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Bacteriological studies on egg yolk forms and different formulations of yolk-citrate semen extender

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Using a tabular design, the bacteriological profile of egg yolk forms and different formulations of yolk-citrate semen extenders across different storage periods was examined. Twenty-one eggs less than 24 h old were randomly selected from 3 crates of eggs. The yolks were harvested and divided into two equal parts. One part was centrifuged for 1 h at 3500 revolutions per minute (rpm) to get clarified yolk. The remaining part was left whole. Each part was divided into three aliquots, one of which was cultured immediately (pre-storage) on MacConkey, Chocolate, *Deoxycholate Citrate* and *Sabouraud Dextrose* Agars and the other two stored each for 24 and 48 h before culture on the same media. The cultures were incubated for 24 h at 37°C. Left over egg yolks were pooled according to forms and kept frozen (-20°C) before being used to formulate extenders. Four different extenders were prepared using the clarified and whole yolks with or without antibiotics. The extenders were treated as previously described for egg yolk. Plates with apparent colonies were subjected to biochemical tests to identify the isolates and antibiotic susceptibility testing after the colonies were counted. Clarified yolk was less contaminated compared to whole egg yolk. *Staphylococcus aureus* was isolated from whole egg yolk and it was sensitive to Streptomycin, Amoxicillin, Neomycin, Penicillin and Cefoxitin but resistant to Gentamicin. Extenders with antibiotics were less contaminated than extenders without antibiotics. *Staphylococcus aureus, Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella* species were isolated from semen extenders without antibiotics. All isolates were sensitive to Gentamicin. In conclusion, clarified yolk and extenders containing clarified yolk with antibiotics had no contamination. Hence, clarified yolk should be used in the preparation of extenders instead of whole yolks. Also, Gentamicin should be included in extender formulations to reduce bacterial contamination.

Key words: Egg yolk, whole yolk, clarified yolk, bacterial culture, yolk-citrate extender, antibiotics.

INTRODUCTION

Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection program. During cryopreservation, the spermatozoa are basically confronted with two major changes. Firstly, the decrease and increase in

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temperature that leads to cold and heat shocks. Secondly, the formation and dissolution of ice results in changes of osmolarity and damage due to ice crystals (Watson, 1995). Cold shock can be reduced by a source of lipoproteins or high molecular weight material such as egg yolk, milk or plant-based lipids (Vishwanath and Shannon, 2000).

Egg yolk is one of the most extensively used cryoprotectant for semen preservation in domestic animals (Amirat et al., 2004). The yolk makes up about 33% of the liquid weight of the egg and contains approximately 60 calories, three times the caloric content of the egg white (USDA, 2010). The fresh egg yolk can be fractionated into 78% plasma and 22% granules (Anton, 2007). It has been shown to have a beneficial effect on sperm cryopreservation as a protectant of the plasma membrane and acrosome against temperature-related injury (Amirat et al., 2004). Egg yolk contains several potent antioxidants such as vitamin E and phosvitin, which have the potential to inhibit chain reactive oxidation, lipid oxidation and peroxidation of the sperm membrane (Surai et al., 1998; Anton et al., 2006).

Despite taking all hygienic precautions, bacterial contamination is an inevitable problem in semen processing and cryopreservation (El-Bahrawy et al., 2010) which necessitates the use of antibiotics as integral components of semen extenders. This study was, therefore, aimed at evaluating the bacteriological profile of egg yolk forms and different formulations of yolk-citrate semen extender.

MATERIALS AND METHODS

Sampling location

Eggs were collected from the Center for Dryland Agriculture poultry unit (Lat. 11.97940°N, Long. 8.43082°E) from hens kept in a battery cage. The unit is located behind the Faculty of Agriculture Teaching and Research farm, Bayero University Kano.

First experimental design

The first experiment was laid out in a tabular design as described by Bledzki and Rybak (2016) with microbial growth (yes/no) as the dependent categorical variable and yolk form (whole/clarified) and storage duration (pre-storage, 24 or 48 h) as the independent variables.

