A rapid method to determine bacterial contamination on hatching eggs. 2. Correlation of the optical-density measurements after incubation to bacterial counts on hatching eggs

A.C.E. PIENAAR, L. COETZEE and R.R. BRAGG

Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria
Private Bag X4, Onderstepoort, 0110 South Africa

ABSTRACT

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The suitability of using optical-density (OD) measurements after a 6-h incubation period to determine bacterial contamination on hatching eggs was evaluated.

A total of 154 hatching eggs, from five different flocks, were examined visually and bacterial counts and OD measurements of egg washings after 6 h of incubation at 37 °C were carried out to determine the levels of bacterial contamination, and these results were compared.

A relationship between the OD measurements of egg washings and the log of the bacterial plate counts, was demonstrated. The OD measurements provide accurate and quantative information to detect bacterial contamination on hatching eggs, as they are simple to perform, repeatable and reliable.

Keywords: Optical density, hatching eggs, bacterial contamination, quantification

INTRODUCTION

The quantification of bacterial contamination on hatching eggs is of the utmost importance to the poultry breeder. Contaminated hatching eggs can lead to a reduction in hatchability. 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. This may lead to severe financial losses to the supplier of day-old chicks.

Different procedures for the evaluation of bacterial contamination of hatching eggs have been followed by various workers. Haines (1938); Haines & Moran (1940) and Board (1964) removed the contents of the eggs and crushed the shell and shell membranes for bacterial isolation and counts. Rosser (1942) made the first attempt to use the intact shell for culturing, by placing eggs over the blades of a "Waring blendor" in

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a washing solution. The solution was then cultured and bacterial counts were performed.

Gentry & Quarles (1972) washed eggs by rubbing them in polyethylene bags filled with phosphate-buffered saline (PBS). Each egg was handled with sterile tongs and wiped with a sterile paper towel to remove any particulate matter and to eliminate contamination during handling. Eggs were subsequently placed into sterile bags, rubbed through the bag for 1 min, allowed to stand for 5 min and rubbed again for 1 min. The eggs were removed from the bag and serial dilutions of the washing solution were made and poured into petri dishes. Tryptose agar was added to each petri dish and the samples were allowed to incubate at 37 °C for 48 h. This method was modified by Whistler & Sheldon (1989) by using 0,1 % peptone water for washing the eggs and plate-count agar. Brake & Sheldon (1990) and Sheldon & Brake (1991) also used 0,1 % peptone water for washing eggs, but included violet-red bile and potato-dextrose agar as culture media.

Arhienbuwa, Adler & Wiggins (1980) used a sterile-tape method for turkey eggs. The sites for the adhesion of the tape were selected at random. The tape was removed from the surface of the egg and at least two impressions from one spot on the egg were made on the agar, on different positions on the agar plates. The media used included tryptose agar, eosin methylene blue plates, SS agar, McConkey agar and triple sugar iron agar. Plates were incubated for 24–48 h at 37 °C and the colonies were counted for each 2,88 cm of the tape. The total bacterial count for the egg surface was then calculated according to the formula for the determination of the surface of an egg described by Dunn & Schneider (1923, as cited in Arienbuwa *et al.* 1980). The advantages of this method are that no laboratory skills are required to carry out the procedures, no sacrifice of eggs is necessary and the method can be used at various stages of incubation. However, lower counts were obtained than those obtained by the polyethylene-bag wash method (10–100 x lower). Other problems encountered, included colonies too numerous to count, coalesence of colonies and, in some cases, no growth.

Sacco, Renner, Nestor, Saif & Dearth (1989) used a method in which a circle with a diameter of 3 cm was drawn on the side of each egg. A swab which had been moistened with PBS was used to obtain a sample. Cultures were done on blood agar and McConkey's agar at 37 °C for 48 h.

All of the above methods involve the culturing and physical counting of bacterial colonies on the different agar plates. This would require overnight incubation of the plates. The counting of bacterial colonies on agar plates is a time-consuming task, limiting the number of eggs that can be tested in this way on a routine basis.

Other methods, not based on physical bacterial counts, which may be considered for the enumeration of bacterial contamination of hatching eggs, include the use of the Malthus system (Malthus Instruments Limited, The Manor, Manor Royal, Crawley, West Sussex, RH 10 2PY, England) and luminescence (BioOrbit Oy P.O. Box 36, SF-20521, Turku, Finland). The Malthus system involves the detection of changes in current in culture medium inoculated with bacteria. The changes in current are measured by a computer and from this data the bacterial concentration in the sample can be calculated. The main disadvantage of this system is the very high cost of the equipment.

