

Original Article

Assessment of different storage conditions for *Staphylococcus hyicus* survival

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Abstract

Introduction: Collecting swabs from skin lesions for bacteriological examination is frequently performed to the diagnosis of exudative epidermitis. This method is fast and non-invasive, but it depends directly on the viability of bacteria in clinical samples, which can be influenced by storage and shipment temperatures and the time of transportation. The aim of this study was to assess the capacity of four commercial transport media and swabs with no transport medium to preserve *Staphylococcus hyicus* (*S. hyicus*) for up to 10 days at room temperature and under refrigeration.

Methodology: Samples were stored in swabs with no transport medium and four transport media (Amies, Amies with charcoal, Cary Blair and Stuart) for 10 days at room temperature and under refrigeration. Swabs were plated in Tween 80 Agar and colonies counted.

Results: Samples kept in transport media showed better performance ($P < 0.05$) under refrigeration. Storage under refrigeration in Amies medium showed better results than all other transport media and swabs ($P < 0.05$). Amies medium and swabs with no transport medium showed comparable results in room temperature ($P > 0.05$). In addition, refrigerated Amies medium and swabs with no transport medium at room temperature showed high performance for up to nine and three storage days, respectively.

Conclusions: The recovery of *S. hyicus* in samples stored in Amies medium under refrigeration was higher when compared to other transport media. In addition, swabs with no transport medium could also be indicated when samples are stored at room temperature within three days.

Key words: Exudative epidermitis; swine; transport media.

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Introduction

Exudative epidermitis is a skin disease caused by *Staphylococcus hyicus* (*S. hyicus*) that affects pigs of all ages but is more frequent in suckling and post-weaned piglets [1]. Clinical signs and lesions are typical, even though variation might occur. The diagnostic is usually performed by bacterial isolation from swabs collected from skin lesions, since it is a fast, inexpensive and non-invasive method [2].

The transportation of clinical specimens to microbiological evaluation is a key step to ensure accurate laboratory results [3]. Bacteriological examination depends directly on the viability of bacteria in clinical specimens, which can be affected by storage or shipment temperature, transport time [4,5], and type of transport medium [6], since there are some bacteria capable of surviving in swabs after long storage

periods [7]. It is usually recommended to hold swab samples in low temperatures and use transport media to ensure bacterial survival when the period between collection and processing at the laboratory is lengthy [7].

Several types of swabs and transport media are commercially available, but there are no evaluations about *S. hyicus* survival in swabs and commercial transport media. Therefore, the aim of the present study was to evaluate four types of transport media and swabs with no medium regarding their ability to maintain *S. hyicus* viability for up to 10 days of storage at room temperature (20-25°C) and under refrigeration (4-8°C).

Methodology

Storage conditions: Four transport media and swabs with no transport media were tested. The following

storage conditions were evaluated: (a) Amies transport medium (Cral-Plast, Cotia, Brazil); (b) Amies transport medium with charcoal (Cral-Plast, Cotia, Brazil); (c) Stuart transport medium (Labor Import); (d) Cary Blair transport medium (Cral-Plast, Cotia, Brazil); (e) swabs with no transport medium (Labor Import, Osasco, Brazil). All transport media and swabs were used following manufacturer’s recommendations.

Inoculum: *S. hyicus* ATCC 11249 was used as inoculum. The inoculums were prepared in Mueller Hinton broth to deliver approximately 1.5×10^8 CFU/mL (equivalent to 0.5 McFarland scale).

Experimental procedures: The Roll-Plate Method [4] was used to compare transport media and swabs. From the inoculums, four dilutions were prepared in 0.85% physiological saline: 1:10 (1.5×10^7 CFU/mL), 1:100 (1.5×10^6 CFU/mL), 1:1,000 (1.5×10^5 CFU/mL), and 1:10,000 (1.5×10^4 CFU/mL). The swabs were placed in the wells containing 100 μ L of three dilutions (10^{-2} , 10^{-3} and 10^{-4}) for 10 seconds and transferred to their tubes and stored at room temperature (20-25°C) or under refrigeration (4-8°C) for 10 days.

After storage time, the swabs were plated in three directions, rotating the plate approximately 60° each time to ensure that all tips contacted the agar surface. The plates were then incubated at 37°C for 20 hours and the CFU/mL was calculated.

The swabs from zero-time were immediately plated to Tween 80 Agar [4]. A Tween 80 Agar plate was inoculated for each type of five transport media, 10 days storage times, two different temperatures, three dilutions, and three replications (swabs).

