsen et al.1997; Kume and Nakai 1988; Nielsen 1986). The aim of the present study was to investigate the presence and prevalence of porcine pleuropneumonia in Turkey.

Materials and Methods

Samples

Lung and tonsil samples were obtained from pigs during slaughter to be used for isolation of *A. pleuropneumoniae*, and blood samples were taken for detection of specific antibodies. Samples were obtained from 384 pigs at 11 different farms in various regions of Turkey by random sampling of the animals. No blood samples from the 16 pigs from which lung and tonsil samples were taken, and no lung and tonsil samples from the 16 pigs from which blood samples were taken could be obtained. Samples were transported to the laboratory under cold chain. Organs were used for isolation immediately and sera samples were stored at -20 °C until use for serology after centrifugation.

Detailed information (origin, number, age, sex, clinical and postmortem findings of the animals) of the samples are given in Tables 1 and 2.

Table 1. Origin, number, age, sex, and clinical and postmortem findings of the animals of which organ samples were obtained

Origin (province-county)	Age	Sex ♀ (n)	Sex ♂ (n)	Number of samples lung and tonsil (n)	Clinical signs	Post mortem finding
Adana	1 to 3 years	17	6	23	a	d
Balıkesir	1 to 12 months	3	36	39	a	d
Edirne-Kesan	1 to 12 months	3	13	16	a	d
	1 to 3 years	-	1	1		
Erzincan	1 to 3 years	21	4	25	a	d
Istanbul- 1	1 to 12 months	17	28	45	a	d
	1 to 3 years	47	20	67		
Istanbul-2	1 to 12 months	1	2	3	a,b	c
	1 to 3 years	19	6	25		
Istanbul-3	1 to 12 months	4	12	16	a	d
Izmir	1 to 12 months	20	16	36	a	d
Tekirdag-Corlu-1	1 to 12 months	22	13	35	a	d
Tekirdag-Corlu-2	1 to 3 years	5	5	10	a	d
Tekirdag-Malkara	1 to 12 months	3	27	30	a	d
	1 to 3 years	7	6	13		
Total		189	195	384		

a: decrease in weight gain, or weight loss
b: lameness
c: necrotic and haemorrhagic lung lesions
d: no macroscopic lesion

Isolation

The lung and tonsil samples were inoculated onto PPLO agar plates with 2µg/ml NAD (Difco-0412-01), 2µg/ml crystal violet (Sigma C-3886), 1µg/ml lincomycin (Sigma L-6004) and 6 IU/ml bacitracin (Sigma B-0125). The plates were incubated for 72 hours at 37 °C microaerobically, and were observed daily for growth. Gram staining was performed for the colonies. Suspicious colonies were transferred onto PPLO agar with NAD, MacConkey agar, and sheep blood agar plates in order to investigate their growth. Identification was performed according to the results of catalase, oxidase, O-F, nitrat reduction, ONPG, gelatinase, H_2S production, indole production, citrate, urease, eskulin hydrolysis, methyl red, voges-proskauer, lysine decarboxylase, arginine dihydrolase, ornitin decarboxylase, phenylalanine deaminase, malonate utilisation, and acids from adonitol, arabinose, dulcitol, fructose, D-galactose, D-glucose, inocitole, inulin, D-xylose, lactose, maltose, D-mannitol, D-mannose, melibiose, rafinose, rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, sucrose and trehalose tests. Motility, synthesis of satellite phenomena, CAMP, and haemolysis on blood agar with 7% defibrinated sheep blood of the isolate were also detected (Fenwick and Henry 1994; Quinn et al. 1994; Sidibe et al.1993).

ELISA

Sera samples were tested for antibodies to *A. pleuropneumoniae* by a commercial ELISA (Cypress diagnostic-Ref. VS035) which is based on the principle of an indirect enzyme immunoassay (EIA). This assay system utilises two recombinant proteins which are coated on the microtiter wells of the ELISA plate. One is an exotoxin virulence

Table 2. Origin, number, age, sex, and clinical and postmortem findings of the animals of which sera samples were obtained