Luminescence utilizes the reaction between ATP, extracted from bacteria, and luceferin to produce a light reaction. It can detect bacterial counts from 10^3 bacteria per mℓ. The sensitivity can be increased by filtration and enrichment procedures which will, however, take more time. Although this is a very rapid procedure, the cost is once again prohibitive and the luminometer can be used only for this purpose.

Another possible method for determining the bacterial concentration on hatching eggs is to measure the optical density of bacterial cultures made from the washing of hatching eggs. The optical density of a suspension increases with bacterial growth (Sokatch 1969). The spectrophotometer is capable of detecting very small changes in optical density and an indication of bacterial growth can be obtained in a very short space of time, eliminating elaborate bacterial cultures.

Pienaar, Coetzee & Bragg (1994) determined that a 6-h incubation period would place most of the common bacteria isolated from hatching eggs in South Africa into the log phase of bacterial growth. They established that the OD of five different cultures, inoculated with the same concentration of bacteria and incubated for 6 h, is repeatable. They further established that there is a direct correlation between the mean OD reading after 6 h of incubation and the log of the bacterial concentration at the start of incubation. Pienaar *et al.* (1994) thus established that this is a suitable method for determining the bacterial concentration at the time of inoculation, with five different bacterial isolates collected from the surface of hatching eggs. This is a report on the use of the system, established previously on pure cultures (Pienaar *et al.* 1994) to detect and quantify bacterial contamination levels on hatching eggs. It is compared to the visual examination of the eggs and the actual physical bacterial count as determined by the plate-count method.

MATERIALS AND METHODS

Collection of hatching eggs

A total of 154 hatching eggs were collected from five different flocks over a period of 2 months. The eggs were collected by workers wearing sterile gloves, and each egg was placed into a sterile bag, and labelled and

stored at 4 °C until it could be examined. Examination of the eggs was completed within 1 week of collection.

Visual examination of hatching eggs

When the eggs were placed into individually labelled, sterile bags in the laboratory, each egg was evaluated visually and placed into one of the following six categories according to the visual estimation of surface contamination:

- Clean (no visible dirt on the egg shell)
- Very small amount of visible dirt (fewer than two small dirty areas on the egg shell)
- Small amount of visible dirt (two to four small areas of visible dirt on the egg shell)
- Fairly dirty (four to six areas of visable dirt on the egg shell)
- Dirty (six to eight dirty areas on the egg shell)
- Very dirty (more than eight dirty areas on the egg shell)

Egg washing

Between ten and 15 eggs in individually labelled, sterile bags were processed each day. A modification of the egg-washing procedure used by Gentry & Quarles (1972) was used to wash the eggs. Each sterile bag, containing a single egg, was filled with 20 mℓ of nutrient broth (NB). The egg was gently washed inside the bag by rubbing the surface of the egg through the plastic bag for 30 s.

Determination of OD measurements

After the eggs had been washed, a 10-mℓ sample of NB was removed by means of a sterile disposable pipette and placed into an appropriately labelled tube. The tubes were incubated at 37 °C for exactly 6 h. Tubes containing 10 mℓ of sterile NB were also incubated at 37 °C for 6 h. After incubation, the OD of each tube was measured on a spectrophotometer (Milton Roy model 1201), at 540 nm, and the incubated sterile medium was used as a blank.

Plate counts

At the same time that the 10-mℓ sample was removed for incubation and OD determination, another 2-mℓ sample was removed from the NB in which the egg had been washed, in order to determine the bacterial counts. Tenfold serial dilutions, up to a 10^{-4} dilution, were made in PBS. Three plate-count (PC) agar (Oxoid) plates were inoculated with 0,1 mℓ of the undiluted solution or 0,1 mℓ of the diluted samples. The inoculum was spread out over the surface of the plate with a sterile bent glass rod to ensure an even distribution of the bacteria across the plate. The plates were incubated at 37 °C for 18 h and bacterial counts were carried out. An average count was determined for each dilution, and the average count per 0,1 mℓ was determined. This count was subsequently related to 20 mℓ.

