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ORIGINAL ARTICLE

Microbiological control in the large-scale production of ascitic fluid rich in monoclonal antibodies in Cuba

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ABSTRACT: The obtaining of the monoclonal antibodies (MAb) in ascitic fluid (hybridoma technique) in Cuba is targeted to guarantee the production of recombinant anti-Hepatitis B vaccine. Given its necessary production free of contaminating microorganisms, its objective was to evaluate the effectiveness of the Microbiological Control Program implemented in production of ascitic fluid rich in MAb in Cuba. For the program evaluation, microbiological (Chi-square, $p<0.05$), productive and quality indicators in the ascitic fluid production between Stage I (design and implementation) and Stage II (evaluation) were analyzed. A lower incidence of microorganisms isolated from ozonized drinking water (1-28%) and bedding material (3-12%) in Stage II was evidenced, compared to Stage I (8-41% from water and 8-38% on bedding material). Likewise, higher values in the productive indicators [MAb (mg/ml) 3.41 (Stage I)/6.73 (Stage II) and crude volume/engrafted animal (ml/animal) with 5.26 (Stage I)/7.05 (Stage II)] were obtained. Similarly, less quantity of Non-conformities and more Customer Satisfaction were obtained in the quality indicators analyzed (Stage II). The Microbiological Control Program implemented in the large-scale production of ascitic fluid rich in MAb in Cuba is effective, ensuring the consistency in the specified characteristics of the final product for its regulatory approval.

Key words: Microbiological Control Program, ascitic fluid, MAb, Cuba.

Control microbiológico en la producción a gran escala de líquido ascítico rico en anticuerpos monoclonales en Cuba

RESUMEN: En Cuba, la obtención de anticuerpos monoclonales (AcM) en líquido ascítico (técnica de hibridomas) está dirigida a garantizar la producción de la vacuna anti-Hepatitis B recombinante. Debido a su necesaria producción libre de microorganismos contaminantes, se propuso como objetivo evaluar la efectividad del Programa de Control Microbiológico implementado en la producción de líquido ascítico rico en AcM. Para la evaluación del programa se analizaron indicadores microbiológicos, productivos y de calidad en la producción de líquido ascítico entre la Etapa I (diseño e implementación) y la Etapa II (evaluación). Se evidenció menor incidencia de los microorganismos aislados en agua potable ozonizada (1-28%) y material de cama (3-12%) en la Etapa II, comparado con la Etapa I (8-41% en agua y 8-38% en material de cama). Asimismo, se obtuvieron valores superiores en los indicadores productivos [AcM (mg/ml) 3,41 (Etapa I) / 6,73 (Etapa II) y Volumen crudo/animal prendido (ml/animal) con 5,26 (Etapa I) / 7,05 (Etapa II)]. Igualmente, disminuyeron las No Conformidades y se incrementó la Satisfacción del Cliente en los indicadores de calidad analizados (Etapa II). El Programa de Control Microbiológico implementado en el proceso productivo de líquido ascítico rico en AcM en Cuba es efectivo y permite asegurar la consistencia en las características especificadas del producto final para su aprobación regulatoria.

Palabras clave: Programa de Control Microbiológico, líquido ascítico, AcM, Cuba.

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INTRODUCTION

The hybridoma technique, used for obtaining monoclonal antibodies (MAb) with a predetermined specificity in ascitic fluid, was introduced in 1975 for the first time by Köhler and Milstein. The widespread use of these compounds in research, diagnosis and disease therapy has created the need for appropriate methods for their production and purification (1). In Cuba, the large-scale production of ascitic fluid is developed by the *in vivo* method at the National Centre for the Breeding of Laboratory Animals (CENPALAB), with the objective of providing the MAb required for the production of the anti-recombinant hepatitis B vaccine.

Regulatory institutional bodies such as the World Health Organization (WHO), the Federation of Food and Drug (FDA) and others establish that this type of product should be free of infectious agents, inherent wastes to the process of obtaining the hybridoma and other impurities proper of the production process (2,3). Therefore, the establishment of an efficient quality management system with the inclusion of the principles of Good Manufacturing Practices (GMP) constitutes an aspect of high relevance in this type of production process, given that the obtaining of biologics is characterized by a great variability for being derived from living organisms (4).

Any change in the manufacturing process leads to changes in the product that may have some impact on its quality, safety and efficacy (4). On the other hand, the possible existence of a cross-contamination phenomenon will threaten with the quality of the final product; hence, it is necessary to employ validated processes, standard operating procedures and control of process stages, from the identification of critical points (5). In this sense, animals are among the contamination potential sources of ascitic fluid (variety of clinical or subclinical infections) (6), as well as the environment quality, the materials used in the ascitic fluid obtaining, the equipment, and the personnel (7).

Keeping this in mind, the adoption of control strategies is a priority as part of a Microbiological Control Program to prevent, minimize or eradicate the dangers of contamination during the production process, optimizing the ascitic fluid quality as finished product (8). In order to ensure the consistency in the specified characteristics of the final product for the regulatory approval, it was proposed as objective to evaluate the effectiveness of the Microbiological Control Program implemented in the large-scale production of ascitic fluid rich in MAb in Cuba.

MATERIALS AND METHODS

For the initial design and implementation of the Microbiological Control Program, the regulations established for the production of biologicals by different national and international organizations such as WHO (3, 9, 10), Regulation 16 (4), FDA (2), as well as the Directive 75/318/EEC (11), EU Guide to Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use (12), ISO 9000 (5), 10002 (13) and 19011 (14), and Regulations on Biosafety (Decree Law 190 and Biosecurity Manual of the National Centre of Biological Security (15, 16)), were consulted.

Besides this, a qualitative assessment of microbiological contamination risk in the production process was developed, a flow chart was designed and 9 Critical Control Points (animal reception, hybridoma reception, animal irritation, hybridoma inoculation, extraction, clarification, mixture prefiltration, filtration and ascitic fluid expedition) were defined.