Counting of dilution 10^{-4} (1.5×10^4 CFU/mL) at zero-time (close to 300 CFU/mL⁻¹⁰) was considered the rate 100% of *S. hyicus* recovery. Survival rates for each swab and transport medium, temperature and storage time were compared with this result, considered a high rate when above or equal to 50% and a low recovery rate when below 50%.

Statistical analysis: Average percentages of recovery rates were compared along the storage time to evaluate the performance of each transport medium using a nonparametric test (Kruskal-Wallis Test) with SAS [8]. The same procedure was used to compare the storage temperatures (Wilcoxon two-sample test) within each transport medium. Statistical difference was considered when $P < 0.05$.

Results

Staphylococcus hyicus survival rates for each swab and transport media at room temperature or under refrigeration are shown in Table 1. Swabs with no transport medium at room temperature showed early readings close to 70% survival rate and successive

Table 1. *Staphylococcus hyicus* survival in five types of swabs and transport media along ten days of storage at room temperature or under refrigeration.

Storage days	Room temperature					Refrigeration				
	Stuart	Cary Blair	Amies with charcoal	Amies	Without medium	Stuart	Cary Blair	Amies with charcoal	Amies	Without medium
1	43.8 ± 29.9bc	7.0 ± 4.5c*	25.4 ± 9.9c*	137.2 ± 0.64a*	71.9 ± 8.1b	29.9 ± 11.0c	39.8 ± 12.0bc*	76.7 ± 10.2b*	158.9 ± 0.3a*	43.0 ± 17.5bc
2	12.4 ± 3.9c	1.3 ± 0.5d*	16.3 ± 4.0cd*	100.7 ± 14.6a	69.5 ± 0.2b	28.9 ± 8.8c	60.0 ± 6.3b*	71.4 ± 10.3b*	128.3 ± 9.9a	35.0 ± 17.4bc
3	1.5 ± 1.1c*	5.9 ± 2.8bc	15.8 ± 5.0b*	11.1 ± 9.2bc*	56.5 ± 0.2a	29.5 ± 4.6b*	7.2 ± 2.7c	71.6 ± 9.6a*	65.7 ± 26.0a*	32.3 ± 13.1ab
4	0.0 ± 0.0c*	5.9 ± 1.6b*	6.9 ± 1.6b*	30.0 ± 17.6ab*	47.6 ± 11.4a	29.7 ± 7.6b*	31.0 ± 10.2b*	39.3 ± 9.2b*	130.6 ± 8.6a*	31.7 ± 14.0b
5	0.0 ± 0.0c*	4.8 ± 0.7c*	15.2 ± 1.6b*	53.5 ± 14.2a*	25.9 ± 11.4ab	42.6 ± 15.3b*	38.7 ± 8.2b*	38.8 ± 5.6b*	152.6 ± 7.2a*	13.7 ± 5.1b
6	0.0 ± 0.0c*	2.5 ± 0.8d*	7.6 ± 2.4d*	67.2 ± 22.7a*	34.1 ± 1.7b	29.5 ± 14.0bc*	13.1 ± 1.4c*	37.9 ± 6.1b*	164.3 ± 0.2a*	17.8 ± 10.8bc
7	0.0 ± 0.0d*	1.9 ± 0.5c*	12.4 ± 3.2b*	38.0 ± 7.6a*	23.5 ± 17.6ab	28.7 ± 9.6b*	21.3 ± 7.4b*	37.3 ± 6.5b*	108.1 ± 8.2a*	7.3 ± 1.6c
8	0.0 ± 0.0c*	2.7 ± 1.3b	6.9 ± 2.3b*	24.1 ± 1.6a*	5.1 ± 3.2b	25.8 ± 4.6b*	20.2 ± 10.4bc	28.2 ± 3.4b*	126.1 ± 20.6a*	7.0 ± 3.5c
9	0.0 ± 0.0c*	2.1 ± 0.2b*	9.4 ± 1.9a*	12.6 ± 2.4a*	7.7 ± 7.1ab	25.6 ± 7.8b*	14.9 ± 4.1bc*	20.5 ± 1.3b*	76.1 ± 16.7a*	8.6 ± 3.8c
10	0.0 ± 0.0d*	3.6 ± 0.8b*	10.4 ± 3.0a	0.9 ± 0.2c*	5.5 ± 2.4abc	24.6 ± 11.2ab*	13.7 ± 3.8b*	16.5 ± 3.7b	30.4 ± 3.5a*	10.0 ± 2.0b

Different lowercase letters (a, b, c, d) represent significant difference ($P < 0.05$) among the types of swabs within the same temperature of storage; * The symbol indicates a significant difference ($P < 0.05$) between room temperature and refrigeration, within the same type of swab; Each condition combining transport medium and storage temperature was replicated three times. Although means ± standard error of mean are shown, non-parametric statistical analysis was used for comparisons between temperatures (Wilcoxon two-sample test) or among types of swabs (Kruskal-Wallis test).

decrease was observed after day 8, reaching less than 20%. Samples refrigerated with no transport medium presented survival rates ranging from 43.0% to 31.7% from day 1 to 4, falling to less than 20% after day 5.