Analysis of data

The data from each flock was evaluated separately. The eggs from each flock were divided into five groups according to the plate-count results. All eggs with a bacterial count below 10^3 were placed in group 1. Eggs with bacterial counts between 10^3 and 10^4 were placed in group 2. Groups 3, 4, 5 and 6 consisted of eggs with bacterial counts between 10^4 and 10^5, 10^5 and 10^6, 10^6 and 10^7 and above 10^7, respectively. The mean bacterial counts were calculated for each group of eggs from each flock.

Once the eggs from each flock had been divided into groups, the mean bacterial counts and SD were calculated for each group. Likewise, the mean OD measurements and SD were calculated for each egg within a particular grouping for each flock. The mean OD measurements were plotted against the log of the mean bacterial counts for each flock. From these graphs, the relationship between the OD and the bacterial counts on hatching eggs from particular flocks was determined.

Graphs were compiled by plotting the log of the mean bacterial count, plus and minus one SD against the mean OD measurements. From these graphs the estimated bacterial-count range for selected OD measurements was calculated. These estimated bacterial-count ranges for the five different flocks were combined and the log of the mean bacterial range was plotted against the OD readings. From these graphs the relationship between the OD reading obtained after 6 h of incubation and the bacterial counts on the hatching eggs, irrespective of the flock from which the eggs had been collected, was established. Once this graph had been drawn, a grouping system based on the OD readings was calculated, which corresponded to the groupings based on bacterial counts. For example, group 2 consists of eggs which had a bacterial count of between 10^3 to 10^4. From this graph the OD readings corresponding to these bacterial counts can be calculated and the upper and lower OD readings for group 2 can be established.

The eggs were thus classified according to three evaluation systems, i.e. physical bacterial count into the grouping discussed above, OD readings according to groupings corresponding to the bacterial counts, and visual evaluation. A comparison of the three different schemes was carried out.

RESULTS

Relationship between OD measurement after 6 h of incubation and bacterial counts on hatching eggs

One hundred and fifty-four eggs were collected from five different flocks of which 39 eggs were collected and examined from flock 1. When the eggs were grouped according to the bacterial-count data, they

were found to fall into groups 2–5 for this flock (cf. Table 1). The mean OD measurement, with standard deviations in brackets, as well as the log of the mean bacterial count (with SD) obtained for each group, can be seen in Table 1. When the mean OD reading was plotted against the mean log of the bacterial counts, a linear relationship was found (cf. Fig. 1A).

In the case of flock 2, 29 eggs were collected and divided into three groups (groups 4–6). The mean OD reading and the log of the mean bacterial counts for this flock were calculated and can be seen in Table 1. The graph of the mean OD readings *v* the log of the mean bacterial count for this flock also resulted in a linear correlation (cf. Fig. 1B).

Bacterial counts done on eggs from flock 3, resulted in only two groups, consisting of 12 eggs in group 3 and 11 eggs in group 4 (cf. Table 1). When the calculated mean OD measurements (cf. Table 1) were plotted against the log of the mean bacterial counts (cf. Table 1), a linear correlation was found (cf. Fig. 1C).

The fourth flock (flock 4) from which eggs were collected had 3 groups (groups 4–6) according to bacterial counts (cf. Table 1). As with the other flocks, a linear correlation between the mean OD measurement and the log of the mean bacterial counts was obtained when these points were plotted (cf. Fig. 1D).

The final flock (flock 5) from which eggs were collected, yielded five groups (groups 2–6) (cf. Table 1). A linear correlation between mean OD measurements and the log of the mean bacterial counts was also found with this flock when the results were plotted (cf. Fig. 1E).

Establishment of bacterial ranges for selected OD measurements

In order to establish the range of bacterial counts for the different OD measurements after 6 h of incubation, graphs of the log of the mean bacterial counts plus and minus one SD were plotted against the mean OD measurements for each flock (cf. Fig. 2A–E).

From these graphs (Fig. 2A–E) the range of bacterial counts (in log) could be calculated for selected OD measurements (cf. Table 2). The mean of these calculated bacterial ranges for the same OD measurement obtained from different flocks could be calculated (cf. Table 2) and plotted against these OD measurements, thus supplying a graph indicating the relationship between the OD readings and the bacterial count, irrespective of the flock from which the eggs had been collected (cf. Fig. 3A and 3B).