Origin (province-county)	Age	Sex ♀ (n)	Sex ♂ (n)	Number of samples blood (n)	Clinical signs	Post mortem finding
Adana	1 to 3 years	17	6	23	a	d
Balıkesir	1 to 12 months	3	36	39	a	d
Edirne-Kesan	1 to 12 months	3	13	16	a	d
	1 to 3 years	-	1	1		
Erzincan	1 to 3 years	21	4	25	a	d
Istanbul-1	1 to 12 months	18	30	48	a	d
	1 to 3 years	47	20	67		
Istanbul-2	1 to 12 months	2	3	5	a,b	c
	1 to 3 years	19	6	25		
Izmir	1 to 12 months	20	16	36	a	d
Tekirdag-Corlu-1	1 to 12 months	22	13	35	a	d
Tekirdag-Corlu-2	1 to 12 months	11	-	11	a	d
	1 to 3 years	5	5	10		
Tekirdag-Malkara	1 to12 months	3	27	30	a	d
	1 to 3 years	7	6	13		
Total		198	186	384		

a: decrease in weight gain, or weight loss b: lameness
c: necrotic and haemorrhagic lung lesions d: no macroscopic lesion

marker (APX I) common only to serotypes 1, 5, 9, 10 and 11, and the other is an antigen common, but highly specific, to all *A. pleuropneumoniae* serotypes - one of the Transferring Binding Proteins (Tbp2) found in the Outer Membrane Protein. Samples were diluted at a ratio of 1/200. The test procedure was performed according to the instructions of the manufacturer. The optical density (OD) was measured on ELISA reader (OT 230, version 1.53) at 405 nm. Results were expressed as an IRPC value (relative index × 100) according to the formula recommended by the manufacturer;

$$\text{IRPC:} \frac{\text{(OD405 Sample - Mean OD405 Negative Control)}}{\text{(Mean OD405 Positive Control - OD405 Negative Control)}} \times 100$$

The values of ≤ 20 were considered negative, in the 20 to 60 range were considered group 1 positive (*A. pleuropneumoniae* serotypes 2, 3, 4, 6, 7, 8, 12) and ≥ 60 were considered Group 2 positive (*A. pleuropneumoniae* serotypes 1, 5, 9, 10 and 11).

Statistical Analysis

In order to determine the contribution of age and sex to the results, Chi-Square (χ^2) test was used (Evrim and Günes 1998).

Results

Culture

As a result of the isolation studies carried out with lung and tonsil samples of 384 pigs, *A. pleuropneumoniae* was isolated from one tonsil sample belonging to a pig at the farm Istanbul- 3. From the remaining samples, the agent could not be isolated in duplicate. The isolate was determined as serotype-12 by Prof. Dr. K. R. Mittal. The results of biochemical characteristics of the isolate are shown in Table 3.

ELISA

Out of 384 serum samples tested by ELISA, 258 (67.2%) were found to be positive for *A. pleuropneumoniae* antibodies; 209 (54.4%) of the positive samples were determined to

Table 3. Biochemical characteristics of the isolates

Test		Test	
Oxidase	-	Adonitol	-
Catalase	+	Arabinose	-
Growth on MacConkey	-	Dulcitol	-
Growth on 7% defibrinated sheep blood agar	-	Fructose	+
CAMP	+	D-Galactose	+
Haemolysis	+	D-Glucose	+
Motility	-	Inocitole	-
H_2S production / gas(TSI)	- / -	Inulin	-
O-F	Fermentative	D-Xylose	-
Nitrate reduction	+	Lactose	-
Urease hydrolysis	+	Maltose	+
ONPG	+	D-Mannitol	-
Methyl red	+	D-Mannose	-
Voges-proskauer	-	Melibiose	-
Indole production	-	Rafinose	-
Citrate	-	L-Rhamnose	-
Eskulin hydrolysis	-	D-Ribose	-
Arginine dihydrolase	-	Salicin	-
Lysine decarboxylase	-	Cellobiose	-
Ornitin decarboxylase	-	D-Sorbitol	-
Phenylalanine deaminase	-	Sorbose	-
Growth on 6.5% NaCl	-	Sucrose	+
Gelatinase	-	Trehalose	-
Malonate utilisation	-		

be group 1, and 49 (12.8%) were determined to be group 2. The positive results of ELISA by farms and ages are given in Table 4.

Statistical Findings

The effects of age and sex of the samples were found to be non-significant as a result of the statistical analysis of ELISA data with the Chi-Square test ($p > 0.05$).

Discussion

Researchers have indicated that the natural habitat of the agent in pigs is in the respiratory passages, and for identification purposes, lung, tonsil and nasal swab samples are suitable (Chiers et al. 2002; Lo 1998; Gram et al. 1996; Sidibe et al. 1993; Mittal et al.1984). By comparing nasal swab and tonsil samples in terms of recovery of the agent, researchers have claimed that isolation rates from tonsil samples are more appropriate than those from nasal swab samples. Therefore, tonsil samples should be used for isolation purposes (Chiers et al. 2002; Sidibe et al. 1993).

