Onderstepoort Journal of Veterinary Research, 61:341–349 (1994)

A rapid method to quantify bacterial contamination on hatching eggs. 1. Correlation of optical density with initial bacterial count

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ABSTRACT

PIENAAR, A.C.E., COETZEE, L. & BRAGG, R.R. 1994. A rapid method to quantify bacterial contamination on hatching eggs. 1. Correlation of optical density with initial bacterial count. *Onderstepoort Journal of Veterinary Research*, 61:341–349

The use of optical-density (OD) readings after a 6-h incubation period, as a suitable method to quantify the bacterial contamination on hatching eggs, was established by the use of pure cultures of five bacterial isolates found to be the most prevalent on the hatching eggs examined. These isolates were identified as *Micrococcus luteus, Staphylococcus gallinarum, Streptococcus epidermidis, Pseudomonas cepacei* and *Bacillus cereus*.

It was established that the OD reading was repeatable when the same inoculum was used to inoculate five different cultures, which were incubated for 6 h at 37 °C. This repeatability was not affected by bacterial isolate, or bacterial concentration of the inoculum, or when mixed bacterial cultures were used.

A direct relationship was established between the OD reading (at 540 nm) after 6 h and the log of the bacterial concentration at the start of incubation.

The OD reading after 6 h of incubation is a repeatable, rapid and simple method to quantify the bacterial concentration at the start of the incubation period.

INTRODUCTION

The quantification of bacterial contamination on hatching eggs is an important aspect of a hygiene-monitoring programme in a hatchery. Contaminated hatching eggs can lead to a reduction in hatchability (Quarles, Gentry & Bressler 1970). The contamination may also spread to other areas in the hatchery and lead to infection in newly hatched chicks. The wet navel of the newly hatched chick acts as a port of entry for environmental contaminants, which lodge in the nutrient-rich yolk sac. This causes omphalitis and yolk-sac infection, which could be the cause of mortalities in newly hatched chicks. It could also result in severe financial losses to the supplier of day-old chicks.

Quarles *et al.* (1970) showed a correlation between bacterial counts in the air in a hatchery and bacterial counts on egg shells. Micro-organisms are distributed in or on egg shells throughout the hatchery by air movement and employee activity, and can lead to contamination and infection of newly hatched chicks (Magwood 1964; Sheldon & Brake 1991). Sheldon &

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Accepted for publication 20 September 1994—Editor

& Ball (1986, as cited in Sheldon & Brake 1991) showed that 75 % of micro-organisms isolated during a survey of hatchery air quality, are of respirable size (< 5 μm).

The egg shells of freshly laid eggs, or eggs recovered from the oviduct, are free from micro-organisms in more than 90 % of cases (Hadley & Chadwell 1916, as cited in Arhienbuwa, Adler & Wiggins 1980; Stuart & McNulty 1943, as cited in Sacco, Renner, Nestor, Saif & Dearth 1989; Mayes & Takeballi 1983). The major source of contamination of egg shells is therefore of external origin and bacteria are the most important contaminants (Mayes & Takeballi 1983). The egg shell is moist when laid, and can therefore easily become soiled. The presence of dirt in the environment increases the number of contaminating organisms. The degree of egg-shell contamination is a function of the cleanliness of the surface onto which the eggs are laid and the manner in which they are handled (Ayres, Kraft, Board, Torrey & Rizk 1967). Temperature, length of storage and treatment (e.g. washing) also have an effect. Contamination varies quantitatively as well as qualitatively in different geographical areas, but is relatively constant over time, and in respect of geographical location and husbandry methods (Mayes & Takeballi 1983). Season also plays a role in the number of organisms and their type, with a higher incidence in summer in the USA (Williams 1970).

Various workers, using culture methods, determined the total bacterial count on hatching eggs:

- Haines (1938) determined a total count of 13 000 –8 million bacteria per egg.
- Rosser (1942) found 10 000 bacteria per egg.
- Forsythe, Ayres & Radlo (1953) reported a figure of 63 000 bacteria per egg.
- Board (1964) reported a total of 10^2–10^8 bacteria per shell, with 10^3–10^6 bacteria per shell in the case of stained and soiled eggs and total counts as high as 10^4–10^7 per shell in the case of badly soiled eggs.