Relationship between plate counts, OD measurements and visual examination for the evaluation of bacterial contamination on hatching eggs

The calculated OD ranges for groups 1–6, as calculated from the graph in Fig. 3, can be seen in Table 3. The numbers of eggs placed in each of the different groupings for each flock can be seen in Table 4.

DISCUSSION

Various workers have investigated different methods to determine the bacterial contamination of hatching eggs (Haines 1938; Haines & Moran 1940; Rosser 1942; Gentry & Quarles 1972; Whistler & Sheldon 1989; Brake & Sheldon 1990; Sheldon & Brake 1991) All of these workers attempted to do bacterial counts in one way or another. Only in 1972 (Gentry & Quarles 1972) were the eggs washed in phosphate-buffered saline (PBS) in a polyethylene bag. This method was adapted by Whistler & Sheldon (1989) who replaced the PBS with peptone water.

Pienaar *et al.* (1994) established that the OD readings after 6 h of incubation are linearly related to the initial bacterial concentration of the suspension before incubation. It was further established that the OD measurement is repeatable if the initial concentration of bacteria in different cultures is similar. Pienaar *et al.* (1994) therefore postulated that it would be possible to use the OD of cultures of egg washings after 6 h of incubation to establish the bacterial contamination on hatching eggs. The use of such a system has a number of advantages over conventional bacterial-plate-count methods. In order to obtain reliable plate counts, serial dilutions of the egg-washing fluid must be made. As the number of contaminating bacteria on the egg is unknown, a number of different dilutions must be plated out to obtain countable colonies. The preparation of the dilutions as well as the agar plates is costly and time consuming. The physical counting of the colonies on the agar plates after incubation is a time-consuming activity, greatly reducing the numbers of eggs which can be tested. The use of OD after 6 h of incubation is cost effective as no agar plates are required, and this method is also less time-consuming, as no dilutions or counting of bacterial colonies is required.

From the graphs of the OD measurements after 6 h of incubation *v* the log of the bacterial counts, it has been established that there is a linear relationship between the OD measurements (at 540 nm) of a culture of NB in which hatching eggs have been washed and incubated at 37 C for 6 h and the bacterial counts of these eggs (cf. Fig. 1A–E). A linear relationship between the OD reading and bacterial concentration at the start of incubation was established previously when pure cultures of bacteria isolated from hatching eggs were used (Pienaar *et al.* 1994). The linear relationship between the OD readings and bacterial counts obtained directly from a representative population of hatching eggs clearly establishes the use of OD measurements (at 540 nm) of nutrient broth in which hatching eggs have been washed and incubated at 37 C for 6 h, as a reliable method to determine the bacterial contamination of these eggs.

TABLE 1 Mean OD reading (at 540 nm) and log of the mean bacterial counts (obtained from plate-count methods) from eggs collected from five different flocks. The standard deviation (SD) is in brackets. "n" equals the numbers of eggs from each flock, assigned to the different groupings

Flocks	Group 1*	Group 2**	Group 3***	Group 4#	Group 5##	Group 6###
1 (n = 39) n/group Mean OD Mean Count		3 0,026 (0,015) 3,717 (3,433)	24 0,038 (0,021) 4,640 (4,375)	9 0,070 (0,020) 5,501 (5,366)	3 0,125 (0,062) 6,360 (5,605)	
2 (n = 29) n/group Mean OD Mean Count				12 0,046 (0,017) 5,676 (5,241)	11 0,136 (0,023) 6,567 (6,320)	6 0,228 (0,037) 7,650 (7,212)
3 (n = 23) n/group Mean OD Mean Count			12 0,032 (0,013) 4,701 (4.292)	11 0,065 (0,039) 5,279 (5,236)		
4 (n = 38) n/group Mean OD Mean Count				12 0,044 (0,025) 5,594 (5,094)	20 0,185 (0,110) 6,588 (6,395)	6 0,524 (0,089) 7,393 (6,717)
5 (n = 25) n/group Mean OD Mean Count		3 0,005 (0,001) 3,166 (3,386)	13 0,029 (0,011) 4,658 (4,354)	3 0,095 (0,000) 5,920 (0,000)	4 0,156 (0,048) 6,520 (6,395)	2 0,262 (0,085) 7,340 (7,164)