The recovery of *S. hyicus* with Amies transport medium was higher than 100% in the first 2 days at room temperature (Table 1). Moreover, high survival rates were observed in the days 5 and 6 (53.5 % and 67.2 %, respectively). From day 5 to 9, higher survival rates ($P < 0.05$) were observed in Amies than in the other swabs with transport medium. Under refrigeration, recovery of *S. hyicus* was above 100% in 7 out of 10 days of storage. Higher recovery rates ($P < 0.05$) were observed for Amies compared to the other systems, on days 1, 2 and on days 4 to 9.

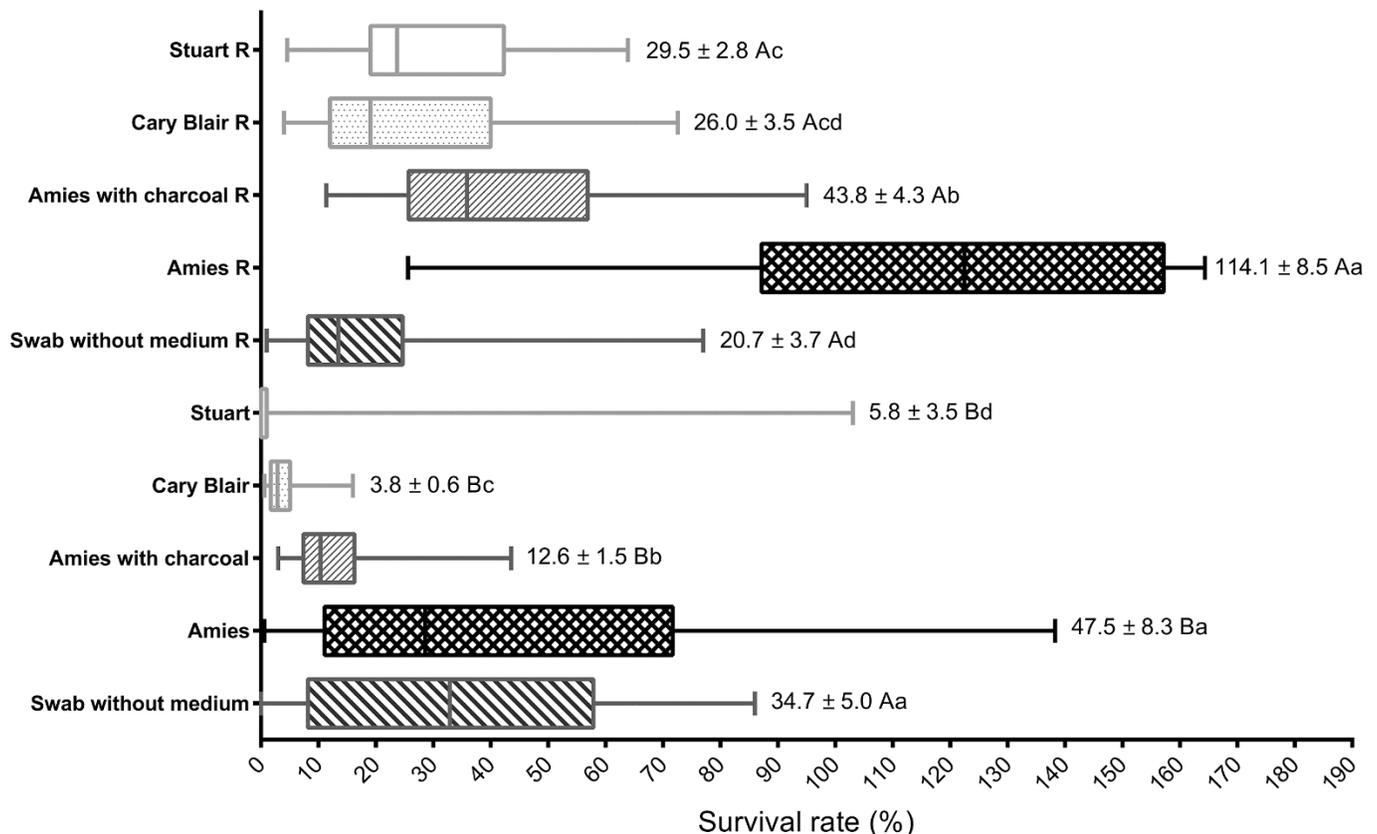
Recovery rates were reduced as early as 24 hours at room temperature (25.4%) in Amies with charcoal transport medium, and subsequent decrease (below 20%) was observed (Table 1). However, viability ranged from 71.4 to 76.7% in the first three days of storage under refrigeration. Recovery rates were close

to 40% from days 4 to 7 and less than 30% in the last three days under refrigeration.