Sample size determination

The resource equation of Mead et al. (2012) was used to determine the sample size. The equation is as follows:

N-1 = T + E

where N = number of experimental units, T = number of degrees of freedom (number of test groups – 1) and E = number that estimates the error (the remainder).

Mead et al. (2012) proposed that E should be between 10 and 20. In this region, the analysis has the most statistical power.

Therefore, 21 eggs constituted the sample size since they gave an estimate of error (E) of 15 that is within the range of 10 to 20 as proposed by Mead et al. (2012).

Sampling

Oral antibiotics were withdrawn from the birds for ten (10) weeks before sampling. Out of 4 complete crates and 1 incomplete crate collected on the sampling day, 3 crates were randomly selected using the hat method. Seven (7) eggs were randomly selected from each crate giving a total of 21 eggs used for the experiment.

Harvesting of egg yolk and storage

The eggs sampled earlier were transported to the laboratory of the Department of Microbiology, Aminu Kano Teaching Hospital for further processing. To release the egg yolk, a modification of the method of Himathongkham and colleagues (Hutchison et al., 2004) was employed. The blunt end of the eggs was dipped into 100% ethanol. The alcohol was then burned off. The end of the egg was broken by firm tapping using a spatula wiped with 70% ethanol. Loose pieces of shell from internal membranes were removed using a pair of sterile forceps. Membranes were sprayed lightly with 70% ethanol before being completely torn off. The yolk was separated from the albumen using the method of Holt and colleagues as cited by Sariozkan et al. (2010) by pouring out the albumen bit by bit carefully until the volume of albumen is reduced to the barest minimum. The yolk when separated from the albumen was rolled on sterile filter paper to remove chalazas and traces of albumen adhering to the vitelline membrane. The vitelline membrane was then disrupted with a sterile scalpel blade and the yolk recovered in a sterile beaker. Yolks from the sampled eggs were pooled and divided into two equal portions (labelled as whole and clarified).

Clarification of egg yolk in 10 ml plastic test tubes was done by centrifugation (Uniscope Laboratory Centrifuge, Model SM112, Surgifriend Medicals, England) at 3500 rpm for 1 h at room temperature as described by Sariozkan et al. (2010) with slight modification. Following centrifugation, the lipid material at the top layer was removed using a pasture pipette and the clear water soluble fraction was retained. The pellet at the bottom of the tube was also discarded. The whole and clarified portions were subsequently divided into three aliquots each per storage period (pre-storage, 24 and 48 h). Not all egg yolks were used in the first experiment. The left over egg yolks from this experiment were pooled according to their form (whole and clarified) and subjected to freezing at -20°C until further use in the next study.

Microbial analysis

Total aerobic bacterial count: Total aerobic bacterial count in whole and clarified egg yolks was determined by the standard plate count. Plates containing growth media were streaked with whole and clarified yolks pre-storage and after 24 and 48 h of storage at 5°C and incubated at 37°C for 24 h. Plates with apparent growth were selected and the number of colonies counted. The number of bacteria per ml (colony forming units per ml, CFU/ml) of diluted sample was calculated using the following:

$$\frac{Number\ of\ colonies\ counted}{Volume\ of\ inoculum} = Microbial\ load\ (CFU/ml)$$

Table 1. Details of antibiotics used for sensitivity testing.

Antibiotic	Code	Disc potency	Diameter of zone of inhibition (mm)	
			Susceptible	Resistant
Streptomycin	S	10 µg	≥ 26	≤ 22
Gentamicin	CN	10 µg	≥ 15	≤ 12
Neomycin	N	30 µg	≥ 26	≤ 22
Cefoxitin	FOX	30 µg	≥ 23	≤ 14
Penicillin	P	10 IU	≥ 29	≤ 28
Amoxicillin	AML	20 µg	≥ 18	≤ 13

Source: Cheesbrough (2006) and CLSI (2011),

Identification of bacteria: Whole and clarified egg yolk samples were each cultured for aerobic bacteria on Chocolate, Deoxycholate Citrate and MacConkey agar. Plates were incubated at 37°C for 24 h. Fungi were ruled out by culturing samples on Sabouraud Dextrose Agar. Microscopic examination for Gram reaction (Claus, 1992) was carried out. The isolates were biochemically characterized using catalase, coagulase, triple sugar iron, citrate and urease tests (Cheesbrough, 2002).