* Less than 10^3 bacteria/egg; ** between 10^3 and 10^4; *** between 10^4 and 10^5
# Between $10^5$and 10^6; ## between $10^6$and 10^7 and ### more then 10^7 bacteria/egg

TABLE 2 Calculated bacterial-count ranges for each of the five flocks from which hatching eggs were collected. Bacterial ranges were read from the graphs in Fig. 2A–E

Flock	OD				
	0,04	0,06	0,08	0,10	0,12
1	4,343–4,895	4,750–5,458	5,204–5,854	5,666–6,146	6,140–6,385
2		5,615–5,964	5,750–6,152	5,893–6,357	6,036–6,607
3	4,590–5,000	4,850–5,370			
4			5,625–6,000	5,750–6,170	5,833–6,333
5	4,143–4,857	4,750–5,286	5,411–5,714	6,321–7,107	5,929–6,289
Mean	4,359–4,917	4,991–5,515	5,498–5,930	5,863–6,445	5,985–6,404

Flock	OD				
	0,14	0,16	0,18	0,20	0,22
1					
2	6,214–6,803	6,505–7,000	6,805–7,232	7,071–7,446	7,339–7,720
3					
4	5,916–6,479	6,042–6,667	6,125–6,813	6,580–7,040	6,250–6,958
5	6,010–6,536	6,100–6,786	6,143–6,964	6,321–7,107	6,500–7,250
Mean	6,047–6,606	6,216–6,818	6,358–7,003	6,657–7,198	6,696–7,309

It can further be seen from these results that both the bacterial counts and the OD measurements obtained for the different flocks, remained more or less constant throughout the collection period. This is particularly noticable in flocks 2 and 3 where only three and two groups of eggs, respectively, were obtained from the bacterial-count data. It can also be seen from the data (cf. Table 1) that the bacterial contamination of some of the flocks (flocks 2 and 4) were higher than that of other flocks.

