Evaluation of survival of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* in four liquid media and two swab specimen transport systems

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Objective—To determine duration and rates of recovery of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* from 4 liquid media and 2 swab specimen transport systems and compare findings with those of *Escherichia coli*.

Sample Population—One strain each of *A pleuropneumoniae* (biovar 1, serotype 1), *H parasuis* (serovar 5), and *E coli* (serotype O149:K91:H19).

Procedure—Strains were incubated in brain heart infusion broth supplemented with horse serum and other nutrients or in horse serum alone, with and without nicotinamide-adenine dinucleotide in both instances, for 150 days at 4°C or room temperature (21°C). Similarly, strains were tested in Stuart and Amies transport systems after storage at room temperature for 8 days.

Results—Colony counts greater than those of the initial inoculum were observed after incubation in horse serum for *A pleuropneumoniae* but not for *H parasuis*. Overall, incubation at 4°C in the 4 liquid media resulted in longer recovery duration and higher rates than at room temperature. Culture of *H parasuis* resulted in lower recovery rates and shorter durations of recovery than culture of *A pleuropneumoniae,* except for culture in horse serum. *Haemophilus parasuis* survived longer than *A pleuropneumoniae* in the transport systems, and all organisms survived longer in the Amies system.

Conclusions and Clinical Relevance—Survival of *A pleuropneumoniae* and *H parasuis* indicated that horse serum prolongs survivability, which may result in exposure of more animals during a prolonged period. The Amies system might be a good choice for collection of clinical samples from animals, especially for recovery of *H parasuis*. (*Am J Vet Res* 2003;64:1176–1180)

Changes in swine management systems, which have resulted in high densities of swine as well as continuous entry of new litters, have contributed to an environment that considerably increases the spread of air-borne pathogens among herds.[1] In this respect, respiratory tract diseases are now regarded as the most serious problem in modern swine production to such a point that it is stated that any pig reaching slaughter weight will have some type of respiratory tract lesion.[2]

Actinobacillus pleuropneumoniae and *Haemophilus parasuis* are 2 of the organisms most commonly involved in swine respiratory tract diseases. *Actinobacillus pleuropneumoniae* causes pleuropneumonia, a highly contagious disease characterized by severe necrotic and hemorrhagic pneumonia with fibrinous pleural adhesions. The organism may cause either acute respiratory tract infection, resulting in high morbidity and mortality rates, or chronic persistent infection, resulting in lower body weight. There are 15 serotypes of biotype 1 differentiated on the basis of antigenic differences in the capsular and lipopolysaccharide antigens.[3] All of the serotypes are capable of causing the disease, although few are highly prevalent in a given area at a given time.[4]

Haemophilus parasuis is the causative agent of Glässer disease, which is a disease characterized by fibrinous polyserositis, polyarthritis, and meningitis that has historically been considered a sporadic, stress-associated disease of young pigs.[5] However, since the establishment of specific pathogen-free herds, increased spread of the disease and increased mortality rates due to *H parasuis* infection have been described. Fifteen serovars of this organism have been identified on the basis of heat-stable antigen preparations tested by immunodiffusion (Kielstein-Rapp-Gabrielson serotyping scheme),[6] and most recent serotyping studies[7,8] have reported the existence of untypeable isolates, indicating the possibility of a larger number of serovars.

Recovery of these 2 organisms from clinical and environmental specimens after transport or storage is a major problem because of loss of viability. For this reason, fresh clinical material must be sent to the laboratory as soon as possible, because freezing is known to have a detrimental effect on the survival of *A pleuropneumoniae*.[9] The fragility of the organisms implies that close contact between infected and susceptible swine is a prerequisite for the spread of the organism; however, protection conferred by respiratory secretions and other organic material may allow prolonged survival and increase chances of infection.[10]

In the study reported here, we evaluated survival of *A pleuropneumoniae* and *H parasuis* in 4 liquid media stored at 4°C and room temperature (21°C) and in 2 swab specimen transport systems and compared results with those obtained with *Escherichia coli*, a gram-negative, nonfastidious bacterium that is common in swine.