According to Mayes & Takeballi (1983), gram-positive bacteria originating from dust, soil and faeces, dominate the microflora found on the surface of the egg shell. Board, Ayres, Kraft & Forsythe (1963) did bacterial isolations from egg shells, fillers and flats and identified gram-positive cocci, including *Micrococcus* spp. and *Staphylococcus* spp. (47,8 % of total), gram-positive rods, including *Arthrobacter* spp. and *Bacillus* spp. (15,3 of total), gram-negative rods, including *Pseudomonas fluoresence, Achromobacter* spp., *Alkaligenes* spp., *Flavobacterium* spp., *Cytophaga* spp., and other unclassified gram-negative rods (26,8 % of total) and *Escherichia* spp., *Aerobacter* spp. and *Aeromonas* spp. (9,5 % of total). Only one strain of *Salmonella* spp. (*Salmonella* Seftenberg) was isolated in this study. Sacco *et al.* (1989) found *Staphylococcus* spp. to be the most prevalent bacterium isolated from egg shells. These workers also isolated *Streptococcus* spp. and a few colonies of *Pseudomonas* spp.

The conventional methods currently used to quantify bacterial contamination of hatching eggs, consist of visual inspection of the egg shell or bacterial cultures on media by means of egg washing or impressions. These methods are unsatisfactory, owing to unreliability and the time involved in carrying out these procedures.

The spectrophotometer measures the optical density (OD) of a bacterial suspension, and there is a direct correlation between increased OD reading and increased bacterial concentration in the suspension as the bacteria replicate (Sokatch 1969). This instrument is capable of detecting very small changes in optical density, and an indication of bacterial growth can thus be obtained in a very short space of time, hence eliminating elaborate bacterial cultures.

The use of OD readings is a suitable method of determining bacterial concentrations in liquid medium (Sokatch 1969). The feasibility of using OD readings to determine the bacterial concentration in nutrient-broth (NB) cultures of egg washings after a fixed incubation period, and of relating these OD readings to the concentration of bacteria on the egg surface, was investigated.

In this first paper, a suitable incubation period was established by obtaining growth curves of five bacterial isolates made from hatching eggs. The repeatability of such a method was investigated by the use of pure cultures as well as mixed bacterial cultures. The correlation between the OD reading after a fixed incubation period, and the bacterial concentration of the inoculum was also investigated and is reported on in this paper.

After these aspects had been established, by the use of pure as well as mixed bacterial cultures *in vitro*, the possible use of this technique on hatching eggs was also established and will be reported on in the second paper of this series.

MATERIALS AND METHODS

Isolation and identification of bacteria from poultry eggs destined for hatchery purposes

Hatching eggs were obtained from two hatcheries, one a layer hatchery and one a broiler hatchery. Two samples of ten eggs each were obtained from a layer hatchery near Bronkhorstspruit, South Africa and three samples of five eggs each, from a broiler hatchery. Sterile gloves were worn to collect the eggs from the setters in a random fashion and to place them

into sterile plastic bags. The eggs were transported on ice to the laboratory.

Moist swabs were taken of large areas of the shell surfaces and plated onto blood tryptose agar (BTA) plates (Onderstepoort Biological Products), MacConkey agar (Oxoid) plates and Xylose Lysine Deoxycholate (XLD) (Oxoid) plates. The plates were incubated for 18 h at 37 °C. Colony morphology and some indication of the prevalence of the different colony types were noted. Pure cultures were obtained from the more prevalent colony types. These were identified by gram-staining, catalase, oxidase, motility, fermentation of different carbohydrates [lactose, maltose, mannitol (aerobic as well as anaerobic fermentation), sucrose and xylose], nitrate reduction and urease fermentation. The bacteria were identified by their fermentation patterns (Krieg & Holt 1984). The identity of the bacteria was also confirmed by Dr M. Henton of the Bacteriology Section of the Onderstepoort Veterinary Institute, Onderstepoort, South Africa.

M. luteus, Staphylococcus gallinarum, Streptococcus epidermidis, P. cepacei and *B. cereus* were selected as suitable candidates for the evaluation of the repeatability and reliability of the use of OD readings for determining bacterial counts of the inoculum.

Establishment of a suitable incubation period for the five selected bacteria

A suitable incubation period for the five bacteria selected, was determined by establishing growth curves for these bacteria. A 200-mℓ volume of nutrient broth (NB) was inoculated with 1 mℓ of a 24-h broth culture of each isolate and incubated at 37 °C. A small volume (10 mℓ) of the culture was aseptically removed every hour for 8 h and the OD of each sample was measured with a spectrophotometer (Milton Roy Spectronic 1201) immediately after collection, at 540 nm. Sterile medium which had been handled in the same manner as the inoculated medium, was collected and used as a blank. This was repeated four times for each of the five bacterial isolates.

A graph was compiled of the OD readings *vs.* time in hours to determine the growth curve of each isolate. A suitable incubation period, when all or most of the isolates were in the log phase of growth, was determined from the growth curves.