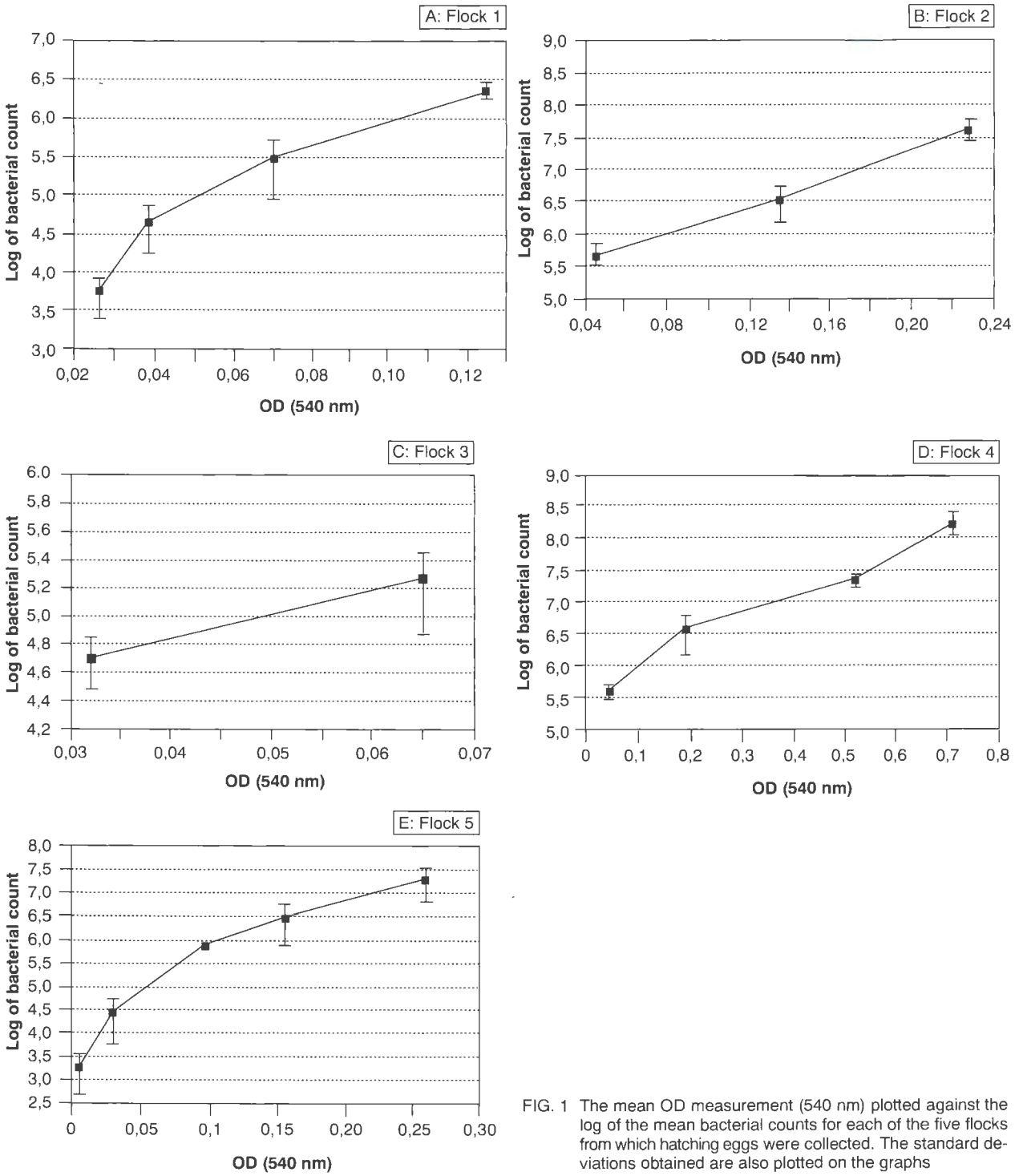

FIG. 1 The mean OD measurement (540 nm) plotted against the log of the mean bacterial counts for each of the five flocks from which hatching eggs were collected. The standard deviations obtained are also plotted on the graphs

A range of bacterial counts for different OD measurements can be calculated when the log of the mean bacterial count, plus or minus one SD, is plotted against the mean OD measurements. This was done for each of the five flocks (cf. Fig. 2A–E). From these graphs the bacterial ranges for OD, such as 0,04, 0,06, 0,08, 0,10, etc. could be calculated. In flocks where the same OD measurements were obtained, mean bacterial ranges were calculated and plotted against these OD measurements. A correlation was found (cf. Fig. 3A and 3B), thus indicating that the relationship between the OD after 6 h of incubation and the number of contaminating bacteria on the shells of hatching eggs is constant across all five flocks tested.