Antibiotic susceptibility test: Isolates were stored at -20°C for 14 weeks before antibiotic susceptibility testing. The identified microorganisms were subjected to antibiotic susceptibility testing using single discs containing Gentamicin, Streptomycin, Penicillin, Cefoxitin, Neomycin and Amoxicillin by the Kirby-Bauer Disc Diffusion method (Bauer et al., 1966).

Briefly, using a sterile wire loop, the pure colonies of bacterial growth were inoculated to Nutrient agar plates by uniformly streaking against the entire agar surfaces. The impregnated antimicrobial discs were then applied to the surfaces of the inoculated plates using sterile forceps 1.5 cm away from the edges of the plates and 3 cm away from each other with the guide of a template placed under the Petri dish. All the discs were gently pressed with forceps to ensure complete contact with the agar surface. The plates were then inverted and incubated aerobically for 24 h at 37°C. The zones of inhibition of bacteria by the antimicrobial discs were measured in millimeter using a ruler on the underside of the plates. The susceptibility of bacteria was determined based on the breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI, 2011). The details of the antibiotics used and the key to interpretation of susceptibility or resistance are presented in Table 1.

Statistical analysis: The distribution of microbial load among different culture media across yolk forms and storage periods was presented in a tabular form. Association among microbial growth, yolk form and storage period was determined using Chi-square test for Independence or Fisher's Exact test as the case may be. Statistical Package for the Social Sciences version 16.0 (SPSS Inc., Chicago, IL, USA) was used throughout the analysis.

Second experimental design

The next experiment was laid out in the same design (Tabular Design) as previous approach. Here, microbial growth (yes/no) was the dependent variable and yolk-citrate semen extenders (clarified yolk extender without antibiotics - CYE, clarified yolk extender with antibiotics - CYEA, whole yolk extender without antibiotics - WYE and whole yolk extender with antibiotics - WYEA) and storage duration (pre-storage, 24 or 48 h) were the independent variables.

Extender preparation and storage

The remaining frozen pooled clarified and whole egg yolks from the first experiment were thawed and used to prepare extenders in the second experiment. Pooled clarified egg yolk was divided into two equal portions. 20 ml of each portion was used in the preparation of clarified yolk-citrate extender with or without antibiotics and these were each divided into three equal parts. The parts were randomly assigned to each of the three storage periods (pre-storage, 24 or 48 h). The pooled whole egg yolks were also subjected to procedures similar to their clarified counterparts. Table 2 shows the formulation of the respective extenders.

Microbial analyses

Total viable bacterial count and identification of bacteria were done as spelled out in the first protocol, the only difference being that isolates were not stored at -20°C for 14 weeks before antibiotic susceptibility testing.

Statistical analysis: The distribution of microbial load among different culture media across yolk-citrate semen extenders and storage periods was presented in a tabular form. Association among microbial growth, yolk-citrate extenders and storage period was determined using Chi-square test for Independence or Fisher's Exact test as the case may be. Statistical Package for the Social Sciences version 16.0 (SPSS Inc., Chicago, IL, USA) was used throughout the analysis.

RESULTS

Bacterial contamination of egg yolk forms across storage periods

There was growth from whole yolk stored for 24 h and cultured on Chocolate agar with a microbial load of 18×10^2 Colony Forming Units (CFU)/ml (Table 3). Whole yolk cultured on Sabouraud Dextrose agar pre-storage and when stored for 24 h had 3.5×10^2 and 12.5×10^2 CFU/ml, respectively, while whole yolk stored for 24 h

Table 2. Composition of test extenders.