Repeatability of OD readings after an incubation period of 6 h

Bacterial cultures to be used as inocula, were obtained by inoculating each of the selected bacterial isolates into tubes containing 10 mℓ NB. These tubes were incubated at 37 °C for 18 h, after which tenfold serial dilutions were made in peptone water. A total of four different dilutions per bacterial isolate were selected as an inoculum and 100 μℓ was used to inoculate 10 mℓ NB in sets of five tubes each. Each bacterial isolate was repeated three times, so that a total of 12 different sets of five tubes were each inoculated with different bacterial concentrations. Each set of five tubes was incubated for 6 h at 37 °C. After incubation, the OD (at 540 nm) was measured for each tube in the set of five. The mean OD, standard deviation (SD) and coefficient of variance (CV) were then calculated for each set of five tubes.

Repeatability of OD readings after incubation of 6 h with different volumes of bacteria in mixed cultures

Samples of the five bacteria selected were inoculated into NB and incubated at 37 °C for 18 h. After incubation, different volumes of bacteria were mixed to a final volume of 100 μℓ in 10 mℓ NB. The procedure for each combination was repeated five times. The different volumes of bacteria can be seen in Tables 2–4. The inoculated tubes of NB were incubated at 37 °C for 6 h, after which the ODs of the different tubes were read and recorded. Statistical analyses (mean, SD and CV) were performed on each combination of bacteria.

Relationship between OD after 6 h and the bacterial concentration of inoculum

The serial dilutions which were used to determine the repeatability of the different bacterial cultures after incubation of 6 h, were also used to determine the concentration of bacteria in the inoculum. The dilution series 10^{-1}–10^{-5}, described above, of each bacterium was plated onto five Standard Plate Count agar (Oxoid) plates per dilution. Each plate was inoculated with 100 μℓ of the diluted bacterial suspension and the inoculum was spread over the plate by means of a sterile, bent glass rod. The plates were incubated for 24 h at 37 °C. After incubation a plate count was performed and the number of bacteria were calculated as colony-forming units.

The number of bacteria in the inoculum of the 10^{-7}–10^{-10} dilutions, used to determine the repeatability of the OD readings after incubation of 6 h, were calculated from the above plate counts. The mean OD reading after incubation of 6 h, obtained from the above repeatability studies, was plotted against the log of the bacterial counts of the inoculum.

RESULTS

Isolation and identification of bacteria from hatching eggs

A number of different bacteria were isolated from the shells of eggs collected from setters at the layer and broiler hatcheries. On most of the eggs, only two or three different colony types were observed. Small,

grey colonies were the most prevalent. Three different types of small, grey colonies were detected and subsequently identified as *M. luteus, Staphylococcus gallinarum* and *Streptococcus epidermidis.*

A larger colony type, identified as *Pseudomonas cepacei*, was found on approximately 55 % of the eggs examined. Another very large colony type, identified as *B. cereus*, was detected on approximately 8–10 % of the eggs examined.

Establishment of the optimum incubation period for the five selected bacteria

Only one of the five isolates (*B. cereus*) reached the stationary phase of growth within the 8-h incubation period (cf. Fig. 1E). This isolate had a very short lag phase (up to 2 h), followed by a log phase of between 2–5 h of incubation. The other four isolates all showed lag phases of between 2–3 h followed by the log phase of growth (cf. Fig. 1A–D). None of these four isolates reached the stationary phase of growth within the 8-h incubation period of this experiment.

An incubation time of 6 h was selected as four of the five organisms were in the log phase of growth at this time. *B. cereus* had already reached the stationary phase after 6 h.

Repeatability of OD readings after an incubation period of 6 h

The OD of each tube, in the set of five tubes inoculated with the same concentration of a bacterial isolate, was read after incubation of 6 h at 37 °C. The mean OD reading and SD for each set of five tubes were calculated (Table 1). From these results the CV for each set of five tubes was calculated (Table 1). In four of the five bacterial isolates, ten different bacterial concentrations yielded OD readings above 0. In all four of these bacterial isolates, two dilutions of bacteria had insufficient bacteria in the inoculum to increase the OD above that of the blank. These sets of tubes, which had OD readings of 0, are not shown in Table 1. Since OD readings of 0 were obtained in three sets of five tubes in one of the bacterial isolates selected, these results are also omitted in Table 1. Consequently, four isolates have ten sets of five tubes each, while one isolate has only nine sets of five tubes per different bacterial concentration.

In all five isolates, 83,7 % of the repetitions had a CV of less that 15 %. In most cases where low numbers of bacteria were present in the inoculum, as indicated by low mean OD readings, a CV of more than 15 % was obtained (Table 1).

The repeatability of the OD reading is therefore very high, with a CV of less than 15 % in 83, 7% of the repetitions and a CV of below 10 % in 59,2 % of the repetitions (Table 1).