The rules and regulations for monitoring the macro and micro environmental factors such as: Standard methods for the examination of water and waste water of the American Public Health Association (APHA) (17), the Federal Standard 209 E (18) about Clean Room and Work Station Requirements, and Controlled Environment, were also consulted.

For the evaluation of the Microbiological Control Program implemented in the large-scale production of ascitic fluid rich in MAb in Cuba, the analysis of microbiological, production and quality indicators were taken into consideration as follows:

Evaluation for microbiological indicators

The microbiological analysis included a period of 13 years, divided into 2 stages; Stage I from 2000-2005 (design and implementation) and Stage II from 2006-2012 (assessment) of the Microbiological Control Program established. The Microbiological Control Program was designed with a planned monitoring system (type and sample quality, sampling frequency and method type) for evaluating the air and surface quality, and the efficacy of disinfectants (effectiveness of the cleaning and disinfection of facilities). Likewise, the ozonized drinking water, feed, bedding material, operator´s clothing and sterilizable materials, as well as the personnel monitoring, the efficacy of the sterilization equipments, the non-finished product, and final product, were analyzed. In order to verify the real effectiveness of the program implemented, an assessment of its microbiological indicators was carried

out. This program was supported by others such as the Cleaning and Disinfection Program, Training and Requalification Program for the Staff, Equipment and Facility Maintenance Program, and Biosafety Program.

The results of the microbiological monitoring and quality controls to the following elements were analyzed:

• *Macroenvironmental factors (facilities, barrier systems and auxiliaries):* The environmental quality (air) was monitored by the passive method of the air particle sedimentation by gravity (plates exposed) weekly, and the opened plates (Plate Count Agar) were exposed during 1 h in different places (immobile stations). Once the time is up, the plates at 30-35°C for 48 h (bacteria) and later at 22-25°C for 72 h (fungi) were incubated. In some cases, the Tryptone Soy Agar was used when necessary, where samples at 35-37°C for 24 h were incubated. The colony forming units (CFU) per plate were counted and identification was carried out by Gram. The action limits (≥1 CFU/m^3) in the critical areas (critical points) and the alert (≥1 CFU/m^3) and action limits (≥3 CFU/m^3) in the support critical areas were established. This procedure was implemented before the start up and every time these facilities were used (17, 19, 20).

To control surfaces, the swab method was used weekly, monthly and before using the barrier and auxiliary systems. Matrixes of aluminum paper with 100 cm^2 of area (with an internal cut area of 25 cm^2) were put on the monitoring surfaces. Later, samples with the swab imbibed in saline solution were collected and transferred to a tube with saline solution. Afterwards, 2 ml of the homogenized sample were deposited in 2 plates (1 ml per plate) with 15 ml of the molten medium (Plate Count Agar or Tryptone Soy Agar) and later mixing was hardened. Plates at the same conditions of temperature and time (bacteria and fungi) as the plates exposed method were incubated. Likewise, the CFU counting and identification were realized. Finally, the CFU number was multiplied per 10 to result in the real quantity in 25 cm^2. The acceptation limits in the Biological Safety Cabinet type II (1 CFU/25 cm^2), wall, roof, transfer, isolators (5 CFU/25 cm^2), and floor (10 CFU/25 cm^2) were established. These controls (both methods) were applied to protected areas, clean rooms, Biological Safety Cabinet type II, transfer, processing equipment, isolators, filters and filtration macroisolators of ascitic fluid (17, 19, 20). The factors were controlled under automation conditions and supervised by the trained personnel.

• *Microenvironmental factors (ozonized drinking water, feed, bedding material, operator´s clothing and sterilizable material):* The microbiological controls (mixed testing method) of the bedding material, feed and operator´s clothing were applied monthly and weekly and each time they (ozonized drinking water) were used. Drinking water (10 ml), feed, bedding material and operator´s clothing (small pack involved in three paper films) were sampled. These samples in Thioglycollate Broth and Tryptone Soy Broth were inoculated and later incubated at 30-35°C (Thioglycollate Broth) and 20-25°C (Tryptone Soy Broth) for 14 days. Turbid tube samples to Blood Agar and Mac Conkey Agar were transferred and later incubated at 37°C for 24-48 h. Likewise, the CFU counting and identification were carried out (17, 20, 21).

The sterility control (direct transference method) to the sterilizable material (before its use) was used. For determining the bacteria and fungi presence, samples in Fluid Thioglycollate Medium and Tryptone Soy Broth directly and after floming covers were inoculated; later they were incubated at 30-35°C (Fluid Thioglycollate Medium) and 20-25°C (Tryptone Soy Broth) for 14 days. Turbid tube samples were considered positive. For mycoplasma determining, a direct transference to Mycoplasma Broth was performed; and later incubated at 35-37°C for 7-14 days. Reactive tube samples to Mycoplasma Agar (supplemented) plates were transferred and later incubated at 37°C for 7 days with 10% of CO_2. After 7 days, the reversed plates in Optic Microscopy (10X) were observed, and the presence of typical Mycoplasma colonies were considered positive (17, 20, 22).

• *Autoclaves:* The sterilization process efficacy was assessed weekly by the microbiological control method with the use of bioindicator bulbs (*Bacillus stearothermophilus* suspension) by QUIMEFA EPB "Carlos J. Finlay" under different times and temperatures. Between 3 or 6 bioindicator bulbs were introduced into the autoclaves during the sterilization process. Later, they were incubated at 55°C for 24-48 hours. Once the time is up, the yellow color presence (pH change) in bulbs indicated that the sterilization process was not effective (violet as original color) (20, 23).

• *Personnel (operator):* The pharyngeal exudates, feces analysis, clean hands (impression method), gloved hands and forearms (swab method) were analyzed. The assessment frequency was quarterly for pharyngeal exudates and feces analysis, while the

other controls were conducted biannually. Pharyngeal exudate samples to Blood Agar and Mac Conkey Agar were transferred and later incubated at 37 °C for 24-48 h.

