Technical Note

Assessment of the viability of 3rd generation cephalosporin-resistant enterobacterial cells during transport under different storage conditions

Evaluación de la viabilidad de enterobacterias resistentes a cefalosporinas de tercera generación durante su transporte bajo diferentes condiciones de almacenamiento

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ABSTRACT: Antimicrobial resistance (AMR) is a growing threat to public and animal health. Monitoring is fundamental in terms of obtaining, compiling and exchanging data and carrying out interventions. The efficient transport of samples is an essential part of the epidemiological research and diagnostic laboratory. The objective of this study was to assess the viability of two 3rd generation cephalosporin-resistant enterobacterial species under different storage conditions for detection of antimicrobial resistance. The preliminary study was based on the principles of the qualitative method published by the CLSI (M-40A2). Swabs were introduced in tubes containing different loads of target microorganisms (Extended spectrum beta-lactamase-producing *Escherichia coli* and third generation cephalosporin-resistance *Salmonella enterica*, clinical isolate), subsequently placed in Cary-Blair transport medium andpreserved at room temperature (28-32°C) and under refrigerated conditions (4-8°C), during 24 and 48 hrs. The results showed recovery for all microorganism / dilution / temperature combinations at 24 and 48 hours, being the refrigerated transport during 24 hours the most appropriate for the storage, preserving initial load of each bacteria. However at room temperature, it is not recommended to keep the samples due to the observed overgrowth.

Key words: antimicrobial resistance, enterobacteria, samples, transport.

RESUMEN: La resistencia a los antimicrobianos (RAM) es una amenaza creciente para la salud pública y animal. La vigilancia es fundamental para obtener, recopilar e intercambiar datos y llevar a cabo intervenciones. El transporte eficiente de muestras es una parte esencial del laboratorio de investigación epidemiológica y de diagnóstico. El objetivo de este estudio fue evaluar la viabilidad de dos especies de enterobacterias resistentes a las cefalosporinas de tercera generación en diferentes condiciones de almacenamiento para la detección de resistencia a los antimicrobianos. El estudio preliminar se basó en los principios del método cualitativo publicado por el CLSI (M-40A2). Los hisopos se introdujeron en tubos que contenían diferentes concentraciones de microorganismos dianas (aislado de *Escherichia coli* productor de beta-lactamasa de espectro extendido y aislado de *Salmonella enterica* resistente a cefalosporinas de tercera generación), que posteriormente se colocaron en un medio de transporte Cary-Blair y se conservaron a temperatura ambiente (28-32°C) y en condiciones de refrigeración (4-8°C), durante 24 y 48 horas. Los resultados mostraron la recuperación de todas las combinaciones microorganismo/dilución/temperatura a las 24 y 48 horas. La estrategia de transporte refrigerado fue la más adecuada para estos microorganismos, preservando la concentración inicial de cada bacteria. Sin embargo, a temperatura ambiente, no se recomienda conservar las muestras debido al sobrecrecimiento observado.

Palabras clave: resistencia antimicrobiana, enterobacterias, muestras, transporte.

Antimicrobial resistance (AMR) represents a growing threat to public and animal health, which is of great concern in all countries and in many economic sectors [1]. Various genetic, biochemical, and physiological mechanisms may be responsible for AMR, but currently resistance to third generation cephalosporin (3GC-R) due to the production of extended spectrum beta-lactamase (ESBL) enzymes or AmpC beta-lactamase- (AmpC-) encoded either by plasmids, transposons and integrons, generates greater significance due to their association with extreme resistance or panresistance. This mechanism usually occurs in Gram negative bacilli such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Proteus* spp., *Citrobacter* spp., *Morganella morganni*, *Serratia marcescens*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, and *Proteus mirabilis* [2]. Due to the ability of enterobacterial species to expand the genes that contribute to resistance to 3GC during the food processing chain of animal origin, surveillance of these bacteria should be a priority [1,2].The permanent monitoring of the prevalence of resistance in zoonotic bacteria such as *E.coli* and *Salmonella* sp. in animal products for human consumption, provides information on the possible reservoir of genes for resistance to antimicrobial agents, which could be transferred to pathogenic strains [1,3].