Repeatability of OD readings after incubation of 6 h with different concentrations of bacteria in mixed cultures

As with pure cultures, the CV of the mixed cultures was also very low, indicating high repeatability. When combinations of two isolates were made, 100 % of the repetitions had a CV of below 15 %, while 93,3 % had a CV of below 10 % (Table 2). Combinations of three isolates yielded a CV of below 10 % in 77,8 % of the repetitions (Table 3). A combination of four isolates resulted in a CV of below 15 % in 100 % of the repetitions, while a CV of below 10 % occurred in 96,3 % of repetitions (Table 4). It can therefore be concluded that the repeatability of the OD readings on mixed cultures is very high.

Relationship between OD after incubation of 6 h and the bacterial concentration of the inoculum

To determine the relationship between OD after incubation of 6 h and the bacterial counts of the inoculum, bacterial counts were made on the inocula to determine the repeatability of OD readings of pure cultures in the above section. The mean OD readings were plotted against the log of the bacterial counts (cf. Fig. 2A–E). In all of the isolates, except *B. cereus* (Fig. 2E), a direct relationship between the OD readings and the log of the bacterial counts of the inoculum was obtained (Fig. 2A–D). In the case of *B. cereus*, a direct relationship was obtained only with low initial concentrations of bacteria (Fig. 2E). At high initial concentrations of bacteria, and with the rapid growth rate of this isolate (Fig. 1E), the bacteria reached the stationary phase of growth with no further increase in OD readings, resulting in the relationship seen in Fig. 2E.

DISCUSSION

Bacteria were isolated from eggs collected from hatcheries, primarily to obtain suitable samples of bacteria to evaluate the reliability and repeatability of OD readings after a fixed incubation period to determine the bacterial concentration on the egg shell. The populations detected on these eggs correspond with the findings of Board *et al.*, 1963) who identified *Micrococcus* spp. and *Staphylococcus* spp. as the most prevalent, as well as with the findings of Mayes & Takeballi (1983), who stated that gram-positive bacteria dominate the microflora on the egg surfaces. In our study, the predominant bacteria isolated from the egg shells were gram-positive and were identified as *M. luteus, Staphylococcus gallinarum, Streptococcus epidermidis* and *B. cereus*. *P. cepacei* was the only gram-negative organism isolated and identified.

Various other colony types were seen on different eggs. Only the predominant colony types, which were