Feces samples to Peptonized Water Regulator Solution (37°C for 18-24 h) and Nutrient Broth (37°C for 24-72 h) were transferred and later incubated. Turbid tube samples to 2 tubes of Selenite Cystine Broth (one at 42°C for 18-24 h and other at 37°C for 24-48 h) were transferred and later incubated. Afterwards, the turbid tube samples to different agar medium plates (Brilliant Green Agar Modified, Salmonella-Shigella Agar Modified, Selenite Cystine Agar, Mac Conkey Agar and Endo Agar) were extended and later incubated at 37°C for 24 h. Likewise, the CFU counting and identification were carried out by different methods (Gram, Oxidase, Coagulase, Catalase and Api tests) (20, 24).

In the operator control, the clean hands (impression method) was performed following the same procedure of the plate exposed method from the initial impression in the Plate Count Agar to the CFU counting and final identification. However, for sampling the gloved hands and forearms (swab method), the procedure of the control surfaces, was followed but starting from the sample collection in saline solution, later its transference to the Plate Count Agar and finalizing with the CFU counting and the final identification (20, 25).

• *Disinfectants:* The evaluation of disinfectant solutions used in the production process was conducted to verify their effectiveness. The evaluation frequency was biannually by the antimicrobial action method. For this method, it was necessary to use the sample (Disinfectant solution) and control (Phosphate Buffering Solution) preparation (20, 26).

The disinfectant sample (9.9 ml) was added to 2 sterile tubes (duplicate), later they were agitated, and 100 µl of microorganism suspension of reference were added in concentration of 10×10^9 CFU/ml. The microorganism suspension was constituted by *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 9642). Afterwards, the mixture was agitated during 30 seconds, and immediately 1 ml of culture was added in 9 ml of Neutralizing Solution. Later, 1 ml of culture was added in 9 ml of Phosphate Buffering Solution, homogenized again and diluted until a dilution of 10^4. Finally, 1 ml of this mixture was spilled in a sterile petri plate, and later 15 ml of fluid Nutrient Agar were added, mixed, cooled, and incubated at 35-37°C for 48 h. Once the time is up, the CFU/ml were counted. The control preparation followed the same procedure of the disinfectant sample, but only diluting the microorganism suspension of reference in Phosphate Buffering Solution until a dilution of 10^5 (20, 26).

The reduction percent (efficacy) of the disinfectant samples was calculated as follows (26):

% of reduction = 100 - ((CFU/ml of sample × 100) / CFU/ml of control)).

A value of $75\text{-}125\times10^6$ CFU/ml in the control preparation was evaluated as valid, as well as the disinfectant sample with 99.999% of CFU reduction in the first 30 seconds was considered as effective (20, 26).

These controls were performed in the Quality Division according to the procedures established in the Manual of Quality Control for the culture and identification of contaminating microorganisms in the production process of ascitic fluid at CENPALAB. Besides these components, the final product was included.

Statistical analysis

The frequency analysis was carried out by using the independence Chi-square test to compare the incidence of biological agents isolated in ozonized drinking water and the bedding material (crumbled bagasse) during the stages of the study, in which one association to $p<0.05$ was considered as statistically significant. The InfoStat v2.0 statistical software was used.

The odds ratio (OR) was calculated to determine the risk magnitude of the absence of preventive measures for the presence of contaminating microorganisms in ozonized drinking water and the bedding material, where a value of OR≥1 indicated the presence of a risk factor; however, an OR<1 indicated to be a protective factor (27).

Evaluation for productive indicators

The control charts for some productive indicators were established according to ISO 7870 (28), as a statistical tool that ensures a continuous monitoring of the process and a compliance with product specifications, allowing its continuous improvement. Warning limits with ± 2 Standard Deviations (SD) and action with ± 3SD were established. For this, the Minitab v. 16 program was used.

The productive indicator behavior according to the Standard Operation Procedure Manual of the ascitic fluid production was analyzed (29), based on the Annual Review of Product as it enabled the analysis of trends in the process data. To determine performance, the following indicators were calculated: performance

by engrafted animal (ml/animal) and batch performance of the final product (mg/ml).

The formulas for calculating these indicators are (30):

• Performance by engrafted animal (ml/animal) = crude volume/engrafted animals

• Performance batch of the final product (mg/ml) = concentration of MAb/ml
ml: milliliter, mg: milligrams.

Evaluation for quality indicators

The behavior of some quality indicators related to the Microbiological Control Program implemented in the ascitic fluid production was analyzed. Among them are:

• *Non-conformities identified in internal and external audits:* In order to evaluate the quality of this bioproduct, 8 internal inspections, 10 internal audits by ISO 19011(14) and 8 external audits were performed, analyzing the non-conformities identified during the evaluation process of the ascitic fluid production.

• *Claims:* Customer´s claims were valued according to ISO 10002 (13). A research and a cause analysis for taking corrective actions were performed.

• *Customer satisfaction:* Customer perception about the quality of this product (Customer satisfaction) was determined by the method of Perceived Quality Index. Survey results sent to the client for 5 years in Stage II were taken into consideration; as far back as 2008 this analysis was inserted for its importance in the production process. The interpretation criterion of this indicator was: PQI≥1: Satisfied/PQI<1: Not satisfied. The following formula was used for calculation (31):

PQI = (5E + 1G + (-1) F + (-5) B) / Total

E: Excellent, G: Good, F: Fair, B: Bad.

Economic evaluation

In order to value the economic impact of this productive process for the institution and country, the total income (resulted of the perceived volume to concept of sales of the final product in the evaluated period), income per man (obtained from relation between the total incomes and total of workers included in process), as well as the income per day, and the percent of income in the institution were determined as efficiency indicators. This monetary value was expressed in Cuban pesos (CUP).

RESULTS AND DISCUSSION

The results analysis of the microbiological, productive and quality indicators will be shown as follows.