Wilson et al. (1987) have indicated that bacteriological culture is specific. However, one or more factors, such as its presence in the carrier in low amounts, localization in a restricted region of the tissue, being dead in the samples obtained, and not using its specific growth media in the laboratory were proposed to result in low isolation ratios. Therefore, despite being specific, bacteriological cultures may not be sensitive.

Levonen (2000) reported that despite the ease of isolating *A. pleuropneumoniae* in acute infections, it may not be possible to isolate the agent from animals with chronic infections.

In this study, lung and tonsil samples were obtained from pigs which did not show any

Table 4. Positive results of ELISA by farms and ages

Farm	Age	Sample (n)	Positive sample (n)			%
			group 1	group 2	Total	
Adana	1 to 3 years	23	10	6	16	69.6
Balıkesir	1 to12 months	39	19	2	21	53.8
Edirne - Kesan	1 to12 months	16	10	1	11	64.7
	1 to 3 years	1	-	-		
Erzincan	1 to 3 years	25	9	-	9	36
Istanbul-1	1 to12 months	48	33	2	81	70.4
	1 to 3 years	67	33	13		
Istanbul-2	1 to12 months	5	1	-	25	83.3
	1 to 3 years	25	14	10		
Izmir	1 to12 months	36	22	5	27	75
Tekirdag- Çorlu-1	1 to12 months	35	21	3	24	68.6
Tekirdag-Çorlu-2	1 to12 months	11	7	2	16	76.2
	1 to 3 years	10	3	4		
Tekirdag- Malkara	1 to12 months	30	16	-	28	65.1
	1 to 3 years	13	11	1		
General Total		384	209	49	258	67.2

clinical respiratory tract disorders during the slaughter. *A. pleuropneumoniae* was isolated from a tonsil of an animal. This is the first *A. pleuropneumoniae* isolation carried out in Turkey. The successful isolation from only one of the tonsil samples, and the unsuccessful isolation from the lung of the same animal point out to the possibilities of a chronic infection, low amounts of the agents in internal organs, or a localisation of the factor in restricted regions of the internal organs.

In a study performed on 120 pigs (62 abscessed and 58 non-lesioned) in a slaughterhouse in Japan, 25 *A. pleuropneumoniae* strains, 20 from abscessed and 5 from non-lesioned lungs, were isolated (Sakpuaram et al. 1989).

Some researchers indicated a possibility of other organisms causing pleural fibrosis junctions that cannot be opened with a knife and chronic lung lesions other than *A. pleuropneumoniae,* and reported that the observations made during the hygiene inspections while slaughtering the animal were not significant, and that even severe lesions caused by *A. pleuropneumoniae* recovered within in a few weeks (Lo 1998; Fenwick and Henry 1994).

Although growth problems and diminished weight gain were observed at all the farms from which samples were collected in this study, walking disabilities, necrosis and oedema in the lungs of pigs were observed only at the farm Istanbul-2. No lesions were detected in the lungs of animals from the farm Istanbul-3, where the isolation was done.

Kume et al. (1984) have reported that pigs that are positive to *A. pleuropneumoniae* in culture may be serologically negative, and pigs that are negative in culture may be positive serologically.

In this research, serological control of the animal on which isolation was performed could not be carried out as its blood sample could not be obtained at the time of slaughter.

Researchers indicated that the number of microorganisms necessary to cause an infection depends on the immune system of the host animal. They reported that when healthy pigs encounter less virulent serotypes or a lower number of microorganisms, a significant clinical situation is usually not observed. However, when large numbers of animals are infected, this can be detected with the increase in serum antibody titres (Fenwick and Henry 1994).

Nicolet (1992) reported that serotypes 1, 5, 9, and 11 of *A. pleuropneumoniae* have high virulence, whereas serotypes 2, 4, 6, 8, and 12 have low virulence.

As the serotype of *A. pleuropneumoniae* isolated in this research is a low virulence serotype 12, this may explain the inability to observe a significant disease course in the animal.

Researchers reported the use of CF, 2-META, and ELISA tests in the serological diagnosis of *A. pleuropneumoniae*, and discussed their advantages against each other (Rycroft and Garside 2000; Fenwick and Henry 1994; Mittal et al. 1984). In the research carried out, the use and reliability of ELISA in the serological diagnosis of *A.pleuropneumoniae* have been approved (Klausen et al. 2001; Habrun et al. 1998a; Habrun et al. 1998b).