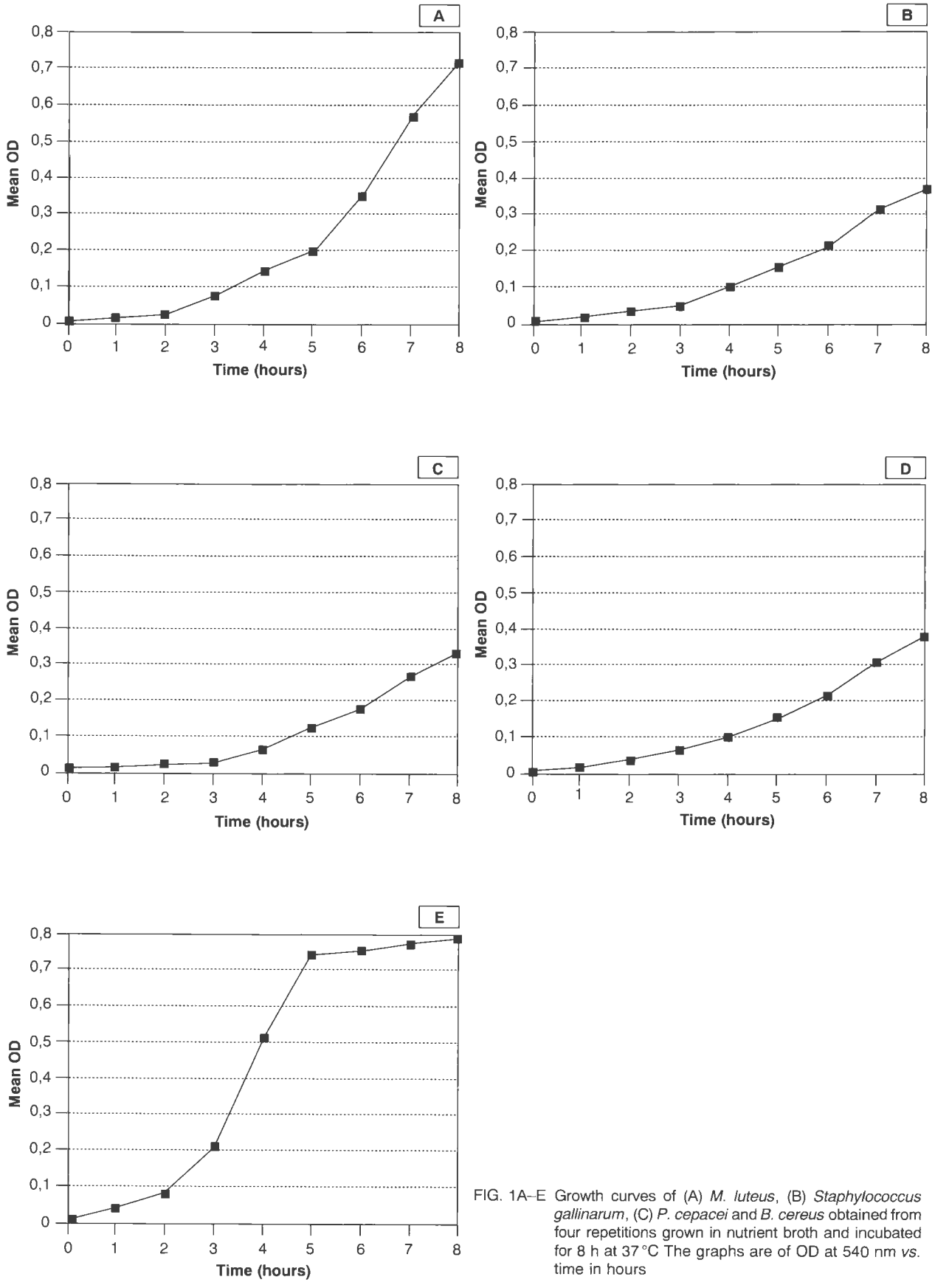

FIG. 1A–E Growth curves of (A) *M. luteus*, (B) *Staphylococcus gallinarum*, (C) *P. cepacei* and *B. cereus* obtained from four repetitions grown in nutrient broth and incubated for 8 h at 37 °C The graphs are of OD at 540 nm *vs.* time in hours

