

Short note

Recovery of long-chain lipopolysaccharides from liquid culture of *Actinobacillus pleuropneumoniae* (serotype 5) for ELISA serodiagnosis

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Summary — *Actinobacillus pleuropneumoniae* (serotype 5) long-chain lipopolysaccharides (LC-LPS) may be used as the antigen for ELISA serodiagnosis of pig pleuropneumonia. A method was developed in order to augment the recovery of this antigen compared to the previously described method. In liquid culture, *A. pleuropneumoniae* was shown to produce 2.4 times more cells than in solid medium. LC-LPS could be recovered by phenol extraction of the crude extract. Only 1 additional phenol extraction was required to produce LC-LPS of the same quality as the one obtained with solid-medium-grown cells, as revealed by SDS-PAGE and ELISA using reference sera. From the same volume of crude extract, approximately 8 times more antigen was present in the aqueous phase of the phenol extraction.

***Actinobacillus pleuropneumoniae* / lipopolysaccharide / pig pleuropneumonia / serodiagnosis**

Résumé — **Obtention de lipopolysaccharides à longues chaînes à partir de culture en milieu liquide d'*Actinobacillus pleuropneumoniae* (sérototype 5) pour le diagnostic sérologique par ELISA.** Le diagnostic sérologique des infections à *Actinobacillus pleuropneumoniae* (sérototype 5) peut s'effectuer par la technique ELISA en utilisant les lipopolysaccharides à longues chaînes (LC-LPS) comme antigène. Une méthode, qui résulte en une plus grande quantité de cet antigène par rapport à la procédure déjà décrite, est présentée. En milieu liquide, 2,4 fois plus de cellules bactériennes ont été retrouvées par rapport à la croissance sur milieu solide. Le LC-LPS a été obtenu suite à une extraction au phénol de l'extrait brut. Une extraction additionnelle au phénol a été nécessaire pour

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obtenir un antigène comparable à ce qu'on obtenait avec le milieu solide, tel que déterminé par SDS-PAGE et ELISA à l'aide de sérums de référence. À partir du même volume d'extrait brut approximativement 8 fois plus d'antigène a été retrouvé dans la phase phénol suite à une partition au phénol.

***Actinobacillus pleuropneumoniae* / lipopolysaccharide / pleuropneumonie porcine / diagnostic sérologique**

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia. The disease causes important economic losses in many countries where swine production is important (Nicolet, 1992). Infection in swine can be fatal but pigs surviving acute infections frequently become carriers. Detection of chronically infected carriers is crucial since those animals act as reservoirs of bacteria (Rosendal and Mitchell, 1981). Various serological assays for *A pleuropneumoniae* have been described. Of those, ELISA is often the most useful since it is faster and easier to perform. Several ELISA have been independently developed using various antigenic preparations during the last few years (Nicolet *et al*, 1981; Bossé *et al*, 1990; Nielsen *et al*, 1991; Trottier *et al*, 1992).

For *A pleuropneumoniae* serotype 5, the capsular polysaccharides have been shown to be responsible for serotype specificity (Inzana and Mathison, 1987); however, obtaining pure material for serodiagnosis is cumbersome. On the other hand, we have previously shown that the use of long-chain lipopolysaccharides (LC-LPS) improved ELISA serodiagnosis over the use of a crude cell extract (Radacovici *et al*, 1992). LC-LPS is now recognized as an antigen showing a very high specificity in ELISA (97%) compared to the crude cell extract (74%) (Gottschalk *et al*, 1994). However, its production in relatively small amounts from solid-medium-grown bacterial cells, although quite accessible, is cumbersome since standardization of each batch is needed. The

aim of this study was to develop a method that would produce a larger amount of LC-LPS than the standard procedure.

MATERIALS AND METHODS

A pleuropneumoniae 81-750 is a local serotype 5b isolate (Faculté de médecine vétérinaire, Saint-Hyacinthe, Quebec, Canada) (Altman *et al*, 1990). Initial cultures were made aerobically at 37°C for 18 h on pleuropneumonia-like organisms (PPLO) (Difco Laboratories) agar plates supplemented with 1 µg/ml nicotinamide adenine dinucleotide (NAD) (Sigma) (PPLO-NAD). After initial growth, bacteria were inoculated on PPLO-NAD plates and grown for 6 h. Cells were harvested with 20 ml of 0.01 M PBS (pH 7.3) (Oxoid) containing 0.05% (v/v) formaldehyde per gram of bacteria. This suspension was kept overnight at 4°C and a volume of 30 ml PBS was added per gram of cells. The suspension was boiled 60 min and centrifuged at 12 000 g for 45 min. The supernatant (crude extract) was collected and filtered on a 0.22 µm pore size filter (Gelman) and stored at -20°C until use.