Survival of *S. hyicus* in Cary Blair transport medium at room temperature was smaller than 10% during the entire storage time (Table 1). At refrigeration, recovery rates were instable, especially on the first five days with values ranging from 7.2% to 60.0%. On the other days, the rates were below or close to 20%.

Recovery with Stuart transport medium was 43.8% after 24 hours at room temperature, decreasing to 12.4% and 1.5% on days 2 and 3; and after day 4 it was no longer possible to isolate *S. hyicus* (Table 1). Survival at refrigeration was stable along storage days, ranging from 24.6 to 42.6% and rates were close to 30% from days 6 to 10. At days 4 to 6, the recovery rates in Stuart were below ($P < 0.05$) to those in Amies but similar ($P > 0.05$) to other transport media or swab with no transport medium. Between days 7 and 10, Stuart refrigerated samples performed similarly ($P > 0.05$) to Cary Blair and Amies with charcoal.

Figure 1. Box-plot representation of average survival of *Staphylococcus hyicus* in five types of swabs and transport media considering ten storage days altogether. Box-plots contain the values corresponding to minimum, first quartile, median, third quartile and maximum. Means plus standard error of means (SEM) followed by different lowercase letters represent significant difference ($P < 0.05$) among the types of swabs within the same temperature of storage. Different capital letters indicate significant difference ($P < 0.05$) between temperatures within the same type of swab. Although means \pm SEM are shown, non-parametric statistical analysis was used for comparisons between temperatures (Mann-Whitney test) or among types of swabs (Kruskal-Wallis test). R= refrigerated.



Overall, refrigerated samples performed better ($P < 0.05$) than specimens stored at room temperature in all transport media. Better performance of refrigerated samples was observed for 8 out of 10 days for Cary Blair and Stuart, and for 9 out of 10 days for Amies and Amies with charcoal (Table 1). However, similar performance was observed in swabs with no transport medium stored at room temperature and refrigerated ($P > 0.05$). Comparing samples at room temperature (Figure 1), there was statistical differences ($P < 0.05$) between all swabs with transport medium, and the higher recovery was observed with Amies medium and the worst performance was observed with Stuart medium. Swabs with no transport medium showed similar performance ($P > 0.05$) with Amies but higher recovery rate ($P < 0.05$) than the other swabs with transport media. At refrigeration (Figure 1), Amies transport medium showed a higher performance than all other swabs ($P < 0.05$), followed by Amies with charcoal medium. Cary Blair medium showed similar result ($P > 0.05$) to Stuart and swab with no transport medium.

Discussion

Shipping clinical samples using transport media may improve diagnostics after swab collection. This method is inexpensive and provides laboratory results at least equivalent to those obtained by usual methods of collection and shipment of tissue specimens [2]. In contaminated samples, the use of transport media may improve recovery of pathogens with low titers, avoiding excessive growth of contaminants able to inhibit the primary agent [2,6]. For *S. hyicus* specifically, no information is currently available on the capacity of transport systems to preserve the viability of this bacterium. In this study, four transport media and swabs with no medium were compared for their capacity to maintain the viability of pure cultures of *S. hyicus* after holding periods ranging from 0 to 10 days.

Most bacteria suffer irreversible damage in dry environment, which can cause cellular death by protein denaturation, membrane rupture or loss of cytoplasm function [9]. An interesting result found in this work was the survival of *S. hyicus* higher than 50% for 72 hours in swabs with no transport medium at room temperature. Gram-positive bacteria (including staphylococci) are usually resistant to low humidity, rupture and cell lysis [1], and it is hypothesized that this characteristic could explain the results of *S. hyicus* recovery under the conditions of the study.