Levonen (2000) conducted a study to determine antibodies formed against *A. pleuropneumoniae* in herds that did not show any significant clinical symptoms related to *A. pleuropneumoniae* infection. The author found that 23.9% and 62.8% of 5,477 colostrum and 675 serum samples (respectively) from female pigs belonging to 154 herds were positive. Levonen (2000) reported that various antibody titres were detected in 129 herds with the percentage of seropositive animals being higher than expected.

In this research, as a result of the ELISA tests carried out for serological diagnosis, 67.2% of the 384 serum samples investigated were found to be positive in terms of *A. pleuropneumoniae* antibodies. For all of the 10 farms investigated serologically, seropositivity was found to be between 36% and 83.3%. The highest seropositivity (83.3%) was detected at the farm Istanbul-2. It is important to note that lung lesions and walking disability were observed only in animals sampled from this farm.

Habrun et al. (1998b) studied the seropositivity in 103 boars (aged 6 - 12 months), 334 sows (over 12 months of age), and 345 gilts (aged 6 months), with ELISA in terms of age and sex of the animals. They reported that the positivity in young males was significantly lower than the positivity in adult females and infants, and proposed that this was caused by the good conditions with separate, air-conditioning, and heat-regulated environments where the young male pigs were kept for optimum efficiency in producing offspring.

According to the results of ELISA tests in this research, the difference in seropositivity ratios of pigs of ages 1 to12 months, and one year and older were found to be non-significant. In addition to this, the association of sex with the positivity ratios was found to be non-significant. The contradiction of the results in this research with respect to the previous research was expected. This is due to the special environment where the young male pigs are kept for breeding in contrast to female, male and infant pigs kept in inappropriate conditions.

Researchers indicated that serological and bacteriological findings are not parallel, and thus yield different results (Levonen 2000; Sidibe et al. 1993; Thielmann 1989). In a research carried out in 5,477 female pigs, Levonen (2000) reported that in 1,307 colostrum (23.9%) and in 424 (62.8%) out of 675 blood serum samples, antibodies were detected but no successful isolation could be carried out.

Thielman (1989) indicated that 141 (21%) out of 679 blood sera were positive to *A. pleuropneumoniae*, and reported that in 2 of the 121 lung samples investigated, successful isolations were carried out.

In this research, serological and bacteriological findings were also found to be unparallel to each other. 67.2% out of 384 serum samples investigated serologically were detected to be positive. However, the causative agent could not be isolated from the lung and tonsil samples of the pigs. *A. pleuropneumoniae* was isolated from only one of the animals, the serum sample of which could not be obtained. Results obtained in this research were similar to findings of previous researchers. Although the high number of positive findings observed increases the probability of observing false positives, the use of Apx I and Tbp2 from the

outer membrane proteins to prevent cross-reactions with bacteria other than *A. pleuropneumoniae* in the ELISA kits, reduced the possibility of observing false positives. No cross-reactions have been reported associated with this kit.

Isolation of *A. pleuropneumoniae* indicates the presence of the infection in Turkey, and high seroprevalence supports this finding.

In conclusion, this study is the first report of the presence of *A. pleuropneumoniae* in Turkey. In accordance, the presence of the infection in Turkey should not be ignored, and necessary prevention and protection programmes should be developed.

Výskyt a séroprevalence *Actinobacillus pleuropneumoniae* u prasat v Turecku

V této studii byl sledován zvýšený výskyt a séroprevalence *Actinobacillus pleuropneumoniae* v různých oblastech Turecka. Proto bylo během porážky náhodně odebráno 384 vzorků plic, tonzil a krve. Prasata nejevila při porážení žádné známky respiratorních infekcí. Vzorky orgánů byly využity k izolaci. Specifické protilátky ze 384 vzorků séra (368 z nich bylo odebráno od zvířat spolu se vzorkem tkáně) byly testovány metodou ELISA (enzyme-linked immunosorbent assay). Vzorky plic a tonzil k izolaci byly inokulovány na PPLO agarové plotny s nikotinamidadenonukleotidem (NAD). Pouze z jednoho vzorku tonzil byl izolován *A. pleuropneumoniae* (sérotyp 12). Ze 384 vzorků krve bylo zjištěno 258 (67,2 %) pozitivních. Dvě stě devět pozitivních vzorků (54,4 %) bylo zahrnuto do skupiny jedna a zbylých 49 (12.8 %) do skupiny dvě. Vliv věku a pohlaví prasat nebyl statisticky významný při analýze výsledků ELISA testu ($p > 0,05$).

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