The success of the surveillance culture on selective medium supplemented with antibiotics depends on the efficient transport of biological samples, the efficiency of the tools used for sample collection and transport and, fundamentally, on avoiding overgrowth of the microbiota without losing the viability of the microorganisms of interest (4,5,6). The rectal exudates and faeces collected from different animal species are the most appropriate samples for the detection of the carrier state of 3GC-resistant enterobacteria (6,7).

Given the diversity of sample types, different devices and conditions can be used for their transport and preservation: commercial swab transport systems, swabs in sterile tubes, or in plastic tubes with transport media (Stuart, Amies, Cary-Blair) which maintain a favorable pH and prevent the drying of the sample. All these transport systems should be under the quality requirements of the standard protocol M40-A published by the International Committee for Clinical and Laboratory Standards (CLSI) (8,9,10). Specimens should be transported and processed as soon as possible, however laboratories are often far from animal husbandry areas, so delays in transport may occur, affecting viability (11). Most protocols guide the conservation of samples under refrigerated conditions. However, considering the difficulties in refrigeration equipment present in laboratories in low-income countries, the purpose of this study was to assess the recovery of different loads of 3GC-R *E.coli* and *Salmonella enteric* by direct plating of swabs previously preserved in the Cary Blair transport medium at different temperatures and time periods.

Pre-sterilized, compact, round cotton-tipped wooden swabs in eppendorf tubes with Cary-Blair transport medium (BiolifeCo., China) were used. The following strains from the collection of the Animal Bacteriology Laboratory of the National Center for Animal and Plant Health (CENSA), Cuba, were included in the study: ESBL-producing *Escherichia coli* (isolate) previously characterized (12) and *Salmonella enterica* (isolate). The non-ESBL-producing organism *Escherichia coli* ATTC 25922 strain was used as control.

The principles of the qualitative method published by CLSI (M-40A2) in 2014, specifically the roll-plate method were followed (9,10). Briefly, three serial dilutions (10^3-10^4-10^5CFU/mL) were prepared from a suspension with a concentration of 10^8 CFU/mL according to the 0.5 MacFarland standard of each of the previously subcultured bacterial strains. Each dilution was prepared in 0.85% saline solution and 100 μL were dispensed in triplicate into a 96-well plate (GreinerBio-One, Germany). Swabs were absorbed in the dilutions for 15 seconds and then placed in the corresponding eppendorf tubes containing 1mL of Cary-Blair medium. Tubes were kept for 24 and 48 hours at room temperature (28.0-32.0°C) and refrigerated (4-8°C), both controlled with a digital thermo-meter (VELCRO®). At the end of the times, bacteriological culture was performed on MacConkey agar (Biocen, 4014) and MacConkey agar plates supplemented with cefotaxime (CTX, concentration 1mg/L, Sigma Aldrich) by spreading the swab over an area of about 2 cm² and then streaking out with a sterile loop. Plates were incubated at 37ºC for 24 hours and then the number of colonies was counted. Growth density was scored semi-quantitatively (<10 colonies; +1(25 colonies); +2(25-250 colonies); +3(25-250 colonies); +4(>250), according to the number of quadrants of the agar plates on which growth was observed.

All strains grew on the control Mac Conkey agar plate. Both 3GC-R strains grew on Mac Conkey agar plate supplemented with cefotaxime; the reference strain did not grow on this medium. The growth of the strains is summarized in Table 1; cells were recovered from all concentrations assayed (10^3,10^4,10^5ufc/mL) on both selective and non-selective media. Growth density showed similar scores on both media for 3GC- R strains. ESBL-producing *E. coli* and 3GC-R *S. enterica* were detected in small numbers, with +1 and less than 10 colonies respectively from tubes with load equivalent to 10^3ufc. A count of more than 250 colonies (+4) was obtained in cultures preserved in Cary Blair medium for 48 hours at temperatures of 28.0-32.0ºC. The conditions used to store the strains did no affect their viability but did favor overgrowth.