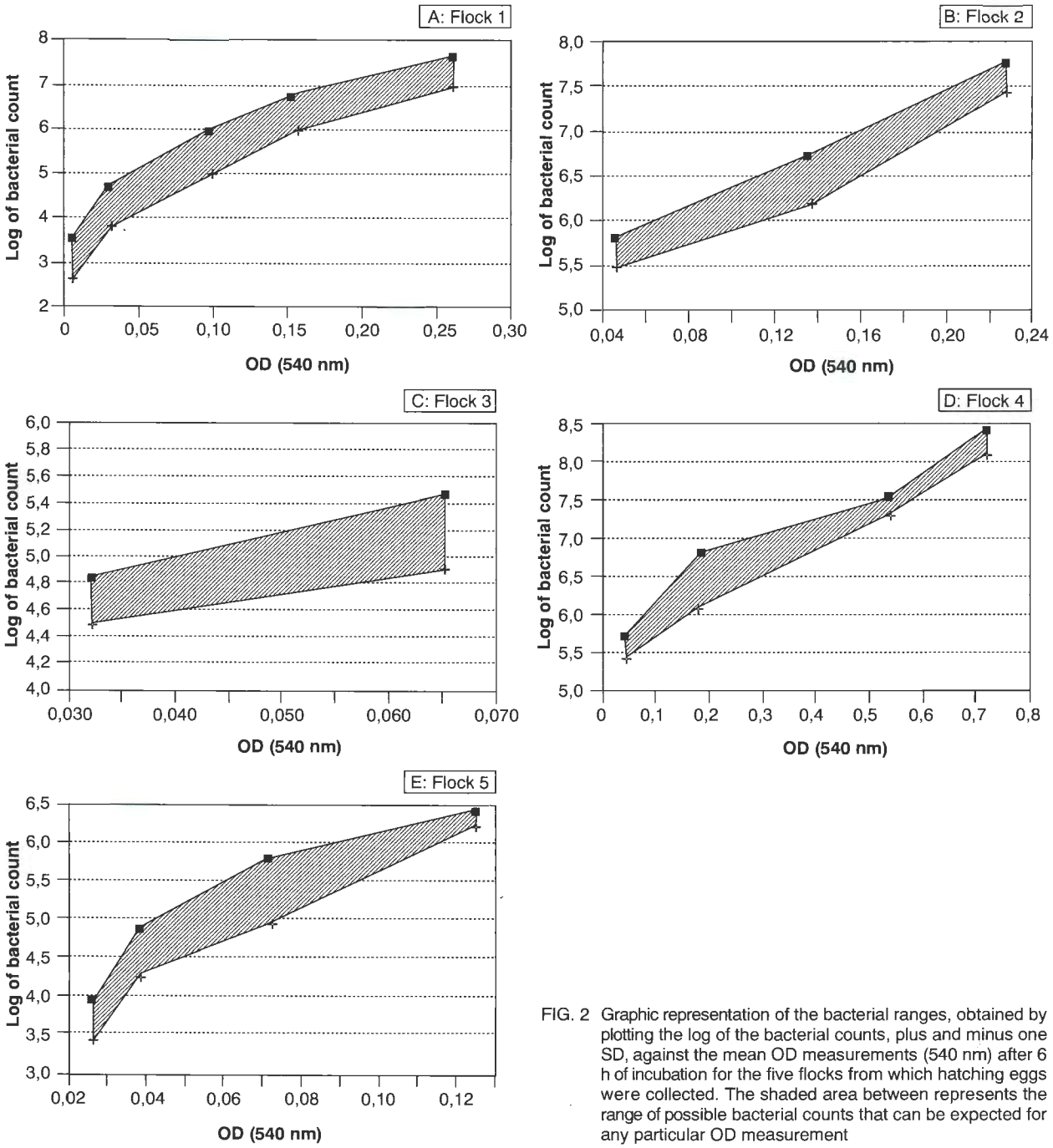

FIG. 2 Graphic representation of the bacterial ranges, obtained by plotting the log of the bacterial counts, plus and minus one SD, against the mean OD measurements (540 nm) after 6 h of incubation for the five flocks from which hatching eggs were collected. The shaded area between represents the range of possible bacterial counts that can be expected for any particular OD measurement

The final stage entailed a comparison between a visual evaluation of the eggs, bacterial counts and OD measurements after 6 h of incubation, as methods for the determination of bacterial contamination. A high degree of similarity was found between the plate counts and the OD reading after 6 h. Both these systems differed from the visual evaluation of the groups. A close correlation between the bacterial counts and the OD measurements was obtained in all five flocks (cf. Table 4). In flock 1, 24 eggs were assigned to group 3 by plate counts while 19 of these eggs were assigned to this group by OD measurements. Only 14 of the eggs were assigned to this group by visual examination. The largest group of eggs (19) was assigned to group 1 by visual examination (cf. Table 4). In flock 2 all the eggs (n = 29) were assigned to either group 4, 5 or 6 by plate count. Only six eggs in group 3 were not assigned to this grouping by OD measurements. On the other hand, only eight eggs were assigned to this range by visual examination (cf. Table

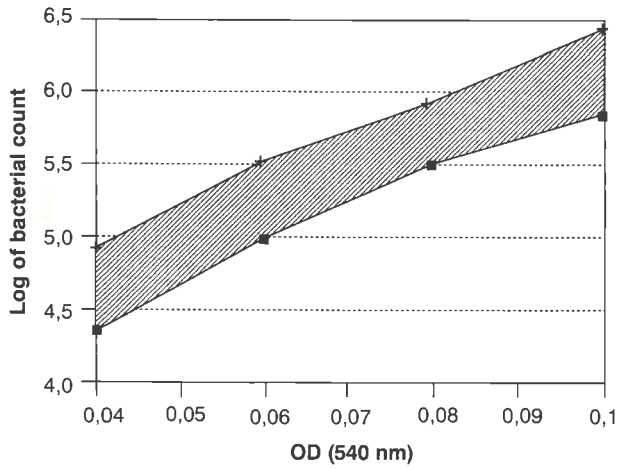

FIG. 3A Graphic representation of the log of the mean bacterial ranges (as calculated from Fig. 2A–E) for all five flocks *v* OD measurements (540 nm) below 0,1. The shaded area represents the bacterial-count range which would be acceptable for a particular OD measurement (at 450 nm)