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Materials and Methods

Bacterial strains—*Actinobacilius pleuropneumoniae* biotype 1, serotype 1 (strain CM5) and *H parasuis*, serovar 5 (strain H413) were obtained from the culture collection of our department. *Escherichia coli* strain 216a (serotype 0149:K91:H19) was isolated from a pig with enteric infection in Spain.[a]

Liquid media—Four liquid media were used. **Brain heart infusion (BHI)** broth with **nicotinamide-adenine dinucleotide (NAD)** consisted of 37 grams of BHI base[b] supplemented with 1% glucose, 5% **horse serum (HS)**, 5% yeast extract, 2.2µM thiamine pyrophosphate,[c] 0.01% hemin,[c] and 0.01% NAD.[c] It was adjusted to a pH of 7.4 and sterilized at 121°C for 15 minutes. Horse serum and NAD were previously sterilized by being passed through a 0.22-µm membrane filter[d] and added to the autoclaved components immediately before use. Complement was removed from the HS by heating it at 56°C for 30 minutes. The other 3 liquid media used were BHI broth prepared as described but without NAD, HS alone, and **HS with 0.01% NAD (HS-NAD)**. Survival of *H parasuis* was only tested on BHI-NAD, HS, and HS-NAD. Bacterial viability was measured on **pleuropneumonia-like organism (PPLO)** agar medium[e] supplemented for BHI-NAD, as described.

Survival in liquid media—To determine when the stationary phase was reached, a standardized growth curve from each test strain was initially calculated in the BHI-NAD medium. Aerobic overnight cultures at 37°C were prepared in BHI-NAD, and bacteria (adjusted to an **optical density [OD]** of 0.1 at 600 nm) were grown on fresh BHI-NAD at the same temperature until reaching the stationary phase (approx OD of 0.6 or 10 hours of incubation for *A pleuropneumoniae* and *H parasuis* and 1.2 or 8 hours of incubation for *E coli*). These suspensions were used to inoculate approximately 10^7 **colony-forming units (CFU)** in each liquid medium and held at 4°C and room temperature. The different media were sampled at 2-day intervals for 150 days after inoculation. The number of viable bacteria was determined by serially diluting each sample in RPMI 1640 medium with L-glutamine,[f] spreading on PPLO agar supplemented with NAD in duplicate, incubating at 37°C for 24 hours (*A pleuropneumoniae* and *E coli*) or 48 hours (*H parasuis*), and counting the colonies.

The percentage of recovery of each strain was calculated by dividing the number of colonies at test time by the original number at time zero and multiplying by 100.[11] All of the studies were carried out in duplicate by use of newly prepared reagents.

Swab specimen transport media—Two commercial swab specimen transport systems for aerobic organism recovery were compared: Stuart medium[g] and Amies medium without charcoal.[h] These transport media were inoculated with approximately 10^9 CFU of test strains/mL.

Survival in swab specimen transport systems—Dilutions of overnight cultures on chocolate polyvitex agar[i] were prepared in sterile saline (0.9% NaCl) solution to obtain approximately 10^9 CFU/mL. Swab specimens of each transport device were inoculated by being dipped vertically into 5 mL of inoculum for 25 seconds, removed, and kept in their respective tubes with transport medium. After daily intervals varying from 0 to 8 days of storage at room temperature, organism survival was evaluated by vortexing each swab in 1 mL of sterile saline solution for 25 seconds, expressing it against the tube wall prior to discarding, and preparing plate counts, as described.

Statistical analyses—Mean values for 2 counts each on triplicate swab specimens were determined for each organism, device, and sample time studied. The percentage of recovery of each strain was calculated, as described. A Student-Fisher test was used to determine whether significant differences existed in the percentage recovery values between the 2 transport systems. Differences were considered significant at $P \leq 0.05$.