Component	Extender			
	I	II	III	IV
Tri-sodium citrate, g	2.9	2.9	2.9	2.9
Fructose, g	0.5	0.5	0.5	0.5
Egg yolk, ml	20 (whole)	20 (whole)	20 (clarified)	20 (clarified)
Sodium penicillin G, IU	100,000	-	100,000	-
Dihydrostreptomycin sulphate, g	0.1	-	0.1	-
Distilled water, ml	To 100	To 100	To 100	To 100

I – WYEA , II – WYE, III – CYEA, IV – CYE. WYEA - whole yolk extender with antibiotics; WYE - whole yolk extender without antibiotics; CYEA - clarified yolk extender with antibiotics; CYE - clarified yolk extender without antibiotics.

Table 3. Microbial load of different culture media across yolk forms and storage periods.

Yolk form	Storage period (h)	Microbial load (×10^2 CFU/ml)	Media
Whole	Pre-storage	0	Choc
		0	DCA
		3.5	SDA
		0	MAC
	24	18	Choc
		0	DCA
		12.5	SDA
		17.5	MAC
	48	0	Choc
		0	DCA
		0	SDA
		0	MAC
Clarified	Pre-storage	0	Choc
		0	DCA
		0	SDA
		0	MAC
	24	0	Choc
		0	DCA
		*ND	SDA
		0	MAC
	48	0	Choc
		0	DCA
		0	SDA
		0	MAC

*Growth seen, ND – Microbial load not determined; Choc – Chocolate Agar, DCA – Deoxycholate Agar, SDA – Sabouraud Dextrose Agar, MAC – MacConkey Agar.

and cultured on MacConkey agar had a microbial load of 17.5×10^2 CFU/ml (Table 3). There was a statistically significant association (χ^2 = 4.800, P<0.05) between microbial growth and yolk form (Table 4). There was no

Table 4. Association between microbial growth and egg yolk form.

Growth	Yolk form		Total
	Clarified	Whole	
Yes	0	4	4
No	12	8	20
Total	12	12	24

χ^2 = 4.800, P<0.05.

Table 5. Association between microbial growth and storage duration.

Growth	Storage duration (h)			Total
	Pre-storage	24	48	
Yes	1	3	0	4
No	7	5	8	20
Total	8	8	4	20

Fisher's Exact Value = 3.545, P>0.05.

Table 6. Gram reaction and biochemical characteristics of isolates across yolk forms and storage periods.

Yolk form	Storage period	Gram reaction	Biochemical test		Isolate
			Catalase	Coagulase	
Whole	Pre-storage	Gram positive cocci	Positive	Positive	*Staphylococcus aureus*
	24	Gram positive cocci	Positive	Positive	*Staphylococcus aureus*
	48	Nil	Nil	Nil	Nil
Clarified	Pre-storage	Nil	Nil	Nil	Nil
	24	Fungal filaments and conidia	Not applicable	Not applicable	*Aspergillus* species
	48	Nil	Nil	Nil	Nil

association (Fisher's Exact Value = 3.545, P>0.05) between microbial growth and egg yolk storage duration (Table 5). Gram-positive cocci (in singles, diploids, tetrads and clusters), fungal filaments and conidia were observed under the microscope after Gram staining. The Gram-positive cocci were confirmed to be *Staphylococcus aureus* (Table 6) and were sensitive to all the antibiotics evaluated except Gentamycin (Table 7) while the fungal filaments and conidia were suspected to be *Aspergillus* species.

Bacterial contamination of yolk-citrate extender across storage periods

On Chocolate agar, whole yolk extender without antibiotics had growth (2.5×10^2 CFU/ml) pre-storage. On the other hand, whole and clarified yolk extenders without antibiotics stored for 24 and 48 h on Chocolate agar, Deoxycholate Citrate agar, Sabouraud Dextrose agar had growth that were too numerous to count. On MacConkey agar, a non-lactose fermenter and a lactose fermenter too numerous to count were seen in whole yolk without antibiotics stored for 24 and 48 h while the clarified yolk recorded only lactose fermenters that were too numerous to count. In whole yolk extender with antibiotics, microbial load of 7×10^2 CFU/ml was recorded pre-storage (Table 8). There was a statistically significant association (Fisher's Exact Value = 26.381, P<0.01) between microbial growth and semen extender formulation (Table 9) as well as a statistically significant association (χ^2 = 7.414, P<0.05) between microbial growth and semen extender storage duration (Table 10).

