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THE EFFECT OF DIFFERENT STABILIZERS ON MAINTAINING VIABILITY OF LYOPHILIZED PASTEURELLA MULTOCIDA STRAINS

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SUMMARY

In the present study, different stabilizers were used. Results indicated that the best viability percentage (70.60 and 68.00), mean death time in mice, microscopical examination and colonial morphology were shown in lyophilized strains stabilized by skimmed milk lactalbumin sucrose peptone medium and Angus medium respectively, followed by 59% and 58% viability in strains stabilized by skimmed milk, lactalbumin, sucrose medium and skimmed milk peptone sucrose medium respectively.

INTRODUCTION

Pasteurellosis is one of the most common diseases of most animals causing economic losses. One way to control and eliminate pasteurellosis is to use effective and safe vaccines (Carter, 1967). Stability of strains used for such vaccines is essential for the production of a high quality product to key target populations (Stanley and Walter, 1999). Artificial method for maintaining viability must thereby reduce a metabolic rate or provide a new environment which is achieved by simple subculture to a fresh medium or addition of stabilizing medium and this media must be cryoprotective, nutritive and buffering (Weiss, 1956). The stabilizing media play a role for maintaining viability of such strains by protecting the contents of bacterial cell specially protein during the lyophilization process as skimmed milk, peptone, lactalbumin and sucrose or by acting as cryoprotective factor as skimmed milk and glycerol (Janthan and Darvall, 2000). So, this work was planned to study the effect of adding different stabilizing media to Pasteurella multocida culture to select the ideal stabilizer that maintains the viability of Pasteurella multocida strains prepared for lyophilization which are used in vaccine production.

MATERIAL AND METHODS

Pasteurella multocida strains:

Different serotypes of Pasteurella multocida A:1 and A:3 standard strains which are used for vaccine production in Aerobic Bacterial Vaccines Department were used in this study.

Laboratory animals:

Rabbits:

New Zealand rabbits of 1.5-2.0 kg b.wt. were used for passage of all strains to maintain their virulence.

Mice:

Swiss mice of 20-25 gm were used for pathogenicity, virulence and determination of mean death time of Pasteurella multocida strains according to Hanson and Brandly (1955).

Preparation of pure culture of standard strains:

Each strain of recently passaged Pasteurella multocida standard strain was grown in tryptose broth medium for 18-24 hours and examined for colonial appearance and microscopical examination.

Stabilizing media:

The following stabilizing media were used:

1. Skimmed milk medium:

It was produced by Asketon Company Linerk Ireland "Wyeth SMA". It was used in different dilutions (5%, 10% and 20%) in distilled water and sterilized by autoclaving, then added to the fluid culture as 1:1, but in case of 20% dilution it was added as 1:1 and 1:3.

2. Sucrose-lactalbumin medium:

It was prepared according to the protocol of attenuation of Rift Valley Fever vaccine, by mixing 0.5% sucrose and 1% lactalbumin (Stanley and Walter, 1999) then added to the fluid culture as 1:1.

3. Sucrose-peptone medium:

It was prepared as the previous medium by mixing 0.5% sucrose and 1% peptone, then added to the fluid culture as 1:1.

B. Mixed stabilizing media:

4. Skimmed milk (peptone+sucrose) medium:

It was prepared by mixing equal amounts of 20% skimmed milk and 1% peptone and 0.5% sucrose, then added to fluid culture as 1:1.

5. Skimmed milk lactalbumin sucrose medium:

It was prepared by mixing equal amounts of 20% skimmed milk, 1% lactalbumin and 0.5% sucrose, then added to fluid culture as 1:1.

6. Skimmed milk lactalbumin sucrose peptone medium:

It was prepared by mixing equal amounts of 20% skimmed milk, 1% lactalbumin 1% sucrose and 1% peptone, then added to fluid culture as 1:1.

7. Modified Angus medium:

It was prepared according to Angus (1984) which consists of enzymatic digest of casein 2.5gm, sucrose 5gm, sodium glutamate 1gm and distilled water 100 ml and modified by El-Ayouby et al. (2000) by addition of skimmed milk 5% and glycerol 0.5% and sterilized by autoclaving and added to fluid culture as 1:1.