Results

Survival in liquid media—Colony counts > 100% were obtained for *A pleuropneumoniae* for 3 days after storage in HS at room temperature and 5 days at 4°C (**Table 1**). The greatest recovery rate above that of the initial inoculum was found after 9 days of storage at 4°C in HS-NAD, and the longest duration of recovery > 100% was 13 days under the same conditions. The HS-NAD medium was able to maintain the viability of this organism at a level greater than half that of the original inoculum for 23 days, whereas no recovery was obtained with the other 3 media at this time. No survival of *A pleuropneumoniae* was detected beyond 29 days. Except for HS-NAD, storage at 4°C yielded longer

Table 1—Median percentages of recovery of *Actinobacillus pleuropneumoniae* strain CM5 tested via 4 media and 2 incubation temperatures

	Median recovery (%)							
	BHI		BHI-NAD		HS		HS-NAD	
Storage time (d)	RT	4°C	RT	4°C	RT	4°C	RT	4°C
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	100.0	98.8	100.0	109.6	102.7	116.4	108.2
3	95.3	93.0	97.7	86.0	104.1	113.7	102.7	108.2
5	77.9	89.5	89.5	88.4	91.8	101.4	105.5	105.5
7	48.8	82.6	64.0	77.9	86.3	89.0	98.6	104.1
9	45.3	72.1	0	72.1	76.7	93.2	91.8	120.5
11	22.1	68.6	0	67.4	42.5	90.4	100.0	113.7
13	30.2	32.6	0	30.2	41.1	89.0	91.8	105.5
23	0	0	0	0	0	0	61.6	69.9
25	0	0	0	0	0	0	42.5	49.3
29	0	0	0	0	0	0	17.8	0
31–150[a]	0	0	0	0	0	0	0	0

[a]Days 31 through 150 every 2 days.
BHI = Brain heart infusion. NAD = Nicotinamide-adenine dinucleotide. HS = Horse serum. RT = Room temperature (21°C). HS-NAD = HS with 0.01% NAD.

Table 2—Median percentages of recovery of *Haemophilus parasuis* strain H413 tested via 4 media and 2 incubation temperatures

	Median recovery (%)					
	BHI-NAD		HS		HS-NAD	
Storage time (d)	RT	4°C	RT	4°C	RT	4°C
0	100.0	100.0	100.0	100.0	100.0	100.0
1	83.2	88.1	91.6	96.8	92.7	93.4
3	20.8	65.3	88.1	94.6	91.2	92.1
5	0	54.5	81.9	89.1	85.7	86.8
7	0	42.6	78.3	85.9	73.6	79.1
9	0	17.8	73.5	84.8	64.8	72.5
11	0	11.9	72.3	78.3	56.0	59.3
13	0	0	71.1	73.9	46.2	48.9
23	0	0	13.3	41.3	0	18.7
25	0	0	0	33.7	0	6.6
31	0	0	0	21.7	0	0
33–150[a]	0	0	0	0	0	0

See Table 1 for key.

Table 3—Median percentages of recovery of *A pleuropneumoniae*, *H parasuis,* and *Escherichia coli* strains tested via 2 swab specimen transport systems

	Median recovery (%)					
	A pleuropneumoniae		*H parasuis*		*E coli*	
Storage time (d)	Stuart	Amies	Stuart	Amies	Stuart	Amies
0	100	100	100	100	100	100
1	79.7 ± 2.23	85.6 ± 3.73	67.1 ± 7.71	91.9 ± 2.62[a]	96.8 ± 2.77	119.7 ± 4.18[a]
2	48.2 ± 3.91	54.2 ± 4.28	70.7 ± 6.45	94.0 ± 0.91[a]	102.2 ± 1.31	112.0 ± 6.70
3	0	0	58.9 ± 0.66	81.4 ± 2.07[a]	100.7 ± 5.47	112.4 ± 4.23
4	0	0	0	71.9 ± 1.34	84.9 ± 2.69	120.4 ± 2.75[a]
5	0	0	0	27.4 ± 2.16	73.4 ± 1.42	108.8 ± 1.99[a]
6	0	0	0	29.7 ± 0.46	87.5 ± 2.86	109.9 ± 4.13[a]
7	0	0	0	0	89.2 ± 3.90	108.3 ± 2.35[a]
8	0	0	0	0	80.5 ± 3.05	108.8 ± 1.61[a]

[a]Significant ($P = 0.05$) difference, compared with the results obtained on the same day with the Stuart transport medium.

recovery duration than at room temperature. Incubation in BHI-NAD at room temperature resulted in the shortest duration of recovery (7 days).