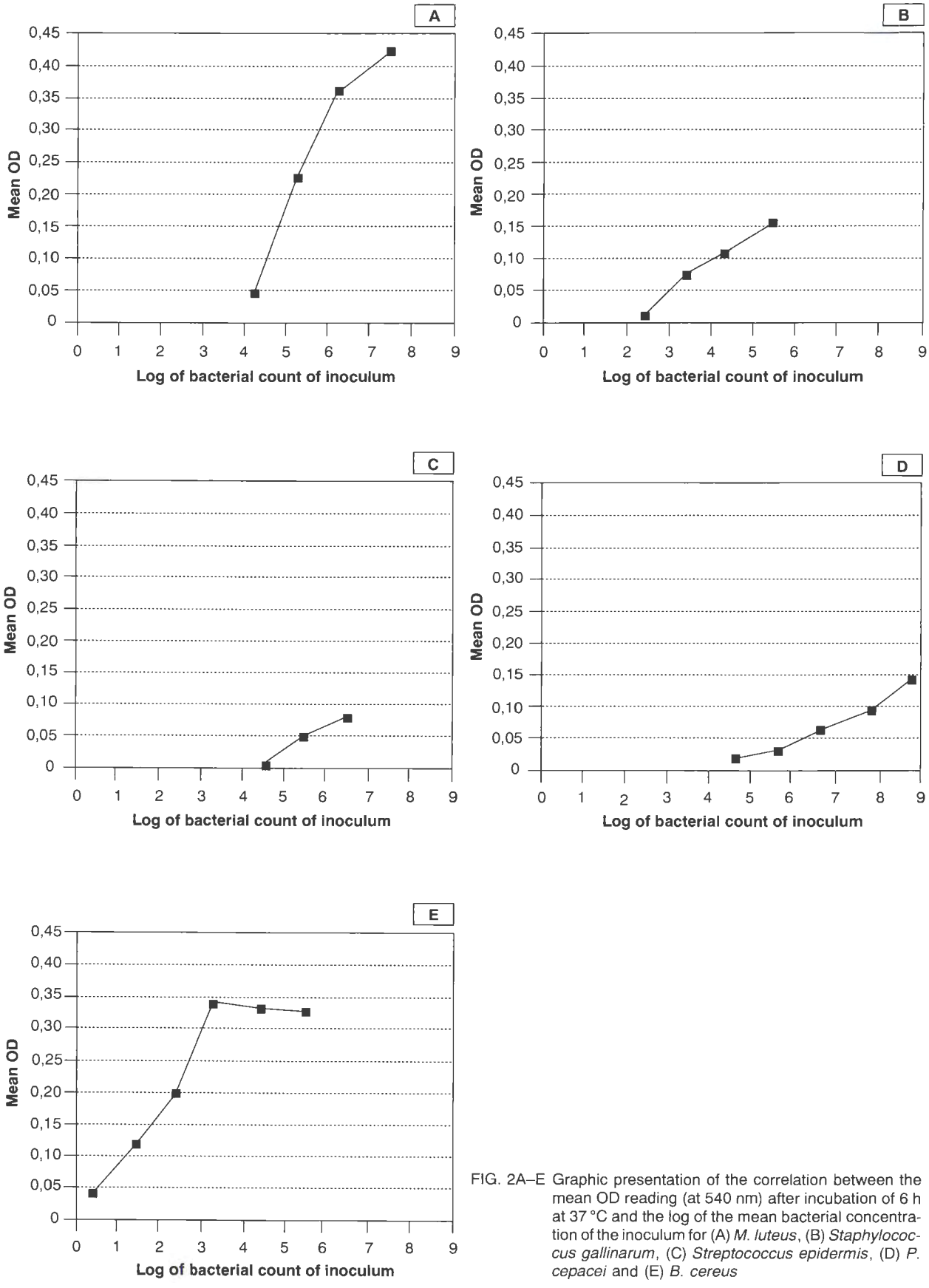

FIG. 2A–E Graphic presentation of the correlation between the mean OD reading (at 540 nm) after incubation of 6 h at 37 °C and the log of the mean bacterial concentration of the inoculum for (A) *M. luteus*, (B) *Staphylococcus gallinarum*, (C) *Streptococcus epidermis*, (D) *P. cepacei* and (E) *B. cereus*

TABLE 1 Statistical analyses of the results obtained when testing the repeatability of the OD (at 540 nm) after a 6-h incubation period at 37 °C in nutrient broth. Each mean OD represents the mean of the OD of five different tubes, each containing 10 mℓ nutrient broth and inoculated with 100 µℓ of the same concentration of bacteria. A total of 12 different bacterial concentrations per selected bacterial isolate was tested and only those cultures which showed an OD reading above the blank are recorded. The SD for each set of five tubes was calculated and from the mean and the SD, the CV was calculated

Staphylococcus gallinarum			*Micrococcus luteus*			*Streptococcus epidermidis*			*Pseudomonas cepacei*			*Bacillus cereus*		
Mean OD	SD	CV	Mean OD	SD	CV	Mean OD	SD	CV	Mean OD	SD	CV	Mean OD	SD	CV
0,362	0,022	6,07	0,300	0,025	8,51	0,140	0,014	10,13	0,085	0,008	9,71	0,319	0,033	10,34
0,358	0,009	2,51	0,199	0,016	8,23	0,096	0,005	4,73	0,059	0,002	4,08	0,315	0,012	3,81
0,345	0,012	3,48	0,157	0,010	6,23	0,046	0,002	4,64	0,009	0,003	30,27	0,078	0,008	10,26
0,249	0,024	8,03	0,144	0,008	5,56	0,017	0,002	11,27	0,077	0,008	10,67	0,143	0,027	18,88
0,128	0,010	7,81	0,044	0,005	11,27	0,082	0,004	5,13	0,058	0,004	6,73	0,066	0,020	30,30
0,060	0,013	21,67	0,199	0,012	5,88	0,062	0,009	14,18	0,078	0,012	15,58	0,022	0,007	31,82
0,133	0,011	8,27	0,137	0,009	6,41	0,151	0,007	4,41	0,052	0,007	13,25	0,295	0,020	6,78
0,091	0,008	8,79	0,084	0,006	7,14	0,105	0,003	3,23	0,085	0,004	4,99	0,275	0,015	5,45
0,055	0,006	10,91	0,037	0,004	9,83	0,050	0,005	10,84	0,058	0,007	12,06	0,104	0,004	3,85
0,004	0,002	50,00	0,005	0,001	20,41	0,142	0,016	10,72				0,137	0,012	8,57

TABLE 2 Mean OD reading (at 540 nm), SD and CV of different combinations of two different bacteria in 10 mℓ of nutrient broth after incubation of 6 h at 37 °C. The mean OD reading was obtained from the OD readings of five tubes of nutrient broth, each inoculated with the same combination of bacteria

No. of tubes	Bacterial concentration		Mean	SD	CV
	A (µℓ)	B (µℓ)			
5	5	95	0,378	0,005	1,275
5	10	90	0,384	0,005	1,396
5	15	85	0,383	0,006	1,488
5	20	80	0,391	0,009	2,226
5	25	75	0,383	0,012	3,180
	C (µℓ)	B (µℓ)			
5	5	95	0,141	0,006	4,46
5	10	90	0,129	0,011	7,59
5	15	85	0,142	0,010	7,04
5	20	80	0,139	0,008	6,04
5	25	75	0,138	0,005	3,64
	D (µℓ)	B (µℓ)			
5	5	95	0,139	0,003	2,40
5	10	90	0,156	0,016	10,47
5	15	85	0,143	0,006	4,41
5	20	80	0,141	0,003	2,24
5	25	75	0,125	0,003	2,29