8. Tryptose broth medium:

It was prepared according to Colee et al. (1989), then added to fluid culture as 1:1.

Lyophilization of strains:

The fluid cultures of Pasteurella multocida standard strains that mixed with different stabilizers were lyophilized by freeze dryness at the same time according to Angus et al. (1977).

Evaluation of strains:

Viability of strains were checked before lyophilization, immediately after lyophilization and after different periods during one year of lyophilization according to Angus et al. (1977).

RESULTS AND DISCUSSION

Bacterial strains prepared for vaccines production were stored in freeze dried (lyophilized) form. The considerable variation in survival of bacteria during lyophilization process depends on the effect of growth media, cooling rate, residual water content, storage temperature, length of storage and thawing rate (Kirsop and Snell, 1984). The culture before lyophilization that contains additive such as stabilizer including protein or other organic compounds extended the shelf life or dating period for the vaccine (Stanley and Walter, 1999).

The results presented in Table (1) and Fig. (1), the survival percentage of lyophilized strains was higher (70.60%) when are using stabilizer as 20% skimmed milk lactalbumin sucrose peptone. These results agreed with Crowe et al. (1987) who reported that disaccharides perform well as stabilizing of proteins during freezing and drying, and have also been shown to stabilize phospholipid membrane during drying. Also, disaccharides are able to stabilize protein during drying by replacing water at hydrogen binding sites. It is believed also that they stabilize membrane by forming favourable interactions with the polar head groups of phospholipids. The survival percentage of lyophilized strains was higher with using modified Angus media. These results agreed with Grout et al. (1990) who proved that during the process of lyophilization the freezing process will damage the bacterial cells and adding of stabilizer containing glycerol and skimmed milk (as cryoprotective agents) prevent the freezing crystallization that damage the bacterial cells before freez-

ing and thus improved the survival rate and maintenance of characteristics.

Evaluation of virulence of strains applied in mice as shown in Table (2) for detecting the mean death time before and after lyophilization showed that the time of death was short before and after lyophilization (about 18, 20 hours) and 20 hours in strains stabilized by skimmed milk lactalbumin sucrose peptone media and stabilized by modified Angus media. The previously mentioned results were the same as those obtained by Swamy et al. (1997) who stated that Pasteurella multocida strains are lethal to mice within 18-20 hours after inoculation.

Results obtained in Table (3) revealed the appearance of compact form of lyophilized Pasteurella multocida strains with stabilizers (skimmed milk lactalbumin sucrose peptone medium and modified Angus media) which was still without change during 1 year post lyophilization. Bacterial count and survival percentage were also not changed. However, the lyophilized strains stabilized by 5%, 10% and 20% skimmed milk were friable just after lyophilization, 2 months and 6 months after lyophilization respectively with decrease in viability percentage. These results nearly agreed with Martindale (1996) who found that some bacterial strains decreased in viability percentage after a period of lyophilization.

Bacteriological characteristics such as colonial and microscopic examination and biochemical reactions of Pasteurella multocida strains did not vary in response to the stabilizers types used in this study. These findings are as that reported by Quinn et al. (2002). So, it could be concluded that the ability of bacteria to withstand adverse conditions necessitate a stabilizer addition in the process of bacterial preservation through lyophilization, as it acts as essential component for maintaining viability, and extends the storage life time of Pasteurella multocida strains used for vaccines production.

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Table (1): Viability of Pasteurella multocida strains with different stabilizers before and after lyophilization