Unlike *A pleuropneumoniae*, the colony count of *H parasuis* did not increase under any of the conditions tested (**Table 2**). Recovery rates > 88% were obtained in the media containing HS after 3 days, whereas recovery was only 20.8% for organisms in BHI-NAD at room temperature. Both the greatest recovery and longest duration of recovery were achieved at 4°C. Overall, *H parasuis* had a shorter duration of viability than *A pleuropneumoniae* with the exception of culture in HS medium at 4°C, which maintained survival of *H parasuis* for 12 days longer than that of *A pleuropneumoniae*, and culture at room temperature, which maintained survival of *H parasuis* for 10 days longer than that of *A pleuropneumoniae*.

Each of the 4 media enhanced the growth of the initial inoculum of *E coli*, independent of the incubation temperature. Rates of > 100% were obtained for 17 days, and viability was maintained through 150 days.

Survival in swab specimen transport systems—Survival of the 3 organisms in aerobic Stuart and Amies culture tubes was determined (**Table 3**). *Escherichia coli* yielded the best results (positive results of cultures after 8 days in both transport systems). The Amies system performed better at maintaining the viability of *H parasuis* and *A pleuropneumoniae* than the Stuart system. After 2 days of incubation, 94.0 and 54.2% of *H parasuis* and *A pleuropneumoniae*, respectively, were recovered in the Amies system, whereas 70.7 and 48.2%, respectively, were recovered in the Stuart system. Contrary to the results in liquid media, *H parasuis* survived longer than *A pleuropneumoniae* in both transport systems. The Amies system maintained 29.7% of the *H parasuis* organisms after 6 days of incubation; however, no recovery was observed with the Stuart system beyond 3 days. Both systems maintained the viability of *A pleuropneumoniae* for only 2 days, although the Amies system again resulted in higher survival rates than the Stuart medium. The recovery rates of *H parasuis* in the Amies system at 1 to 3 days were significantly higher than in the Stuart system, and similarly, those of *E coli* were significantly higher at 1 and 4 to 8 days.

Discussion

Survival of microorganisms is a prerequisite for infectivity. All factors known to influence survival must affect infectivity as well.[12] The survival of airborne microorganisms, such as *A pleuropneumoniae* and *H parasuis*, is influenced by many physical and biochemical factors, including conditions of growth,

methods of aerosol generation and collection, climate, and radiation.[13] *Actinobacillus pleuropneumoniae* and *H parasuis* have traditionally been considered fragile organisms, because they do not survive particularly well during routine laboratory practice unless subcultured regularly. However, it is well known that after being shed from infected animals into the environment, organisms can maintain their viability and even multiply outside the host inside a variety of organic matter present in swine facilities; thus, swine can be exposed over a sustained period.[10]

For this reason, a broth medium containing a large amount of organic matter, especially proteins, was selected for our study. It also contained sodium chloride and glucose, additives known to maintain and enhance survival rates in *A pleuropneumoniae* and *Pasteurella multocida* (another airborne organism also belonging to the family *Pasteurellaceae*).[14,15] In addition, undiluted serum was used because it has been chosen as a model of organic matter in another study[16] and because of the good survival results reported for *A pleuropneumoniae* in aerosols.[15] The results obtained for growth of this organism appeared to depend on nutrients, because it multiplied in HS but not in BHI; the addition of NAD also enhanced recovery rates. Although NAD-dependent growth is a characteristic of *A pleuropneumoniae* biotype 1,[17] our results indicated that strain CM5 (biotype 1, serotype 1) can multiply slightly in HS without this supplement for a few days. A certain degree of growth has also been reported for other serotypes of this biotype on NAD-restricted media.[18] In an earlier investigation of *Bordetella bronchiseptica*[19] (another respiratory tract pathogen of swine), 8-fold increases were observed in low-nutrient fluids (various natural water samples) for the first 72 hours, which is in accordance with the multiplication period reported in our study.