Gram-positive cocci (in singles, diploids, tetrads and clusters) were observed under the microscope after Gram staining. They were confirmed to be *S. aureus* which was sensitive to only Gentamycin. Lactose fermenting Gram-negative bacilli confirmed to be

Table 7. Susceptibility of *Staphylococcus aureus* to various antibiotic discs.

Isolate	Antibiotic susceptibility discs					
	Streptomycin	**Penicillin**	**Gentamicin**	**Amoxicillin**	**Neomycin**	**Cefoxitin**
Staphylococcus aureus	S	S	R	S	S	S

S= Sensitive; R= resistant

Table 8. Microbial load of different culture media across yolk-citrate extender formulations and storage periods.

Extender	Storage period (h)	Microbial load (× 10^2 CFU/ml)	Media
WYE	Pre-storage	2.5	Choc
		0	DCA
		0	SDA
		0	MAC
	24	TNTC	Choc
		TNTC	DCA
		TNTC	SDA
		TNTC (NL, L)	MAC
	48	TNTC	Choc
		TNTC	DCA
		TNTC	SDA
		TNTC (NL, L)	MAC
WYEA	Pre-storage	0	Choc
		0	DCA
		0	SDA
		7	MAC
	24	0	Choc
		0	DCA
		0	SDA
		0	MAC
	48	0	Choc
		0	DCA
		0	SDA
		0	MAC
CYE	Pre-storage	0	Choc
		0	DCA
		0	SDA
		0	MAC
	24	TNTC	Choc
		TNTC	DCA
		TNTC	SDA
		TNTC (L)	MAC

Table 8. Cont'd

	48	TNTC	Choc
		TNTC	DCA
		TNTC	SDA
		TNTC (L)	MAC
	Pre-storage	0	DCA
		0	SDA
		0	MAC
CYEA	24	0	Choc
		0	DCA
		0	SDA
		0	MAC
	48	0	Choc
		0	DCA
		0	SDA
		0	MAC

WYE - Whole yolk extender without antibiotics; WYEA - whole yolk extender with antibiotics; CYE - clarified yolk extender without antibiotics; CYEA - clarified yolk extender with antibiotics; Choc - Chocolate Agar, DCA - Deoxycholate Agar, SDA - Sabouraud Dextrose Agar, MAC - MacConkey Agar; TNTC - too numerous to count; NL - Non-Lactose Fermenters; L - Lactose Fermenters.

Table 9. Association between microbial growth and yolk-citrate semen extender formulations.

Growth	Yolk-Citrate extenders				Total
	WYE	WYEA	CYE	CYEA	
Yes	11	1	8	0	20
No	3	11	4	12	30
Total	14	12	12	12	50

Fisher's Exact Test Value = 26.381, P<0.01. WYE-Whole yolk extender without antibiotics; WYEA - whole yolk extender with antibiotics; CYE - clarified yolk extender without antibiotics; CYEA - clarified yolk extender with antibiotics.

Table 10. Association between Microbial Growth in Yolk-Citrate Semen Extender and Storage Duration.

Growth	Storage duration (h)			Total
	0	24	48	
Yes	2	9	9	20
No	14	8	8	30
Total	16	17	17	50

$\chi^2 = 7.414$, P<0.05.

Klebsiella pneumoniae was sensitive to Gentamicin and Cefoxitin. The non-lactose fermenter confirmed to be *Salmonella* species was susceptible to only Gentamicin. Another lactose fermenting Gram-negative bacilli confirmed to be *Escherichia coli* was sensitive to Gentamicin, Cefoxitin and Streptomycin (Tables 11 and 12).

DISCUSSION

An assortment of culture media was employed in the cultivation of bacteria in the current study. Media used include Chocolate, Deoxycholate Citrate, Sabouraud Dextrose and MacConkey agars. Depending on the specificity of the media for the organism in question,

Table 11. Gram reaction and biochemical characteristics of isolates across yolk forms and storage periods.