The accuracy and diagnostic value of the tests carried out for detecting microorganisms depends on the quality of the sample and the type of assays. Therefore, a poorly performed or transported collection will determine a possible failure in the recovery of microorganisms (13,14). Lopardo *et al.* (4) stated that the survival of bacteria in the transport medium depends on several factors: type of bacteria, transport time, storage temperature, concentration of bacteria in the sample and formulation of the transport medium. In this study, it was shown that for the recovery of both species, the cotton-tipped wooden swab system, transported in eppendorf tubes with Cary-Blair medium at refrigerated temperature (4-8°C), guaranteed their viability for a period of 24 and 48 hours.

Several commercial companies have developed a wide range of swab systems, which adapt to the new needs of the market and facilitate the task for the microbiology laboratories (Σ-Transwab®, e-swab®, Copan, Eurotube, Mantacc, PurFlock Ultra®, Hydra-Flock®, Deltalab, among others). Many laboratories use various commercial transport devices without being compromised by cost, and thus optimize the collection and transport of microbiological samples according to their particularities, (5,11,15,16).

Warnke *et al.* (17) report that the method recommended by CLSI depends on individual technical skills and is therefore only standardized within certain limits. Furthermore, this technique contributes to low recovery rates since microorganisms can only be

Table 1. Viability of enterobacteria species on Mac Conkey agar media recovered from cotton-tipped woodens swabs under different conservation conditions. / *Viabilidad de las especies de enterobacterias en los medios de agar Mac Conkey recuperadas a partir de los hisopos de madera con punta de algodón en diferentes condiciones de conservación*

Bacterial species / Culture medium	Holding temperature	Cell load range (UFC)					
		24 hours			48 hours		
		10^3	10^4	10^5	10^3	10^4	10^5
Escherichia coli ATCC / (MacConkey)	4°C	+2	+2	+3	+3	+3	+4
	28.0-32.0°C	+3	+3	+4	+4	+4	+4
Escherichia coli ATCC (MacConkey+CTX)	4°C	NG	NG	NG	NG	NG	NG
	28.0-32.0°C	NG	NG	NG	NG	NG	NG
ESBL-Escherichia coli (MacConkey)	4°C	+2	+2	+4	+3	+3	+4
	28.0-32.0°C	+3	+3	+4	+4	+4	+4
ESBL-Escherichia coli (MacConkey+CTX)	4°C	+1	+3	+3	+3	+3	+3
	28.0-32.0°C	+3	+3	+3	+3	+4	+4
3GC-R *Salmonella enterica* (MacConkey)	4°C	+3	+3	+3	+3	+3	+3
	28.0-32.0°C	+4	+4	+4	+4	+4	+4
3GC-R *Salmonella entérica* (MacConkey+CTX)	4°C	<10	<10	+2	+1	+2	+2
	28.0-32.0°C	<10	+2	+4	+3	+4	+4

- CTX: cefotaxime
- CFU colony forming units

  NG Not Growth

transferred to the agar surface by direct contact and the interaction between swab tip materials and microorganisms has a physical and chemical influence. To reduce these effects, new swab materials with improved release capabilities have recently been introduced for routine diagnostic procedures, thereby improving microbial recovery rates (5,11,12,18,19); as well as a variant introducing a homogenization step of the swab tips inoculated *in vitro*, with a Precellys high-throughput homogenizer (20). However, for surveillance studies conducted in resource-limited settings, the medium and swab can be prepared by using locally available materials.

As for the temperature of the test, it had a very significant effect on the preservation of the microorganisms in the transport device used, varying the qualitative and quantitative content of the bacterial load. A similar effect was reported by Nys *et al.* (19), when an accelerated growth of the microorganisms tested at room temperature was observed. Regarding the temperature of the test, it had a very significant effect on the preservation of microorganisms in the transport device used, varying the qualitative and quantitative content of the bacterial load. However, in the studies carried out by Van Horn KG *et al.* (11), the temperatures tested had favorable results according to the M40-A standard (9,10). Based on the above and taking into account the tropical climate of Cuba, it is advisable to keep the samples in a refrigerator from the moment they are taken until they are transferred to the laboratory, and to continue their refrigeration for a maximum of 48 hours until their analysis.