In comparison with *E coli*, *A pleuropneumoniae* and *H parasuis* must be considered fragile organisms, because they do not survive more than 1 month under the best conditions tested. In a previous investigation,[20] the viability of *P multocida* in several liquids (eg, distilled water, artificial seawater, nutrient broth,[j] sterile pig slurry, and nasal washings) was quite similar to that observed here for *A pleuropneumoniae* and *H parasuis*. Similarly, *B bronchiseptica* remained viable for at least 3 weeks in PBS solution and in lake water at 37°C[19]; this is a finding similar to ours, although the nutrient content of the fluids used in our study was considerably greater. In contrast, Bredy and Botzler[14] found that *P multocida* survived for > 1 year in water supplemented with soluble proteins (175 µg/mL) and 0.5% sodium chloride. In a previous report,[21] *H parasuis* had the least viability, compared with *A pleuropneumoniae*, *P multocida,* and *E coli*, in low-nutrient fluids (saline solution and sodium PBS solution); this is in agreement with our findings. The viability of *A pleuropneumoniae* and *H parasuis* in all liquid media is enhanced by refrigeration at 4°C. This result is in disagreement with that obtained in another report[20] in which *P multocida* survived better at higher temperatures in tryptose broth, a medium with composition quite similar to BHI.

Proper collection and transport of specimens is mandatory for appropriate recovery and further identification of any infecting bacteria, especially the most fragile organisms. Specimens are often received by laboratories 24 to 48 hours after collection, but unexpected transport delays may impair the timely culture of these samples; therefore, the transport system needs to be adequate to maintain the viability of organisms during extended periods. In addition, an ideal transport system must support viability without promoting bacterial multiplication to avoid overestimation of bacterial counts. To the authors' knowledge, there are no previous reports evaluating commercially available culture swab specimen systems for transporting *A pleuropneumoniae* and *H parasuis* species.

Stuart and Amies transport systems are commonly used for mailing veterinary and human samples; thus, both were selected for this study. Only incubation at room temperature was selected, because most references recommend holding specimens at room temperature rather than at refrigeration temperature. Arbique et al[22] determined that there are no differences in the recovery rates of different organisms when the Amies medium is stored refrigerated or at ambient temperature.

The Amies system performed better than the Stuart system for the 3 organisms studied. Unlike our results, those of an earlier study[23] revealed better performance for the Stuart medium than for Amies medium when testing *E coli*, but not when testing *H influenzae*. The survival of *H influenzae* in different commercial transport systems has been reported to be shorter or similar to that observed in our study.[23-25] Similarly, the viability of *P multocida* described previously[26] was quite similar to that of *A pleuropneumoniae* and *H parasuis* determined in our study. The fact that the Amies system is more effective in maintaining viability of these organisms can be explained by the different composition of these 2 transport systems; in fact, the Amies medium is a modified form of the Stuart medium and also contains a phosphate-balanced salt solution in a semisolid medium.

Our survival results for the 4 liquid media suggest that organic matter plays a role in the maintenance of viability of *A pleuropneumoniae* and *H parasuis*. These agents may be protected in the environment, thus prolonging exposure time for susceptible swine, which could result in wider spread of infection. Conversely, although the submission of intact tissues or fluid samples rather than swab specimens is recommended for routine isolation of *A pleuropneumoniae* and *H parasuis* (when submission of this material is not possible), the Amies system might be a good choice for collection of clinical samples that are destined to be plated within 48 hours (eg, nasal mucus, intratracheal washes, and joint aspirates). Beyond this time, the Amies system appears to be useful only for recovery of *H parasuis*.

[a]Courtesy of Dr. Balnco, Faculty of Veterinary Medicine of Lugo, University of Santiago, Spain.
[b]Brain heart infusion broth, Gibco Laboratories, Detroit, Mich.
[c]Nicotinamide adenine dinucleotide, Sigma Chemical Co, St Louis, Mo.
[d]Millex GP, Millipore, Molsheim, France.
[e]Pleuropneumonia-like organisms agar, Biolife, Milano, Italy.

[f]RPMI 1640 medium with L-glutamine, Gibco Laboratories, Detroit, Mich.
[g]Stuart medium, Biomedics, Madrid, Spain.
[h]Amies medium without charcoal, Copan, Italy.
[i]Chocolate polyvitex agar, BioMérieux Inc, Marcy l'Etoile, France.
[j]Bacto tryptose broth, Difco Laboratories, Detroit, Mich.

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