Extender	Storage period (h)	Gram reaction	Biochemical test							Isolate
			Catalase	Coagulase	TSI			Citrate	Urease	
					S	B	GP			
	Pre-storage	Gram-positive cocci	+	+	NA	NA	NA	NA	NA	*Staphylococcus aureus*
WYE	24	Gram-negative bacilli	NA	NA	Y	Y	+	+	+	*Klebsiella pneumoniae*
		Gram-negative bacilli	NA	NA	R	Y	+	+	-	*Salmonella* species
	48	Gram-negative bacilli	NA	NA	Y	Y	+	+	+	*Klebsiella pneumoniae*
		Gram-negative bacilli	NA	NA	R	Y	+	+	-	*Salmonella* species
WYEA	Pre-storage	Gram-positive cocci	+	+	NA	NA	NA	NA	NA	*Staphylococcus aureus*
	24	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	48	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
CYE	Pre-storage	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	24	Gram-negative bacilli	NA	NA	Y	Y	+	-	-	*Escherichia coli*
	48	Gram-negative bacilli	NA	NA	Y	Y	+	-	-	*Escherichia coli*
CYEA	Pre-storage	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	24	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	48	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

WYEA - Whole yolk extender with antibiotics; WYE - whole yolk extender without antibiotics; CYEA - clarified yolk extender with antibiotic; CYE - clarified yolk extender without antibiotics; TSI - triple sugar iron; S - slant; B - bottom; GP - gas production; R - red; Y - yellow; NA - not applicable.

microbial counts do vary. This could be responsible for the uneven incubation outcome recorded across culture media in the present study. In other words, some culture media encourage the growth of a particular bacterium while others hinder it. For example, Chocolate agar encourages the growth of *S. aureus* but not coliforms while MacConkey agar encourages the growth of coliforms. Ordinarily, MacConkey agar should not encourage the growth of *S. aureus*, but in the current study it was isolated from MacConkey agar. According to Smith (2019), MacConkey agar is selective for Gram-negative organisms and helps differentiate lactose fermenting Gram-negative rods from non-lactose fermenting Gram-negative rods. It contains crystal violet which generally inhibit Gram-positive bacteria (Acharya, 2013). The commercial modification of MacConkey agar base used in the present study lacked crystal violet as part of its composition. This particular modification of MacConkey agar permits growth of *S. aureus* (Acharya, 2013).

Clarified egg yolk was less contaminated when compared with whole egg yolk. This could be as a result of the centrifugation it underwent. Centrifugation in essence involves compacting

Table 12. Susceptibility of the isolatesto various antibiotic discs

Isolate	Antibiotic susceptibility disc					
	Streptomycin	Penicillin	Gentamicin	Amoxicillin	Neomycin	Cefoxitin
S. aureus	R	R	S	R	R	R
E. coli	S	R	S	R	R	S
Salmonella species	R	R	S	R	R	R
K. pneumoniae	R	R	S	R	R	S

S = sensitive; R = resistant.

bacteria into a pellet, causing collisions against each other that result in shear forces on the bacterial cell surface, which may easily lead to cell surface damage (Peterson et al., 2012). According to Liu et al. (2017), total coliform and *E. coli* densities in the liquid phase of processed manure were all reduced after centrifugation. Ratnam and March (1986) established that after centrifugation, most bacteria were concentrated in the sediment and that only very few remain in the supernatant in an uneven distribution throughout the fluid column. Centrifugation is also used to produce a concentrated amount of low-density lipoprotein which makes up 85% of clarified egg yolk. The lipid-mediated antimicrobial activity of the low-density lipoprotein (Brady et al., 2002) could also explain the lesser degree of contamination in clarified egg yolk. In the current study, fungal filaments and conidia were seen in clarified egg yolk stored for 24 h. Although *Aspergillus* spp. was suspected, no quantification and confirmatory tests were carried out because the reagents needed for the confirmatory tests were not available.

Whole and clarified yolk extenders without antibiotics had more contamination when compared with whole and clarified yolk extenders with antibiotics. This agrees with the work of Rustenov et al. (2013) who established that high decontamination level in extended semen indicates that antibiotics in semen extenders act synergistically against semen microflora. However, it is not known whether antibiotics in semen extenders could compromise the quality of spermatozoa in semen since extenders produced in the current study were not used in extending semen.