Bacteria were also grown in brain heart infusion (BHI, Difco) supplemented with 1 µg/ml of NAD and in Mueller-Hinton broth (MHB); 500 ml of fresh broth were inoculated and grown for 18 h. Cells were harvested by centrifugation (8 000 g for 15 min) and resuspended in 200 ml PBS containing 0.05% (v/v) of formaldehyde. The cells were then treated as described earlier. The volume of crude extract was reduced to a fifth of the initial volume with an Amicon system (Amicon Corp) using a YM 30 membrane and stored at -20°C until use. Volumes of 30 ml of crude extract obtained either from solid or liquid-media-grown cells were treated with phenol as previously described (Radacovici *et al*, 1992) in order to obtain LC-LPS.

To assess the quality of the LPS antigen obtained, sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) in a mini-slab apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). Samples were separated on 12.5% acrylamide gels. Gels were stained with Coomassie blue and silver nitrate according to the method of Tsai and Frasch (1982).

In addition, using LC-LPS obtained following the standard method with solid-medium-grown cells, an ELISA was done to compare the quality of the antigen obtained with liquid-medium-grown cells. Serum 380 served as a target serum with the crude extract of strain 81-750 grown on solid medium. Volumes of 50 μ l of a 1:200 dilution of the crude extract was added to each well and the reaction was stopped when serum 380 reached a value of approximately 1.5. For LC-LPS, 1 μ g (dry weight) was immobilized in the wells and the reaction was stopped when serum 380 reached approximately 1.5. All samples were examined in triplicate and simultaneously for comparison purposes.

An indirect ELISA was done according to the method described previously (Radacovici *et al*, 1992). Briefly, crude extract or LC-LPS were coated directly in a 96-well U-shaped Cooke Microtiter polystyrene plates (Dynatech Laboratories) using 0.1 M carbonate buffer (pH 9.6). Pig sera were diluted 1:200 in PBS-Tween (20 mM phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20). Horseradish peroxidase-labelled immunoglobulin G fraction of goat antiserum raised against porcine IgG (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA) diluted 1:1 000 in PBS-Tween was added. The reaction was visualized using 2 mM hydrogen peroxide and 0.4 mM 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 50 mM citrate solution (pH 4.0). Optical density was read at 410 nm using an automated plate reader (MR 5000, Dynatech).

Pig sera were produced as previously described (Radacovici *et al*, 1992) and are listed with their characteristics in table I.

RESULTS

To obtain the maximum number of cells (weight basis), the bacteria were grown overnight (18 h) in liquid medium. Two different liquid media were compared; BHI was

found to result in more cell production than MHB. On average, we obtained 6 g/l (wet weight) of bacteria with liquid medium (BHI) compared to 2.5 g/l on solid medium (PPLO-NAD). After phenol extraction of 30 ml crude extract (liquid production), we obtained an average of 80 mg (dry weight) of LC-LPS compared to 10 mg for solid-medium-grown bacterial cells. However, an additional extraction was needed with the crude extract obtained from the liquid compared to the solid-media-grown cells in order to have LC-LPS of the same quality. The quality of LC-LPS was evaluated using ELISA and SDS-PAGE (fig 1). LC-LPS were stained with silver nitrate but the preparation was not stained with Coomassie blue. We also fortuitously observed that bacteria grown in liquid media possessed a shorter O-chain (an estimated shift of 7 000 Da) than those grown on solid media as revealed by SDS-

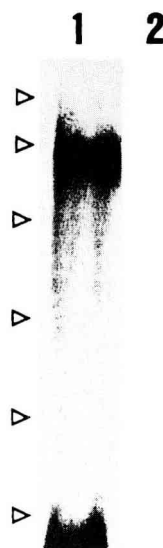


Fig 1. SDS-PAGE (12.5% acrylamide) of long-chain lipopolysaccharides obtained by phenol extraction of liquid media grown cells. Lane 1: silver-stained gel; lane 2: Coomassie-blue-stained gel. The arrow heads indicate the molecular mass standards (from top to bottom; 94, 67, 45, 30, 20.1 and 14.4 kDa).

