

## A protocol for microbiologically safe preparation, storage, and use of autologous serum eye-drops in low-income countries

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### Abstract

**Introduction:** The study aimed to investigate whether the preparation, storage, and use of autologous serum in insulin syringes is microbiologically safe.

**Methodology:** Blood samples (10 mL) were obtained from 10 volunteers. After centrifugation, the supernatant serum was removed and distributed in 5 sterile insulin syringes for each sample; syringes were numbered 0 to 4 and labelled with the subject's details. Syringes were immediately transported to the microbiology laboratory and stored in a refrigerator at +4°C. The "0" labelled syringes were separated from the others and 100 µl of serum from each syringe was immediately seeded on chocolate and Sabouraud agar plates, which were incubated aerobically at 37°C for 96 hours to detect any bacterial and/or fungal contamination. In the next 4 days, the same procedure was repeated for the remaining syringes: on day 1, the "1" labelled syringes were analyzed; on day 2, the "2" labelled ones, and so on. In a second experiment, blood samples were obtained from 5 different volunteers. The same procedure as above was followed, but each syringe was used for repeated cultures at 2-hour intervals, for a total of 12 cultures/day. The needle was removed and replaced for each inoculation and the syringes were stored in the refrigerator after use.

**Results:** Under these experimental conditions, none of the cultures showed microbial growth.

**Conclusions:** Results suggest that, under the protocol described, preparation, storage and use of undiluted autologous serum in insulin syringes is inexpensive, fast, and microbiologically safe. This is of great importance for low-income countries.

**Key words:** autologous serum; preparation; storage; administration; microbiological investigation.

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### Introduction

Eye-drops made from autologous serum (AS) have been reported to be effective in the management of ocular surface disorders, such as persistent epithelial defects and severe dry eye syndrome, and after ocular surface reconstruction [1-6]. Their beneficial effect on the damaged ocular surface is related to the supply of a series of epitheliotropic factors [1]. Since AS eye-drops have a high protein content, there is some risk of microbial colonisation. Added preservatives are usually avoided because of their toxicity, which can negatively affect the epitheliotropic properties of serum. Furthermore, the addition of bacteriostatic agents may not be necessary, because the serum itself has several anti-microbial agents, including lysozyme,

complement, and immunoglobulins. The published protocols for the preparation and storage of AS eye-drops are incomplete or vary significantly [1-9]. With the increasing use of AS eye-drops, it is important to have information on their sterility, as ocular surface infections during AS use have been reported [2,3]. Furthermore, the availability of a cheap and rapid method would be of great importance for low-income countries, where ocular surface disorders needing treatment with AS are common.

As far as we know, no report assessing insulin syringe use for AS eye-drop preparation has been previously published. The purpose of this study was to investigate whether the preparation, storage, and use of AS in insulin syringes is microbiologically safe.

## Methodology

A blood sample was obtained from 10 healthy volunteers (5 males and 5 females) during the same morning. Under aseptic conditions, venesection was performed at the antecubital fossa and 10 mL of whole blood were collected in sterile vacutainer tubes without additive. The blood was left to clot at 4°C for 2 hours before centrifuging at 302 g for 10 minutes. The supernatant serum was removed in a laminar flow cabinet and distributed in five sterile insulin syringes (0.5 ml/syringe, ~12-13 drops) for each sample, numbered 0 to 4 and labelled with the subject's details. The syringes were then re-capped with a new, sterile needle with its cap. Each batch of syringes was put in a separate thermal container at +4°C, immediately transported to the microbiology laboratory and stored in a refrigerator at +4°C, while the "0" labelled syringes were separated from the others. After the needle was removed, 100 µl of serum from the "0" labelled syringes was immediately seeded on

chocolate agar with no antibiotics and Sabouraud agar with chloramphenicol and gentamicin; the remaining serum was discarded. The plates were incubated aerobically at 37°C for 96 hours to detect any bacterial and/or fungal contamination. In the next four days, the same procedure was repeated for the remaining syringes: the "1" labelled syringes were analyzed on day 1; the "2" labelled syringes on day 2, and so on.

In a second experiment, blood samples were obtained from five healthy volunteers. The same procedure as above was followed, but each syringe was used for repeated cultures at 2-hour intervals on the same day, for a total of 12 cultures/day for each syringe. In the next four days, the same procedure was repeated for the remaining syringes. The needle was removed and replaced for each inoculation and the syringes were stored in the refrigerator after use, until they were finally discarded after the last instillation.

In both experiments, one *Staphylococcus aureus* and one *Pseudomonas aeruginosa* reference strain

**Table 1.** Cultures results after repeated seeding of autologous serum on chocolate and Sabouraud agar plates at 2-hour intervals.