A = *Bacillus cereus*
B = *Staphylococcus gallinarum*
C = *Pseudomonas cepacei*
D = *Streptococcus epidermidis*

TABLE 3 Mean OD reading (at 540 nm), SD and CV of different combinations of three different bacteria in 10 mℓ of nutrient broth after incubation of 6 h at 37 °C. The mean OD reading was obtained from the OD readings of five tubes of nutrient broth each inoculated with the same combination of bacteria

No. of tubes	Bacterial concentration			Mean	SD	CV
	A (µℓ)	B (µℓ)	C (µℓ)			
5	5	90	5	0,311	0,060	19,19
5	5	85	10	0,326	0,010	3,15
5	5	80	15	0,319	0,018	5,52
5	10	85	5	0,283	0,085	29,99
5	10	80	10	0,286	0,064	22,51
5	10	75	15	0,301	0,080	26,56
5	15	80	5	0,322	0,008	2,41
5	15	75	10	0,323	0,014	4,42
5	15	70	15	0,322	0,006	1,69
	A (µℓ)	B (µℓ)	D (µℓ)			
5	5	90	5	0,359	0,011	3,20
5	5	85	10	0,348	0,013	3,80
5	5	80	15	0,368	0,017	4,54
5	10	85	5	0,362	0,013	3,48
5	10	80	10	0,373	0,017	4,54
5	10	75	15	0,371	0,011	2,95
5	15	80	5	0,364	0,011	3,01
5	15	75	10	0,374	0,009	2,37
5	15	70	15	0,350	0,018	5,18
	C (µℓ)	B (µℓ)	D (µℓ)			
5	5	90	5	0,154	0,044	28,50
5	5	85	10	0,120	0,003	2,46
5	5	80	15	0,123	0,001	0,88
5	10	85	5	0,120	0,002	2,04
5	10	80	10	0,115	0,001	0,98
5	10	75	15	0,120	0,004	3,61
5	15	80	5	0,118	0,004	3,43
5	15	75	10	0,170	0,053	31,10
5	15	70	15	0,120	0,008	6,90

TABLE 4 Mean OD reading (at 540 nm), SD and CV of different combinations of four different bacteria in 10 mℓ of nutrient broth after incubation of 6 h at 37 °C. The mean OD reading was obtained from the OD readings of five tubes of nutrient broth, each inoculated with the same combination of bacteria

No. of tubes	Bacterial concentration				Mean	SD	CV
	A (µℓ)	B (µℓ)	C (µℓ)	D (µℓ)			
5	5	5	5	85	0,348	0,010	2,99
5	5	5	10	80	0,372	0,005	1,40
5	5	5	15	75	0,368	0,010	2,77
5	5	10	5	80	0,364	0,008	2,28
5	5	10	10	75	0,355	0,012	3,49
5	5	10	15	70	0,368	0,011	2,89
5	5	15	5	75	0,364	0,008	2,14
5	5	15	10	70	0,372	0,020	5,45
5	5	15	15	65	0,373	0,010	2,59
5	10	5	5	80	0,374	0,011	3,02
5	10	5	10	75	0,375	0,029	7,67
5	10	5	15	70	0,361	0,017	4,70
5	10	10	5	75	0,385	0,006	1,62
5	10	10	10	70	0,359	0,035	9,86
5	10	10	15	65	0,380	0,003	0,88
5	10	15	5	70	0,376	0,018	4,74
5	10	15	10	65	0,377	0,010	2,68
5	15	5	15	60	0,379	0,012	3,05
5	15	5	5	75	0,378	0,005	1,39
5	15	5	10	70	0,370	0,005	1,43
5	15	10	15	65	0,378	0,003	0,76
5	15	10	5	70	0,374	0,006	1,49
5	15	10	10	65	0,353	0,012	3,34
5	15	10	15	60	0,317	0,024	7,54
5	15	15	5	65	0,303	0,017	5,68
5	15	15	10	60	0,324	0,011	3,43
5	15	15	15	55	0,378	0,048	12,70

A = *Pseudomonas cepacei*
B = *Bacillus cereus*
C = *Streptococcus epidermidis*
D = *Staphylococcus gallinarum*

found on a significant number of eggs, were selected for identification. This study should therefore not be seen as a definitive study on the bacterial population of hatching eggs.