Table 1. ELISA values (OD at 410 nm) obtained with crude extract and long-chain lipopolysaccharides obtained from strain 81-750 (serotype 5b) of *Actinobacillus pleuropneumoniae*.

Serum number	Strain (serotype)	Solid medium		Liquid medium	
		Crude extract	LC-LPS	Crude extract	LC-LPS
380	81-750 (5b)	1.58 ^a	1.49	1.10	1.02
381	81-750 (5b)	1.75	1.53	0.76	1.10
391	81-750 (5b)	1.17	0.88	0.97	0.74
250	K17 (5a)	1.49	1.31	1.09	1.03
392	L20 (5b)	1.21	1.06	0.53	0.78
386	1421 (3) ^b	1.48	0.07	0.37	0.01
384	Negative ^c	0.18	0.12	0.03	0.03

^a Values are the means of at least 2 determinations; ^b a local serotype 3 isolate inducing cross-reacting antibodies (Radacovici *et al*, 1992); ^c serum from a pig in a minimal disease herd free of *A pleuropneumoniae*.

PAGE and silver staining (data not shown). With the same quantity of antigen (1 µg/well), the optical densities were lower with the LC-LPS from the liquid production. Nevertheless, the results were positive with all the serotype 5 antisera tested (250, 380, 381, 391 and 392) (table 1). Serum 386 gave a negative result with the LC-LPS from the liquid production. The negative control remained negative.

DISCUSSION

For serodiagnosis purposes, the ease with which an antigen can be obtained and the amount of antigen recovered are of prime importance. In addition, having to standardize each batch of antigen can be time-consuming. The method we previously developed for obtaining LC-LPS gave, on average, 10 mg (dry weight) per 30 ml of crude extract from bacterial cells grown on solid media. Of this antigen preparation, approximately 1 µg per well was required for serodiagnostic purposes in ELISA. Thus, 10 mg of antigen could be used to coat

numerous ELISA plates but several batches still had to be made when diagnosis required testing hundreds of sera daily. In order to circumvent this problem, and because phenol extraction is time-consuming, we tried to increase the quantity of LC-LPS obtained without having to treat larger quantities of crude extract.

The amount of bacteria recovered was an important limiting factor when using solid medium; it thus seemed more appropriate to grow the bacteria in liquid medium. The cost of solid media being superior than that of liquid media, and the ease with which those cells are recovered, are also to be taken into account when designing a way to purify antigens for diagnostic purposes. In fact, from the same volume of medium the cell wet weight was approximately 2.5 times higher in liquid medium. One of the problems we had to solve with liquid-medium-grown cells was the larger volume of crude extract. To reduce the volume of crude extract we had to handle, this extract was concentrated on an Amicon system using a YM 30 membrane. This step was slow but did not involve a lot of manipulations and

the LC-LPS were not lost or affected in any way, as shown by SDS-PAGE and ELISA. This step also permitted the reduction of the amount of proteins of smaller molecular weight (< 30 kDa) and LPS with short polysaccharidic chains. This could be observed in ELISA with the crude extract as it gave lower values with serum 386 (table I). It was previously demonstrated that this serum gave cross-reactions with the crude extract of serotype 5 due to LPS with no or short polysaccharidic chains (Radacovici *et al*, 1992). After phenol extraction of the crude extract (liquid production), we obtained an average of 80 mg (dry weight) of LC-LPS. This is 8 times more than the previously described method using solid-medium-grown cells for the extraction of the same volume (30 ml) of crude extract. These LC-LPS were tested in ELISA and showed similar results (table I). The shift in molecular weight of LC-LPS of cells grown in liquid medium was observed on whole cells taken after 18 h of growth and before any treatment was performed on the cells. The shorter O-chains could be the result of either the type of culture (solid *versus* liquid) or the composition of the media used. However, this shorter O-chain possessed the same antigenicity as it gave positive results with all the antisera raised against homologous and heterologous strains of *A pleuropneumoniae* serotype 5.

In conclusion, we have developed a method that allows recovery of LC-LPS from liquid-medium-grown bacterial cells. From 30 ml of crude extract, approximately 8 times more LC-LPS could be recovered from the liquid-medium-grown cells. Considering the cost of the media and that only one extra phenol extraction was needed to recover LC-LPS of the same quality, liquid culture represents an improvement over the previously reported method.

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