Despite some fluctuations in survival rates among different days (especially low titers on the third day

under refrigeration), a satisfactory capacity to maintain stability of *S. hyicus* was observed in Amies medium for up to 9 days in refrigeration. High performance of Amies medium on survival of aerobic and anaerobic bacteria up to 24 hours was already described [10]. Moreover, survival of methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in Amies transport media for up to 14 days regardless of the transportation temperature [3]. The large amount of salts in Amies medium might have contributed to *S. hyicus* survival, since electrolytes can promote bacterial metabolism and help osmotic control [11,12]. Sodium hydrogen phosphate is one of Amies components, which acts like a buffer [10], avoiding acidification which can occur under excessive bacterial growth. Another advantage in Amies medium is the presence of a second buffer (potassium dihydrogen phosphate), which provides pH stability in the medium. Our results showed that Amies medium could maintain *S. hyicus* viable for large periods, especially when stored under refrigeration.

Charcoal may absorb inhibitors and bacterial metabolites, such as toxins, which can accumulate during storage, helping bacteria survive for prolonged periods [12]. Amies with charcoal showed lower performance (average recovery rate of 12.6% at room temperature and 43.8% refrigerated) when compared to Amies, at both storage temperatures. Intense decrease in *S. aureus* survival with Amies with charcoal on the first 24 hours of storage at room temperature was already reported [13]. Moreover, it is suggested that charcoal could be toxic for Gram-positives, what may explain the performance of this medium on *Staphylococcus* survival [14].

The Cary Blair medium is a modification of Stuart medium, replacing the sodium glycerophosphate buffer by inorganic phosphate. It is used to preserve Gram-negative and anaerobic bacteria, especially in rectal swabs and fecal specimens [15]. In the present study, samples of *S. hyicus* held in Cary Blair medium at room temperature showed a decrease in the recovery rate after 24 h, which was the worst performance in all swabs and media used at this temperature (average recovery rate of 3.8%). Low survival rates of *S. aureus* were already reported after 24 hours (20%) and 48 hours (4%) of storage at room temperature [13]. On the other hand, refrigerated specimens presented recovery rates higher (average of 25.5%) than swabs held at room temperature, but unstable for most of the storage time. The results of this and other studies showed that Cary Blair medium is not recommended for staphylococci, especially when kept at room temperature.

Staphylococcus hyicus did not show satisfactory survival rates in Stuart medium, both at room temperature (average recovery rate of 5.8%) and under refrigeration (average of 29.5%). Barber *et al.* (1998) observed that Stuart medium was efficient to preserve *S. aureus* specimens for 24 hours at room temperature, ranging from 81% to 141% of survival, and on the second day ranged from 29% to 166%. However, the samples were no longer evaluated, so it was not possible to determine the duration of *S. aureus* recovery. Considering the results of our study, Stuart medium would not be recommended for shipment of *S. hyicus* specimens.

In this study, storage at low temperatures contributed to better performance of all swabs with transport medium, like previous reports [5,6,16-18]. At room temperature, the metabolism of most bacteria is hasty, resulting in multiplication and negative variations in bacterial populations, like accumulation of bacterial toxins, pH changes and nutrient depletion. However, the results of our study showed that swabs with no transport medium did not present significant differences in *S. hyicus* recovery between temperatures (average recovery rates of 34.7% and 20.7% at room temperature and refrigerated, respectively), which was an unexpected result. It is possible that a reduction of bacterial metabolism happened by swab dissection, with no relationship with storage temperature. Additionally, variations in the volume absorbed by cotton tips, present in swabs with no transport medium, were observed in our experiment when compared to rayon swabs, present in the swabs with transport medium. This fact could have had an influence on the inoculum of *S. hyicus* transferred to agar surface and on recovery rate observed in swabs with no transport medium. In order to control the effect of each swab, the NCCLS (2003) recommends the usage of triplicates and the use of CFU averages obtained from each type of swab, each temperature and storage day.

We found no data about the effect of temperature and transport media on the survival of *S. hyicus*. According to our results, storage in Amies medium under refrigeration was efficient and could be the best choice to preserve *S. hyicus* specimens. Preserving specimens is a critical step in a country with a large territory, since it is not always possible to transport and streak samples in a short time after sampling. Our results also showed that exudative epidermitis specimens can be shipped in swabs with no transport medium, since they had high recovery rates (higher than 50%) for up to three days at room temperature. This technique has low cost and shows good performance,

and can be indicated in clinical routine in pig farms when it is possible to transport specimens to the laboratory in less than 48 h.

Conclusion

The *S. hyicus* recovery may be influenced by the type of transport medium or storage temperatures. Amies medium under refrigeration was the most effective transport system to maintain a long-term viability of *S. hyicus*. Moreover, recovery of *S. hyicus* in swabs with no transport medium was satisfactory when specimens were maintained at room temperature for up to three days.

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