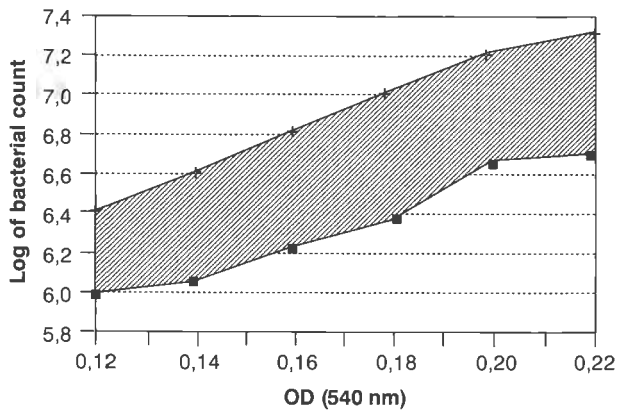

FIG. 3B Graphic representation of the log of the mean bacterial ranges (as calculated from Fig. 2A–E) for all five flocks *v* OD measurements (540 nm) above 0,1. The shaded area represents the bacterial-count range which would be acceptable for a particular OD measurement (at 450 nm)

TABLE 3 Grouping criteria used for placing hatching eggs into various categories for visual examination, bacterial counts and OD measurements, at 540 nm

Group no.	Counts	OD	Visual
1	$< 10^3$		Clean
2	10^3–10^4	up to 0,006	Very small amount of dirt
3	10^4–10^5	0,007–0,044	Small amount of dirt
4	10^5–10^6	0,045–0,092	Some dirt
5	10^6–10^7	0,093–0,170	Dirty
6	$> 10^7$	> 0,171	Very dirty

TABLE 4 The numbers of hatching eggs placed into various categories according to the grading system and criteria set out in Table 3

Flock		Groups					
		1	2	3	4	5	6
1	Count	0	3	24	9	3	0
	OD	0	2	19	13	4	1
	visual	19	2	14	4	0	0
2	Count	0	0	0	12	11	6
	OD	0	0	6	7	9	7
	visual	6	5	10	0	8	0
3	Count	0	0	12	11	0	0
	OD	0	0	13	5	4	1
	visual	20	1	3	0	0	0
4	Count	0	0	0	12	20	6
	OD	0	1	10	3	8	16
	visual	10	2	9	0	10	7
5	Count	0	3	13	3	4	2
	OD	0	3	10	2	3	7
	visual	11	3	5	0	0	6

4). In flock 3 all the eggs (n = 23) were assigned to groups 3 and 4 by plate count. Only five eggs were not assigned to this range by OD measurements, while only three eggs were assigned to this range by visual examination. Similar results were obtained for flock 4 with all the eggs being assigned to the group range 4–6 by plate count (cf. Table 4). In this flock 11 eggs (ten in group 3) were not assigned to the range. Visual examination assigned 21 eggs outside the range obtained by plate count, with ten of these in group 1. A wide group range was obtained with flock 5, (group 2–6). The largest concentration of eggs was assigned to group 3 by plate count (13) while ten eggs were assigned to this group by OD measurements. Only five eggs were assigned to this group by visual examination (cf. Table 4).

It can be concluded that there is a linear correlation between the OD measurement (at 540 nm) of a NB culture in which hatching eggs have been washed and incubated for 6 h at 37 °C, and the bacterial counts made from these eggs. It can also be concluded that the use of OD to determine bacterial contamination is repeatable and reliable, as very similar results were obtained for each flock over the collection period.

From this data it is clear that the amount of bacterial contamination varies from flock to flock, and that flocks with high bacterial contamination of the hatching eggs can easily be detected and management steps initiated to reduce the bacterial contamination.

Finally, the main advantages of using OD measurements, after 6 h of incubation, to determine bacterial

contamination of hatching eggs, are a great reduction in cost and a very large reduction in the time taken to perform this test, thus allowing for the evaluation of more hatching eggs. The use of OD measurements to determine bacterial contamination of hatching eggs will be a useful tool in the management of breeding flocks, when identifying and controlling flocks with serious bacterial contamination problems. The use of this method can also reduce the bacterial load in the setters and hatchers, resulting in better quality day-old chicks.

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