It is concluded that the selection of bacteria, made for the evaluation of the use of OD readings to determine bacterial concentration on the shells of hatching eggs, is a representative sample of the bacteria occurring on these eggs. This can be concluded from both our rough estimation of bacterial incidence and the published data on bacterial populations on hatching eggs (Board *et al.* 1963; Mayes & Takeballi 1983).

The isolation of *Staphylococcus gallinarum* was interesting, as it is the first recorded isolation of this organism in South Africa.

Typical bacterial growth curves (cf. Fig. 1A–E) were obtained for all of the bacteria examined. Only in the case of *B. cereus* was the full growth curve, consisting of a lag phase, a log phase and a stationary phase, obtained within 8 h of incubation. The other four bacteria (Fig. 1A–D) completed only the lag phase and a section of the log phase within this incubation period. The experiment was not continued to reach the stationary phase for all of the bacteria, as only the incubation time during which the bacteria were still in the log phase of growth, was desired for these experiments. An incubation period which would allow most or all of the bacteria to reach the stationary phase of growth, was not desirable as this would adversely affect the sensitivity of the test. After an incubation period of 6 h, the most prevalent species (*M. luteus, Staphylococcus gallinarum* and *Streptococcus epidermidis*) detected on the shells of eggs would be in the log phase of growth. A 6-h incubation period also allows for the test to be completed within one working day.

Before any attempts could be made to evaluate the use of OD readings to determine bacterial contamination of hatching eggs, it was important to establish whether the OD reading after incubation of 6 h is repeatable when the initial concentration of bacteria is the same. When pure cultures of bacteria were used (Table 1), low CVs were obtained. These low CVs are an indication of the repeatability of the OD reading of a culture after incubation of 6 h when the inoculum for each culture is the same. Of the five isolates tested, the highest mean CV (n = 10) was 13,01 % for *B. cereus*. The only other isolate to have a mean CV of over 10 % was *Staphylococcus gallinarum*. In both cases, high individual CVs were obtained when very low numbers of bacteria (as seen by the mean OD readings in Table 1) were used. It can be concluded from these results that the OD reading after incubation of 6 h is repeatable when the same concentration of a pure bacterial culture is inoculated into the culture medium.

In the above section, the repeatability of the OD readings was established with the use of pure cultures of bacteria. If OD readings are to be used to determine bacterial contamination of hatching eggs, it is important to determine whether the OD reading is also repeatable when mixed bacterial cultures are used. To evaluate the effects of mixed cultures on the repeatability of the OD after 6 h, cultures of the five more prevalent bacteria were mixed at different concentrations, in an attempt to mimic the population dynamics found on the egg surfaces. Thus, in most cases, the gram-positive cocci were in the majority. When two isolates were mixed, the mean CV (n = 15) (Table 2) was 4,0 %, thus indicating a very high level of repeatability. A mean CV (n = 27) (Table 3) of 8,46 was obtained when three isolates were mixed,

and a mean CV (n = 27) (Table 4) of only 3,77 was found when four isolates were mixed. It can therefore be concluded from these results that the OD reading after incubation of 6 h is highly repeatable when the same bacterial inoculum is used. This is a very important aspect of the project and indicates that the use of OD readings after a fixed incubation period will be similar when the concentrations of bacteria in the inocula (or on the surfaces of the hatching eggs) are similar.

The final and most important aspect of this work was to determine whether the OD reading after incubation of 6 h correlates with the bacterial counts of the inoculum.

As the bacterial concentration of the inoculum can be calculated from the plate counts, a correlation between the bacterial concentration at the start of incubation, and the OD reading after 6 h could be established. When the mean OD reading after 6 h is plotted as a function of the log of that bacterial concentration at the start of incubation, a direct correlation is obtained (cf. Fig 2A–E).

A direct correlation between the mean OD reading after 6 h and the bacterial concentration at the start of incubation was obtained for all five of the most prevalent bacteria isolated from hatching eggs. It must be noted that when the bacterial concentration of *B. cereus* is too high at the start of incubation, a direct correlation is not obtained (cf. Fig. 2E). This is due to the fact that *B. cereus* is a very fast-growing organism, as can be seen from the growth curve in Fig. 1E. When the bacterial concentration is too high to start with, the bacterial population reaches a stationary phase of growth.

It can therefore be concluded that the OD reading after incubation of 6 h is a repeatable method to determine the bacterial concentration at the start of incubation in both pure cultures and mixed cultures. It can further be concluded that there is a direct correlation between the OD readings after incubation of 6 h and the bacterial concentration at the start of incubation.

It is therefore possible that the determination of the OD after incubation of 6 h is a suitable method of determining the bacterial concentration on hatching eggs. This method would have to be evaluated on hatching eggs.

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