Sample	Time	Day 0 (syringes 0)	Day 1 (syringes 1)	Day 2 (syringes 2)	Day 3 (syringes 3)	Day 4 (syringes 4)
A	baseline	no growth	no growth	no growth	no growth	no growth
	2 h	no growth	no growth	no growth	no growth	no growth
	4 h	no growth	no growth	no growth	no growth	no growth
	6 h	no growth	no growth	no growth	no growth	no growth
	8 h	no growth	no growth	no growth	no growth	no growth
	10 h	no growth	no growth	no growth	no growth	no growth
B	baseline	no growth	no growth	no growth	no growth	no growth
	2 h	no growth	no growth	no growth	no growth	no growth
	4 h	no growth	no growth	no growth	no growth	no growth
	6 h	no growth	no growth	no growth	no growth	no growth
	8 h	no growth	no growth	no growth	no growth	no growth
	10 h	no growth	no growth	no growth	no growth	no growth
C	baseline	no growth	no growth	no growth	no growth	no growth
	2 h	no growth	no growth	no growth	no growth	no growth
	4 h	no growth	no growth	no growth	no growth	no growth
	6 h	no growth	no growth	no growth	no growth	no growth
	8 h	no growth	no growth	no growth	no growth	no growth
	10 h	no growth	no growth	no growth	no growth	no growth
D	baseline	no growth	no growth	no growth	no growth	no growth
	2 h	no growth	no growth	no growth	no growth	no growth
	4 h	no growth	no growth	no growth	no growth	no growth
	6 h	no growth	no growth	no growth	no growth	no growth
	8 h	no growth	no growth	no growth	no growth	no growth
	10 h	no growth	no growth	no growth	no growth	no growth
E	baseline	no growth	no growth	no growth	no growth	no growth
	2 h	no growth	no growth	no growth	no growth	no growth
	4 h	no growth	no growth	no growth	no growth	no growth
	6 h	no growth	no growth	no growth	no growth	no growth
	8 h	no growth	no growth	no growth	no growth	no growth
	10 h	no growth	no growth	no growth	no growth	no growth

obtained from the American Type Culture Collection (ATCC 43300 and ATCC 27853, respectively; ATCC, Manassas, VA) and one clinical isolate of *Candida albicans* were used as positive controls. Sterile phosphate buffered saline solution (PBSS) was used as negative control. 0.5% McFarland standard suspensions [ $10^8$  colony-forming units (CFU)/mL] of *S. aureus*, *P. aeruginosa*, and *C. albicans* were prepared. Twenty  $\mu$ l of each suspension and 20  $\mu$ l of sterile PBS were plated on chocolate agar and Sabouraud agar with antibiotics concurrently with the serum cultures.

Approval from the Ethics Committee/Institutional Review Board of the Department of Surgical, Microsurgical, and Medical Sciences, University of Sassari, Sassari, Italy, was obtained and the study was conducted in complete accord with the principles of the Declaration of Helsinki. Each participant received detailed information and provided written informed consent before inclusion.

## Results

In the first assay, 50 serum syringes were prepared (5 syringes from each blood sample). A total of 100 cultures (50 on chocolate agar and 50 on Sabouraud agar with chloramphenicol and gentamicin) in 5 days (20 cultures/per day) were performed. Under the conditions used in this experiment, none of the chocolate and Sabouraud agar plates seeded with undiluted serum showed any microbial growth after aerobic incubation at 37°C for 96 hours.

In the second experiment, 30 serum syringes were prepared (6 syringes from each blood sample). For each blood sample, one syringe per day was used. Repeated undiluted serum cultures were performed at 2-hour intervals, for a total of 12 cultures/day per syringe (6 on chocolate agar and 6 on Sabouraud agar with antibiotics) for a total of five days. On the whole, 300 cultures in 5 days (60 cultures per day) were obtained. In this case too, all cultures yielded no microbial growth (Table 1).

In both experiments, all the chocolate agar plates seeded with *S. aureus* and *P. aeruginosa* and all the Sabouraud agar plates seeded with *C. albicans* (positive controls) showed microbial growth, whereas no growth was observed in the corresponding plates inoculated with sterile PBSS (negative control).

## Discussion

AS eye-drops have been used for the treatment of ocular surface disorders, such as Sjögren syndrome- and graft vs host disease (GvHD)-related keratoconjunctivitis sicca, superior limbal keratoconjunctivitis, recurrent erosion syndrome, persistent epithelial defects resulting from rheumatoid arthritis, neurotrophic keratopathy, or dry eye, as well as an adjunctive treatment in ocular surface reconstruction following Stevens-Johnson syndrome, toxic epidermal necrolysis (TEN), and cicatricial pemphigoid [1-6].