Evaluation for microbiological indicators

Among the microenvironmental elements monitored in both stages (water, bedding material, food, clothing and other sterilizable materials), only the non-compliant results in water and the bedding material were obtained (21). The rest of the macroenvironmental factors controlled (facilities, barrier and auxiliary systems) behaved in correspondence with the acceptance limits defined (19).

The incidence of microorganisms isolated from ozonized drinking water during Stages I and II is shown in Table 1.

Among the agents identified, *Bacillus* sp. (environment microbiota) and *Staphylococcus* sp.

TABLE 1. Incidence by stages of microorganisms isolated from ozonized drinking water./ *Incidencia por etapas de los microorganismos aislados en agua potable ozonizada.*

Microbial Entity	Stage	S	Proportion				X^2	p	OR
			(-)	(+)	(-)	(+)			
Staphylococcus sp.	I	130	106	24	0.82	0.18	5.31	0.0212	2.05
	II	231	208	23	0.90	0.10			
Enterococcus sp.	I	130	120	10	0.92	0.08	14.84	0.0001	19.17
	II	231	230	1	0.99	0.01			
Escherichia coli	I	130	119	11	0.92	0.08	2.59	0.1073	-
	II	231	221	10	0.96	0.04			
Bacillus sp.	I	130	77	53	0.59	0.41	6.03	0.0141	1.76
	II	231	166	65	0.72	0.28			

S: Samples, (-): Negative, (+): Positive, OR: Odds ratio.

(skin microbiota) were considered environmental contaminants, while *Enterococcus* sp. and *Escherichia coli* were classified as opportunistic pathogens (skin or mucosal microbiota) (9, 32, 33). These findings coincided with others obtained in previous studies by Riera (34), who also obtained isolates of these bacterial entities from ozonized drinking water, being *Bacillus* sp. and *Staphylococcus* sp. the ones with the highest incidence (37.5 and 15.6%, respectively).

Likewise, Caorsi *et al.* (32) evidenced that *Staphylococcus* sp. was the most frequently environmental contaminant in the sterile preparation units from the Chilean Clinical Hospital studied, followed by *Micrococcus* sp. and *Bacillus* sp. In this sense, Donatién (35) demonstrated an incidence of 48% of *Staphylococcus* sp. as environmental contaminant in Cuba, while Hernández and Marín (36) reported the presence of this microbial entity in environmental controls in human meeting places (library) in Colombia.

The opportunistic pathogens were naturally present in the environment. Not considering them as defined pathogens may eventually cause human diseases whose mechanisms of local or systemic defenses were deficient. If drinking or bath water contains a significant number of microorganisms, it can be a source of skin and mucosal infection. This can be confirmed in reports or studies carried out by WHO (9) and Llop *et al.* (37), where they referred to *Pseudomonas aeruginosa*, *Escherichia coli* and to a lesser degree species of *Flavobacterium*, *Acinetobacter*, *Klebsiella*, *Serratia* and *Aeromonas*, as well as some slow-growing mycobacteria, as major opportunistic pathogens in water.

Taken into consideration that the treatment of drinking water with ozone is considered an effective mechanism for sterilizing bacteria and viruses according to the statements made by Bataller *et al.* (38), the presence of the isolated bacteria could be the result of contamination of the passageways colonized within the water distribution network. This coincided with Allen (39), who noted that *Enterobacter*, *Staphylococcus*, *Pseudomonas* and *Klebsiella* species frequently colonized the inner surfaces of water pipes and storage tanks (often called “flare”) and grew into biofilms when conditions were favorable, such as the presence of nutrients, warm temperatures, low concentrations of disinfectants and long storage, among others.

The isolated agents may have been transmitted to the water as a result of the condition of the ducts and pipes of the treatment system and their distribution according to what was stated by WHO (10), possibly having a negative influence on the quality and production process stability. Likewise, Goya and Wilde (40) reported that the construction defect or structures of deposits, as well as the absence or irregularities in the maintenance of facilities, constituted causes for the presence and growth of microorganisms from different sources.

These isolates in ozonized drinking water used in the productive process entailed the establishment of a maintenance plan as part of the Technology Management System implemented in Gnotobiotic Rodent Management. It consisted on circulating water with high concentrations of ozone in the pipes for 1 hour; besides including the checking and cleaning of deposits and ozone generating equipment with monthly frequency.

The incidence of isolated contaminant microorganisms in ozonized drinking water is shown in Table 1. *Bacillus* sp. had the major incidence in both stages due to its high resistance in environment, as dust and soil microbiota, followed by *Staphylococcus* sp. as another common environment contaminant but from the human and animal skin microbiota (32). However, the results achieved in Stage II showed an improvement in the quality of ozonized drinking water in relation to the previous stage, since *Enterococcus* sp. was scarcely reported and nor the incidence of the rest of the isolated agents or other contaminants. These findings demonstrated that the implementation of the Microbiological Control Program improved the microbiological quality of this contaminant source through a considerable reduction. Analyzing the statistical processing (Chi-square) of the results obtained in both stages, statistically significant differences ($p<0.05$) were observed in the percentage values of *Staphylococcus* sp., *Bacillus* sp. and *Enterococcus* sp. incidence in ozonized drinking water with a significant decrease in Stage II. Unlike the percentage values of *Escherichia coli* showed no significant differences although its incidence was lower (Table 1).

Water contamination and changes in its chemical composition can affect the health of animals and the results of the final product quality, since it is used in all the activities of the productive process, cleaning and preparation of solutions, as well as in the staff bath to enter the protected area, and in the maintenance of rodents as they constitute important factors to control (29).

The statistical analysis confirmed the effectiveness of the measures taken to achieve improvements in the quality of the water used in the process, since a significant reduction of microorganisms was identified.

Likewise, it was necessary to implement other actions to eradicate the bacterial agents isolated in the water which could potentially affect the quality of the finished product.