Types of stabilizing media	Bacterial count / ml			
	Before lyophilization	After lyophilization	Reduction %	Survival %
Modified Angus medium	2.06×10^{12}	1.40×10^{12}	32 %	68 %
Skimmed milk medium 5%	2.06×10^{12}	0.93×10^{12}	55 %	45 %
Skimmed milk medium 10%	2.06×10^{12}	0.98×10^{12}	52 %	48 %
Skimmed milk medium 20% in ratio 1:1	2.06×10^{12}	1.03×10^{12}	49.61 %	50.39 %
Skimmed milk medium 20% in ratio 1:3	2.06×10^{12}	1.07×10^{12}	48 %	52 %
Skimmed milk lactalbumin sucrose medium	2.06×10^{12}	1.23×10^{12}	41 %	59 %
Skimmed milk peptone sucrose medium	2.06×10^{12}	1.19×10^{12}	42 %	58 %
Skimmed milk lactalbumin sucrose peptone medium	2.06×10^{12}	1.45×10^{12}	29.40 %	70.60 %
Sucrose lactalbumin medium	2.06×10^{12}	1.13×10^{12}	45 %	55 %
Sucrose peptone medium	2.06×10^{12}	1.09×10^{12}	47 %	53 %
Tryptose medium	2.06×10^{12}	0.09×10^{12}	52 %	48 %

Table (2): Mean death time of Pasteurella multocida strains with different stabilizers pre and post lyophilization

Types of stabilizing media	Mean death time (per hours)	
	Before lyophilization	After lyophilization
Modified Angus medium	18.00	20.00
Skimmed milk medium 5%	20.00	22.00
Skimmed milk medium 10%	19.30	21.20
Skimmed milk medium 20% in ratio 1:1	19.30	21.20
Skimmed milk medium 20% in ratio 1:3	19.80	22.00
Skimmed milk lactalbumin sucrose medium	19.30	22.00
Skimmed milk peptone sucrose medium	20.00	22.00
Skimmed milk lactalbumin sucrose peptone medium	18.00	20.00
Sucrose lactalbumin medium	20.00	21.50
Sucrose peptone medium	18.90	23.50
Tryptose medium	19.50	24.00

N.B.: Mean death time was calculated as described by **Hanson and Brandly (1955)** according to the following formula:

$$\frac{\text{N. of dead at (X hours) X (X hours) + No. of dead at (Y hours) X (Y hours) etc.}}{\text{Total No. dead}}$$

X hours: The first time death was recorded
Y hours: The second death was recorded.

Table (3): Changes in characteristics of lyophilized Pasteurella multocida strains with different stabilizers during one year

Types of stabilizing media	Forms (after lyophilization)		Changing in forms of lyophilized strains during 1 year	Chainging in viability during 1 year	
	Friable	Compact		Mean bacterial count/ml	Mean survival (%)
Modified Angus medium	-	+++	No change	1.40×10^{12}	68 %
Skimmed milk medium 5%	+++	-	Friable just after lyophilization	0.61×10^{12}	30 %
Skimmed milk medium 10%	++	+	Friable after 2 months	0.82×10^{12}	40 %
Skimmed milk medium 20% in ratio 1:1	++	+	Friable after 6 months	0.87×10^{12}	42 %
Skimmed milk medium 20% in ratio 1:3	-	+++	No change	1.07×10^{12}	52 %
Skimmed milk lactalbumin sucrose medium	-	+++	No change	1.23×10^{12}	59 %
Skimmed milk peptone sucrose medium	-	+++	No change	1.19×10^{12}	58 %
Skimmed milk lactalbumin sucrose peptone medium	-	+++	No change	1.45×10^{12}	70.60 %
Sucrose lactalbumin medium	-	+++	No change	1.13×10^{12}	55 %
Sucrose peptone medium	-	+++	No change	1.09×10^{12}	53 %
Tryptose medium	-	+++	No change	0.09×10^{12}	48 %

N.B.:

+++ : Very clear form of lyophilization (very compact).
++ : Clear form of lyophilization (compact).
+ : Less clear form of lyophilization (less compact).
- : No clear form of lyophilization (friable).

Mean bacterial count and mean survival % are calculated from 12 reading.

SP: Skimmed milk peptone sucrose medium
SLP: Skimmed milk lactalbumin sucrose peptone medium
SL: Sucrose lactalbumin medium
SP: Sucrose peptone medium
T. Tryptose medium

MA: Modified Angus medium
SS: Skimmed milk medium 5%
S10: Skimmed milk medium 10
S20 (1): Skimmed milk medium 20% 1:1
S20 (3): Skimmed milk medium 20% 1:3
SL: Skimmed milk lactalbumin sucrose medium

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