Serum is the fluid component of the full blood that remains after clotting. It contains a large variety of growth factors, including epidermal growth factor (EGF) and transforming growth factor-beta (TGF-beta), immunoglobulins, and vitamins, some in higher concentrations than in natural tears [1]. The growth and migration-promoting effects of serum on human corneal epithelial cells are well known and its therapeutic effect on the damaged ocular surface is thought to be related to the supply of a series of epitheliotropic factors [4-10]. In the ophthalmic literature, there are a number of protocols for preparation and storage of AS eye-drops, which vary considerably from each other [1-9]. While it is obvious that serum from various patients will not be identical, other factors, including centrifugation and addition of diluents and/or preservatives or antibiotics, may significantly affect its biochemical, immunological, and epitheliotropic properties, thus reducing its beneficial effect on the damaged ocular surface [1].

The frequency of administration of AS eye-drops has not been standardized so far. In different studies, it ranged from as often as 1/4 hourly to 3 times daily [1-6]. Serum is usually instilled as a preservative-free, autologous product, thus lacking antigenicity. The addition of preservatives or antibiotics is usually avoided, because they can negatively affect some of the beneficial effects of AS and worsen the ocular surface damage. On the other hand, even though the serum itself has some anti-microbial properties due to the presence of lysozyme, complement, and immunoglobulins, the lack of added preservatives or antibiotics may expose it to the risk of microbial contamination during preparation, storage, and use. Repeated administration of preservative-free AS eye-drops from the same container with refrigerator storage between applications may increase the risk of microbial contamination, especially if the container is used for a prolonged period of time. As a result, some authors strongly recommend to avoid using the same

container for more than a week or, preferably, replacing it every day [1,7,11,12].

In a previous study, Sauer *et al.* assessed the contamination of AS eye-drops containers in a hospital setting [8]. These authors failed to find any contamination in the samples analyzed on the day of preparation and in the eye drops tested four days later; however, they found that three (7.5%) out of 40 containers were contaminated with *Staphylococcus epidermidis* after a week's use. Similarly, Garcia Jimenez *et al.* [13] reported no contamination of AS containers tested on the day of preparation, but disclosed contamination rates of approximately 30% in containers from home-treated patients after five days of use. In another hospital-based survey, Lagnado *et al.* [7] observed bacterial growth in 13 (8.8%) out of 134 cultures from AS samples, with *S. epidermidis* as the most common isolate (84.6%). Furthermore, in a study by Leite *et al.* [14], microbiological analysis revealed that six out of 11 AS samples were contaminated after 30 days of use, with *Klebsiella pneumoniae* and *Streptococcus viridans* as the predominant isolates. More recently, López-García and García-Lozano [9] reported that only one (2.1%) out of 48 AS containers with an adapted sterilizing filter (ABAK, Théa, Clermont-Ferrand, France) resulted contaminated with *S. epidermidis* after 1 month of treatment, whereas the contamination rate among 128 conventional AS containers was 28.9% after 7 days of treatment.

In agreement with other studies [7-9], we failed to find any contamination in samples analyzed immediately after preparation of the serum eye-drops or during the follow-up of samples kept in the refrigerator at 4°C for 1-5 days. The results of our second experiment on repeated AS instillations and culturing at 2-hour intervals confirm that contamination rarely occurs during the handling and storage of serum eye-drops, if they are done correctly. Time of use and incorrect container handling by patients or personnel responsible for AS administration are the main factors involved in microbial contamination of AS [7-9]. If contamination of correctly used AS eye-drops is uncommon [7-9], infectious complications related to AS use are even rarer [1], probably because in most cases the antimicrobial agents contained in the serum, along with the concomitant use of topical antibiotics, could overcome an infection in its initial phase and prevent the development of true conjunctivitis and/or keratitis.

## Conclusion

The results of this study suggest that, under the protocol described, preparation and storage of undiluted AS in insulin syringes is inexpensive, fast, and microbiologically safe, at least for the first five days after preparation and for a maximum of six instillations a day. Thus, this protocol may be of great importance in low-income countries, where ocular surface disorders needing treatment with AS are common. Even though, in the clinical setting, repeated use of eye-drops from the same container may increase the risk of bacterial and fungal contamination, this risk is expected to be minimal, if the container is stored in a refrigerator at 4°C between applications, handled correctly, and discarded 8 hours after the first use.

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