The odds ratio values obtained in both stages (Table 1) showed (in Stage II) a decrease in the risk magnitude of water contamination by such agents, since lower values of this indicator were obtained compared with Stage I. This defined the absence of preventive measures as a risk factor for the contamination of *Enterococcus* sp., *Staphylococcus* sp. and *Bacillus* sp. in 19.17; 2.05 and 1.76 times higher for this type of sample respectively; compared with the presence of the measures to prevent water contamination by these microbial entities (27).

The incidence of isolated agents in crumbled bagasse (alternative source of bedding material) during the stages studied is shown in Table 2. It can be seen that in Stage I *Bacillus* sp. and *Escherichia coli* were the most isolated microbial entities; followed by *Proteus* sp. and in a lower percentage of isolation *Pseudomonas aeruginosa*. The presence of these agents in the bedding material could have been obtained from the origin source or storage conditions, which can favor contamination when they are not adequately controlled. According to the statements made by Francis *et al.* (41), cellulose and sucrose content in its composition, and its residual moisture (about 8%) favored the multiplication of these microorganisms when the sterilizing process was not effective.

In the analysis of the results with the statistical processing (Chi-square) of the sterility control in the bedding material in the two stages of the study, significant statistical differences ($p<0.05$) were observed in Stage II, with a marked decrease in the incidence of bacteria that had been identified in Stage I (Table 2). However, *Pseudomonas aeruginosa* had a lower incidence in the second stage, although a statistically significant difference was not evidenced as compared to Stage I.

These isolations coincided with the approach made by Riera (34) in studies carried out about the microbiological behavior in dehydrated crumbled bagasse as bedding material for rodents free of specific pathogens, and subsequent works related to the health monitoring and maintenance of microbiological hygienic condition in experimental rodents.

The presence of these bacteria entities in the bedding material showed the need to make adjustments to the sterilization process which was made from the microbiological results obtained in Stage I (Table 2), on adjustments in sterilizing time for this material. The drying stage and a maximum storage time for a week was made longer, which allowed to obtain better results in Stage II; therefore a significant decrease was observed as mentioned above.

Analyzing the odds ratio values obtained (Table 2), it was found that the corrective measures implemented enabled to reduce the magnitude of the contamination risk by these entities in Stage II, since the absence of preventive measures increased the contamination risk by *Proteus* sp., *Bacillus* sp. and *Escherichia coli* in 8.29; 5.17 and 3.06 times respectively; compared with the presence of measures to prevent the contamination of the bedding material for these microbial entities (27). Based on these arguments, this indicator confirmed the effectiveness of the measures applied, directing

TABLE 2. Incidence by stages of microorganisms isolated from the bedding material./ *Incidencia por etapas de los microorganismos aislados en material de cama.*

Microbial Entity	Stage	S	(-)	(+)	Proportion (-)	Proportion (+)	X^2	p	OR
Bacillus sp.	I	86	53	33	0.62	0.38	22.20	<0.0001	5.17
	II	121	108	13	0.89	0.11			
Proteus sp.	I	86	67	19	0.78	0.22	17.97	<0.0001	8.29
	II	121	117	4	0.97	0.03			
Escherichia coli	I	86	60	26	0.70	0.30	10.07	0.0015	3.06
	II	121	106	15	0.88	0.12			
Pseudomonas aeruginosa	I	86	79	7	0.92	0.08	1.48	0.2241	-
	II	121	116	5	0.96	0.04			

S: Samples, (-): Negative, (+): Positive, OR: Odds ratio.

the use of corrective actions to other alternatives that allowed obtaining a completely free of contaminant bedding material.

In the bedding material, *Bacillus* sp. had the major incidence in Stage I, again due to its high resistance in environment such as dust and soil microbiota (32), as it was previously mentioned. However, once more it is demonstrated that the implementation of the Microbiological Control Program improved the microbiological quality of this another contaminant source through a significant reduction.

Another control carried out as a part of the Microbiological Control Program was the health monitoring of the staff. In this case, the highest prevalence of microorganisms identified was in the pharyngeal exudates (Fig. 1), being the results of the stool analysis and vaginal exudates insignificant in the laboratory analysis (24). The positive staff was separated from the productive activity and subjected to treatments with subsequent rechecking.

During the two stages, high *Staphylococcus aureus* prevalence values were evidenced and identified through all the years studied (Fig. 1). This result coincided with the findings made by Riera (34), who reported this bacterium as the most prevalent in the staff pharyngeal exudates, reaching percentages of isolation up to 50%. This microorganism is referred to in the literature as normal microbiota of the pharynx, although it is also considered an opportunistic pathogen that could eventually cause inflammatory processes, not only in humans, but also in animals. Therefore it is controlled and measures are taken (9, 42), deriving to the positive cases of *Staphylococcus aureus β* hemolytic which could be considered by a physician to value the needs of a specific antibiotic treatment.

Pseudomonas aeruginosa and *Escherichia coli* had the major annual persistence after *Staphylococcus aureus* in the pharyngeal exudate samples (Fig. 1). The results obtained showed that these microorganisms have been considered as major opportunistic pathogens in the environment, particularly in drinking