Another influencing factor is the transport medium used for these type of samples: Stuart, Amies, Cary Blair (13,20). Cary Blair medium offers better maintenance performance; it is prepared with a minimum nutrients to facilitate the survival of the organisms without multiplying. Its semi-solid consistency provides ease of transport, and the prepared medium can be stored at room temperature (17). This system avoids desiccation and preserves the existing bacteria, especially if they are scarce; as is the case of the low prevalence reported for ESBL-producing enterobacteriacea such as *Samonella* sp., while avoiding overgrowth of other unwanted bacteria of the microbiota that are usually found in this type of samples.

In this work, 10^3ufc/mL were assayed as minimal bacterial concentration, however there is still no established knowledge about the minimum bacterial load of 3GC-R-enterobacteria that is necessary for a stable colonization of gut in different animal species and to guarantee their detection in the laboratory. The normal colonization density of *E. coli* as commensal bacteria of the intestinal tract is approximately 10^7 to 10^8 cfu per gram of feces; the intestinal population of *E. coli* includes different clones, and the relative abundance of each clone varies. Generally, antibiotic-susceptible *E. coli* constitute the dominant population, whereas resistant *E. coli* are subdominant, although the proportion of resistant clones increases with antibiotic exposure (21). A study aimed to mimic the natural infection pathway with ESBL-and AmpC-producing Enterobacteriaceae pointed out that (i) a colonization dosage of 10^2cfu *E. coli* per animal was sufficient for a successful longtime colonization of broiler chickens and (ii) a ratio of 1:5 inoculated seeder birds to non-inoculated sentinel birds was sufficient to colonize an entire group of broilers, even in the absence of antimicrobial selection pressure (22).

Subsequently, it is necessary to use selective media, which prevent the growth of the sensitive microbiota to ensure the optimal performance in the recovery of ESBL-producing enterobacteriacea from rectal swabs and faeces. Worldwide, the culture procedures used for this detection range from the direct seeding on a differential medium such as MacConkey or chromogenic agar (with and without antibiotics), up to the use of a previous non-selective or selective enrichment step (incorporating antibiotics) and its successive culture on a selective and differential medium. Different media such as MacConkey or Driglasky agar supplemented with cefotaxime (1 mg/L) have demonstrated their usefulness (23).

The European Union Directive (2013/652/EU) (24) recommends the use of harmonized protocols for the surveillance of antimicrobial resistance in zoonotic and commensal bacteria. Specifically, the protocol described by Hasman *et al.* (23), uses selective Mac-Conkey agar (cefotaxime 1mg/L) and a non-selective pre-enrichment of the sample in peptone water buffer (BAP) is performed prior inoculation (1 g $\pm$ 0.1 g of the sample in 9 mL BAP; incubation at 37°C $\pm$ 1°C for 18-22 h). This counteracts negative results, as a result of a low initial quantity or possible death during storage under refrigerated conditions.

An increase in the concentration of each microorganism was observed at different times and at both temperatures. Cotton-tipped swabs in Cary-Blair medium at 4°C maintained the viability of an initial bacterial load of 10^3-10^5ufc/mL in the initial inoculum in the strains up to 48 h. At room temperature, it is not recommended to keep the samples due to the observed overgrowth.

To conduct significant surveillance studies for 3GC-R enterobacterial carriage, it is essential to ensure that primary swab samples are processed efficiently with optimal bacilli recovery and that the transport and storage media maintain the viability of the swab samples. During 2016-2017, a pilot study was completed following the methodology described in this work. A total of 215 rectal swab samples were collected from healthy pigs in farms and slaughterhouses located in Mayabeque and Matanzas, Cuba. Rectal swabs were transported in 1mL of Cary-Blair medium in a cooler containing ice packs from each facility to the Laboratory of Animal Bacteriology at the National Centre for Animal and Plant Health. Samples were directly subcultured on Mac Conkey agar supplemented with cefotaxime for no longer than 24 hours after collection. This methodology allowed the recovery of 96/215 isolates of Gram-negative bacteria, corresponding to the Enterobacteriaceae family. Subsequent evaluation corroborated that 27/96 isolates corresponded to ESBL-producing *E. coli* (25).

This study demonstrated that the swabs preserved at 4-8°C ensured viability and limited overgrowth than those preserved at room temperature. Further studies with a wider range of clinical samples are required to confirm the usefulness of the conditions, taking into account the possible occurrence of variations in the epidemiology of 3GC-R species

ACKNOWLEDGMENT

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