S. aureus, *K. pneumoniae*, *Salmonella* species and *E. coli* were isolated from extenders while only *S. aureus* was isolated from egg yolk forms. It could be that *E. coli*, *K. pneumoniae* and *Salmonella* spp. were present in the yolk but did not have enough time to grow or were contaminants picked up during preparation and storage of the extender. According to Yániz et al. (2010), *E. coli* and *S. aureus* were part of the isolates frequently recovered from ram semen. Also, Althouse et al. (2000), Althouse and Lu (2005), Bresciani et al. (2014) and Mitra et al. (2016) isolated these organisms from semen. Ahmed et al. (2015) isolated *E. coli*, *Klebsiella* and *Salmonella* spp. from poultry semen. Since the present study did not look at microbes prevalent in semen, little could be inferred in this regard. It is, however, possible that if these yolk forms were used to prepare extenders for extension of semen they could have served as additional sources of contamination to the extended semen.

Bacteria are known to enter a viable but non-culturable (VBNC) state upon starvation (Xu et al., 1982). A bacterial cell can be demonstrated to be metabolically active while being incapable of undergoing the sustained cellular division required to form a colony on a plate (Oliver, 1993). This phenomenon could be responsible for the no growth response on Chocolate agar recorded in the whole egg yolk form after 48 h storage despite growth after 24 h storage in the same medium. The growth was subsequently identified as *S. aureus* and study by Watson et al. (1998) showed that *S. aureus* is capable of exhibiting VBNC as a result of glucose, amino acid, phosphate, or multiple-nutrient limitation. They further reported a loss of *S. aureus* viability of about 99 to 99.9% of the population within two days (48 h).

All isolates from yolk-citrate semen extender formulations were sensitive to Gentamicin. Only *E. coli* were sensitive to Streptomycin. *E. coli* and *K. pneumoniae* were sensitive to Cefoxitin. It could be that Gentamicin, being broad spectrum, is more effective against Gram-positive and Gram-negative bacteria (Ball et al., 1987; Shin et al., 1988). *S. aureus* isolated from egg yolk was resistant to only Gentamicin while that isolated from the extender was sensitive to only Gentamicin. According to Fridman et al. (2014), changes in the lag-time in order to develop tolerance is the first change made by bacteria in response to antibiotic stresses. This ‘tolerance by lag’ allows bacteria to survive under high antibiotic concentrations, and may facilitate the subsequent evolution of antibiotic resistance. It could also be a resistant strain as strains isolated clinically, especially methicillin-resistant *S. aureus* (MRSA), exhibit multiple antibiotic resistance (Grundmann et al., 2006; Deurenberg et al., 2007). Also, growth rate and the growth phase of the culture play an important part in determining antibiotic

susceptibility (Desai et al., 1998). Moreover, *S. aureus* cells in stationary growth phase are less vulnerable to antibiotics than those in exponential phase, arguably due to elevated persister levels (Wiuff et al., 2005).

S. aureus isolated in the second experiment was not sensitive to Streptomycin and Penicillin but no contamination was recorded in clarified yolk extenders with these antibiotics across all storage periods (pre-storage, 24 and 48 h). However, in whole yolk extender with these antibiotics, contamination was only recorded pre-storage. This organism (*S. aureus*) most likely survived the freezing and thawing process to which the egg yolk forms from the first experiment were subjected. Also, the antibiotics in the extender probably had less time to act on it since whole yolk extenders with antibiotics stored for 24 and 48 h had no contamination. Therefore, the question here is whether results of antibiotic sensitivity tests could inform the choice of antibiotics in extender formulation.

Conclusion

This work shows that clarifying egg yolk by centrifugation reduced bacterial load in egg yolk and inclusion of antibiotics in extender formulation reduced or eliminated contamination. *S. aureus, E. coli, K. pneumoniae* and *Salmonella* spp. were isolated from yolk-citrate semen extenders. These organisms were susceptible to Gentamicin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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