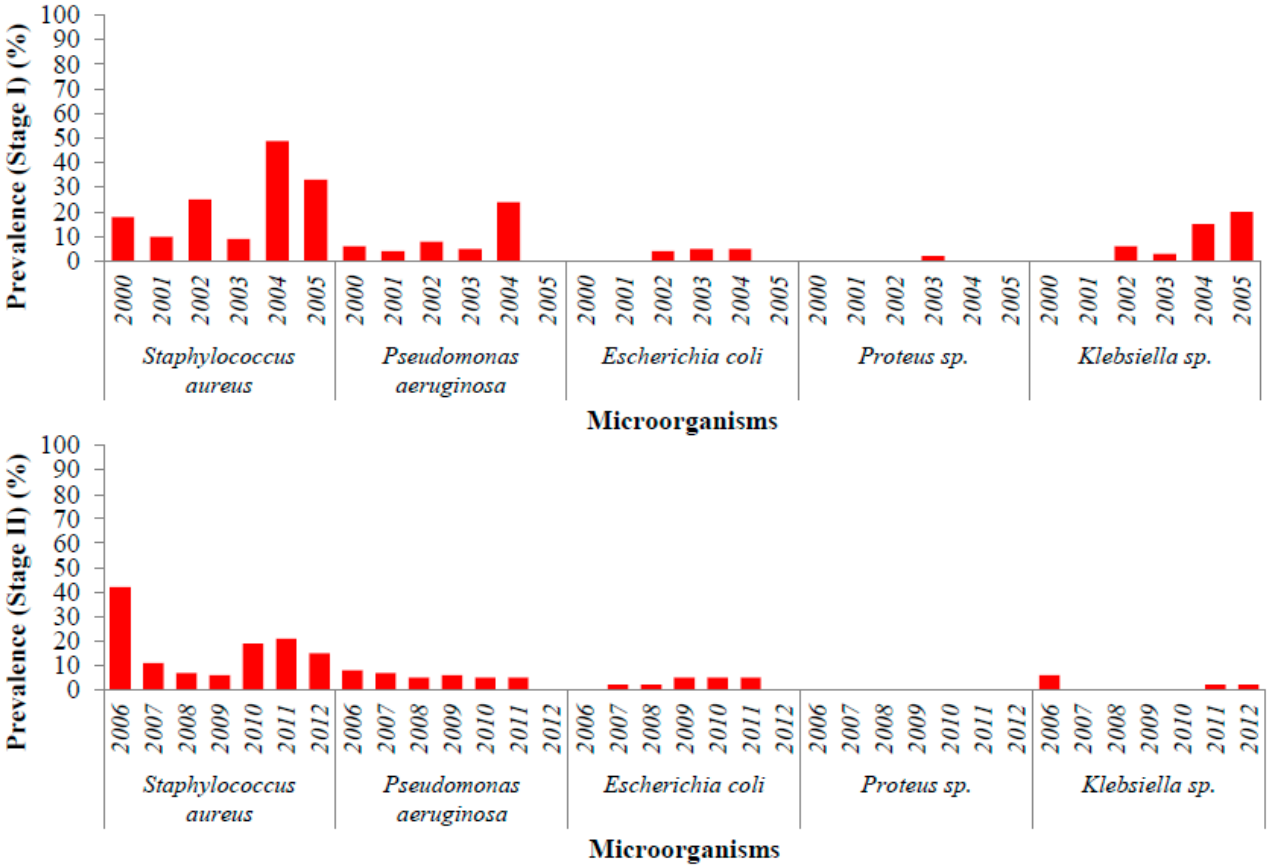

FIGURE 1. Prevalence by stages of microorganisms isolated in pharyngeal exudates from the staff./*Prevalencia por etapas de los microorganismos aislados en exudados faríngeos del personal.*

water (9, 37). In this sense, the strict application of corrective measures in the drinking water treatment is one of the most important actions to reduce the future contaminations of facilities and personnel (17).

In another sense, the analysis of the operator control was acceptable in the majority of the samplings (25). The sterilization process efficacy (autoclaves) (23), sterility control of the sterilizable materials (22) and the evaluation of disinfectant solutions (26) were considered satisfactory. These findings demonstrated that the measures taken after the implementation of the Microbiological Control Program improved the microbiological quality of these contaminant sources in Stage II.

Regarding the quality of the finished product with the implementation of the Microbiological Control Program, a product free of contaminating microorganisms was obtained, which ensured the quality specifications fulfillment, allowing the release of all the batches produced.

Evaluation for productive indicators

Monitoring the productive performance of each operator starting by the analysis of these indicators was of great importance since it has been highly demonstrated that the way productivity increased when ensuring an adequate health maintenance and handling of animals (8).

As can be seen in Fig. 2, the performance of the ascitic fluid, both in crude volume/animal engrafted and MAb, increased considerably in Stage II (2006-2012) after the implementation of the Microbiological Control Program, demonstrating its effectiveness.

This is made clear by taking into consideration the comment of the previous paragraph, plus the fact that once the contamination of the final product is demonstrated, it should be reprocessed so that part of the volume and concentration of MAb produced is lost. That is why the strict application of controls and preventive measures ensured their entire microbiological quality of the process.

The lowest performances described as alert (-2SL) and action limits (-3SL) may be indicatives of the presence of a subclinical infection affecting the productive potential of these animals. This is quite common in laboratory rodents according to the statements made by Shek (6), who referred that there was a wide variety of microorganisms affecting experimental rodents, where most of the infections had no clinical symptomatology. Therefore, there was a risk of not being detected to interfere in the research results and processes in which these biomodels were used.

In contrast with these mentioned criteria, the superior performances defined as alert (+2SL) and action limits (+3SL) indicated a surplus of these productive indicators for the efficacy of the Microbiological Control Program implemented, as the tendency to increase in Stage II (2006-2012), shown in Fig. 2. Thus, in fulfilling the objectives, plans and predicted goals, delivery of MAb in the quantity and quality required for its regulatory approval were ensured.

Evaluation for quality indicators

The non-conformities results detected in inspections, internal and external audits, as well as claims and evaluations of customers´ satisfaction,

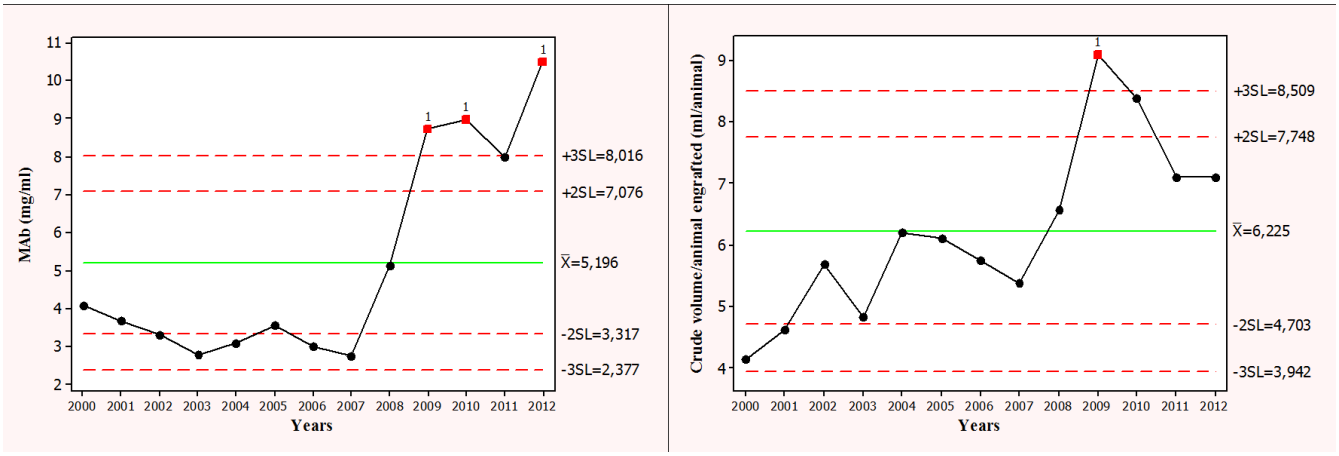

FIGURE 2. Performance evaluation in crude volume/animal engrafted and MAb of the ascitic fluid./ *Evaluación del rendimiento en Volumen crudo/animal prendido y AcM del líquido ascítico.*

and the quality indicators promoting the continuous improvement of processes will be shown as follows.

Non-conformities detected in internal and external audits

In this productive process, the Quality Management established annually the Internal Audit Program, whose objective was to assess the compliance of the Good Production Practice (GPP) and verify the aspects of the Quality Management System. For these audits, there was a competent group of independent experts who were not related to the process that constituted the audit team.

The non-conformities detected during inspections and internal audits conducted by the Quality Management to the ascitic fluid production rich in MAb from 2000 to 2012 are shown in Fig. 3, showing a decreasing tendency, which evidence the improvements in the process, as well as the proper performance of the GPP.

The detection of non-conformities allowed to design an Improvement Program made up of corrective measures directed to optimize the documentary system, the constructive condition of the facilities, the calibration and verification of the equipment, and the purchase of the necessary equipment for the control of harmful elements like ammonia and ozone.

This allowed advancing in the productive process integrity since many of these non-conformities could negatively influence on the effectiveness of the Microbiological Control Program, as they directly affected the micro and macroenvironmental factors already mentioned above. At the same time, this allowed the establishment of action priorities to eliminate those causes that threatened the product quality in each stage of the process.

Other institutions have also conducted numerous audits to this productive process, among them are those made by the Centre for the Quality State Control of Drugs, Equipments and Medical Devices

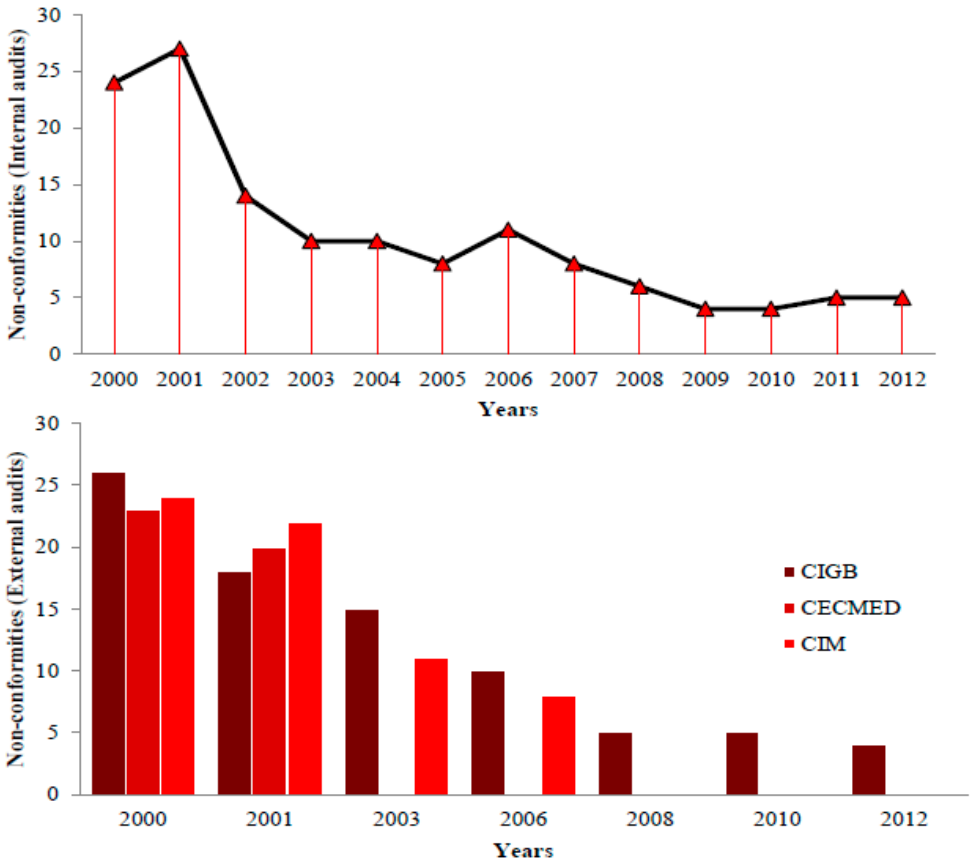

FIGURE 3. Behavior of the non-conformities detected during the internal and external audits performed on the ascitic fluid production process./*Comportamiento de las No Conformidades detectadas en auditorías internas y externas realizadas al proceso de producción de líquido ascítico.*

(CECMED) to grant the Production License, as well as the Supplier audits conducted by the Center for Genetic Engineering and Biotechnology (CIGB), and the Center of Molecular Immunology (CIM). The major non-conformities detected in these audits are referred to aspects in which large investments (equipment to control harmful elements, automation, etc.) are required. These identified deficiencies have been resolved gradually, which are evidenced by the significant reduction of the non-conformities through the years, mainly in Stage II as shown in Fig. 3.

In general, the importance to eliminate the non-conformity is in guaranteeing the improvement of the ascitic fluid production, as the automated equipment optimizes the control on the process, at the same time favoring the effectiveness of the Microbiological Control Program established, conditioned by the strict control over the quality of the environment for animals and the treatment of drinking water and food.

The stated aspects do not invalidate the GPP, as the Production License was granted by the CECMED in 1999, and the acceptability by WHO of the production of anti-Hepatitis B vaccine in 2001, 2002, 2004, 2006, 2008, 2010 and 2012, making possible that our country commercializes vaccines and other drugs through institutions of the United Nations (UN) to other regions and countries of the world.

Claims

This indicator also starts to be evaluated as part of the continuous improvement process, agreeing with the points made by Sosa (8), who stated that the organization should continually improve the effectiveness of its system or process with the use of some tools such as results of inspections, audits, corrective and preventive actions, surveys of customer satisfaction and data analysis. The behavior of claims´ number is shown in Fig. 4.

During all the years of the study, this indicator was below the acceptable value determined by the institution (1%), highlighting that during recent years the claims have been from 0.15 to 0.10% of the total batch issued. The causes of these claims are related to transportation (delivery time), lack of communication between the parties, but not to the quality specifications of the product (8).

Customer satisfaction

Surveys were conducted in order to know the perception of the customers about the ascitic fluid production rich in MAb issued for the production of recombinant anti-Hepatitis B vaccine, since annual surveys were conducted in 2008. Their results provided the necessary information to take actions to improve the process.

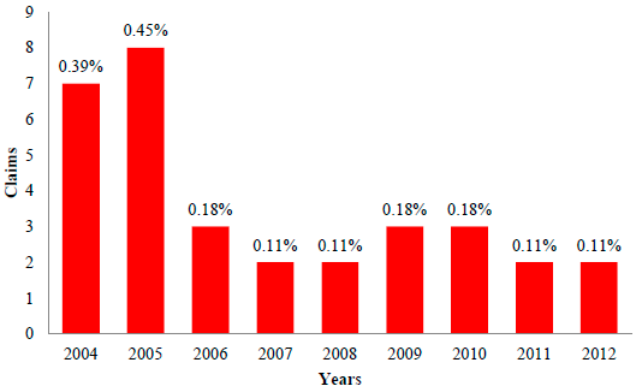

FIGURE 4. Behavior of the claims in the ascitic fluid production./*Comportamiento de las Reclamaciones en la producción de líquido ascítico.*

The values of the Perceived Quality Index (PQI) calculated from the survey results made to the customers evidenced a PQI of 1.6 (2008), 2.6 (2009), 3.3 (2010), 2.2 (2011), and 1.7 (2012) for the centers evaluated.

The answers given by the clients were fully compliant, allowing to have the perception estimation of these customers satisfied (PQI ≥ 1) with the quality of MAb obtained at CENPALAB for its use in the production of the vaccine. As it can be appreciated, the overall satisfaction degree remained as a satisfactory performance, even though the regulations and requirements for this type of product increase every year (13, 31).

Economic evaluation

Table 3 shows the behavior of some efficiency indicators in the productive process, as a result of the Microbiological Control Program effectiveness implemented with the support of other established programs, mentioned above.

The results achieved, in relation to the total volume obtained (Table 3), made the country save about $ 21 million annually, according to the current prices in the world market for this type of product. In addition, the productivity achieved was outstanding, since the group of technicians and specialists involved in the process was reduced in a half since 2004, maintaining and improving the productive results with a high level of expertise and a significant reduction of salary costs.

TABLE 3. Efficiency indicators of the ascitic fluid production./*Indicadores de Eficiencia de la producción de líquido ascítico.*

Indicator	Value	Indicator	Value
Total ascitic fluid (l)	9 454.98	Total income (CUP)	$28 565 875.621
Ascitic fluid/man (l)	620.499	Income/man (CUP)	$1 904 391.70
Ascitic fluid/day (l)	2.65	Income/day (CUP)	$78 262.67
Ascitic fluid/m^2 (l)	9.394	Income/Center (%)	36.24

l: Litres, m^2: Meters squared, CUP: Cuban Pesos.

On the other hand, it was not necessary either to import any animals needed for obtaining this biological product, whereof prices in the world would have been uneconomical, since large volumes of MAb for the production process of the anti-Hepatitis B vaccine are required, therefore the need to import a large number of animals to produce the volume required.

In relation to the incomes obtained for marketing this biological product, it should be noted that this productive process has contributed since its establishment until today with 36.24% of the total income of the institution; generating an income/peso spent of $3.48; in addition to the added value of the medicines generated and evaluated from this bioproduct. It contributes to the development of biotech drugs produced by other institutions (CIGB and CIM), whose exports provide substantial contributions to the Cuban economy.

The adoption of control strategies as part of the Microbiological Control Program constituted a priority to prevent, minimize or eradicate the contamination dangers during the production process, optimizing the ascitic fluid quality as a finished product.

Ensuring that this program contributes to the safety, efficacy and stability of the final product was the major impact in the productive process of ascitic fluid, guaranteeing the consistency in its specified characteristics for the regulatory approval. In this sense, the obtaining of major productive performances stimulated the diversification of the MAb production, generating superior economical profits for our institution. Likewise, in this production diversity, major uses in the Cuban Biotechnology Industry were obtained, not only in the preventive and therapeutic vaccine productions for the infectious diseases, but also in the diagnosis, following and the treatment of cancer, as well as the T lymphocytes quantification in the peripheral blood, among others (29).

CONCLUSIONS

Based on the results obtained, it is concluded that the Microbiological Control Program implemented in the productive process of the ascitic fluid rich in MAb in Cuba is effective, allowing to ensure consistency in the specified characteristics of the final product for the regulatory approval.

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