A rapid method to determine bacterial contamination on hatching eggs. 3. Use of commercial DNA probe kits for detection of specific pathogens after six hours of incubation

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ABSTRACT

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The usefulness of commercially available DNA probe kits for the detection of *Escherichia coli* and *Salmonella* spp. after only 6 h of incubation, was determined.

It was established that the commercially available probe kits used could detect *E. coli* at initial levels of approximately $4,5 \times 10^2$ colony-forming units (cfu) per mℓ after only 6 h of incubation in nutrient broth (NB). Initial bacterial levels as low as $4,5 \times 10^{-1}$ cfu/mℓ could be detected when the NB was incubated for 18 h. *Salmonella* Enteritidis, at initial levels of $2,86 \times 10^2$ cfu/mℓ could be detected after 6 h of incubation at 37 °C in NB, while initial levels as low as $2,86 \times 10^{-1}$ cfu/mℓ could be detected after 18 h at 37 °C in both NB and selected media, as specified by the manufacturers of the probe kits.

Commercially available DNA probe kits can therefore be used to detect specific pathogens on the surface of hatching eggs and these probes can be used in conjunction with an egg-washing system, which is used to determine total bacterial contamination, although a longer incubation period greatly improves the sensitivity of these tests.

Keywords: Bacterial contamination, hatching eggs, *Escherichia coli, Salmonella,* DNA probe kits

INTRODUCTION

The detection of specific pathogens, especially *Salmonella* spp., on the surface of hatching eggs, has become extremely important in the light of the current problem with *Salmonella* Enteritidis in poultry flocks. It is important to the producer of day-old chicks to ensure that this pathogen is not introduced into customers' flocks via the hatchery.

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Mellor & Banwart (1965) evaluated various methods for the recovery of *Salmonella* Derby from the surface of egg shells. These authors stated that the interference of organic matter caused no problems in the isolation of *Salmonella* spp. from the surfaces of egg shells, but that interfering organisms, especially low numbers of *Salmonella* organisms, could pose a problem. They used a soak method in which eggs were left in jars containing broth media for 1 h, after which the eggs were removed and the broth incubated at 37 °C. They found that the best recovery was obtained when

cultures were soaked in lactose broth for 16 h, and then in selenite F-cystine for 8 h of enrichment, and streak plated onto brilliant green sulpha agar. Similar results were also obtained by Board, Ayres, Kraft & Forsythe (1963) who used the same medium, followed by stab inoculation of dulcitol lysine iron agar slants and suspension of colonies in polyvalent 'O' antiserum for identification.

Berrang, Cox, Bailey & Blackenship (1991) washed broken-out egg shells and membranes in buffered peptone water in a plastic bag for 30 s, after which a swab was taken from the bag and plated onto brilliant green sulpha with naladixic acid.

The use of the commercial DNA probe kits for the detection of *Salmonella* spp. and *Escherichia coli* on egg shells should provide a rapid, sensitive method for the detection of these potential pathogens. The use of probes eliminates the need for a pure culture—thus saving time. Detection is done at the genetic level, which enhances the specificity of the method. The occurence of false positive results due to cross-reactions is therefore minimal, and false negative results are reduced, owing to the high specificity of the method. Sensitivity is enhanced by the fact that hybridization occurs in solution.

The objective of this experiment was to determine the sensitivity of commercially available probe kits on pure cultures of bacteria incubated at 37 °C for 6 h. Pienaar, Coetzee & Bragg (1994; 1995), established that reading the optical density (OD) of a culture of nutrient broth in which hatching eggs had been washed, after 6 h of incubation, was a suitable method for determining bacterial contamination levels on these hatching eggs. It would be most convenient if commercially available probe kits could be used to detect specific pathogens in the culture fluid after only 6 h of incubation.

MATERIALS AND METHODS

The Genetrak DNA probe-kit system (Gene-Trak Systems, Massachusetts, USA) was used to evaluate the sensitivity of DNA probes for use in the established system of a 6-h incubation period in nutrient broth (NB).

To determine the lowest number of bacteria which could be detected after a 6-h incubation period, pure cultures of *Salmonella* Enteritidis and *E. coli* were inoculated into NB and incubated at 37 °C for 18 h. After incubation, tenfold serial dilutions of the different bacteria were made (to 10^{-10}). Plate counts were performed by the inoculation of 0,1 mℓ of the different dilutions onto three Plate Count (PC) agar plates (Oxoid) per dilution. The inoculum was spread over the plate with a sterile bent glass rod and the plates incubated at 37 °C for 18 h, after which colony counts were performed.

The sensitivity of the commercially available probe kits was established by inoculating 1 mℓ of each of the diluted bacterial suspensions into separate tubes containg 9 mℓ NB and incubating them for 6 h at 37 °C. After incubation, the procedures for the detection of the different pathogens by the use of commercially available probe kits were performed according to the manufacturer's protocol, for each of the dilutions, and the optical density (at 450 nm) was read on a Milton Roy spectrophotometer after completion of the protocol. Each bacterial isolate was tested four times.

In order to evaluate the sensitivities of the commercially available probe kits for incubation periods of longer than 6 h, two different approaches were followed. Firstly, the tubes containing NB that had been inoculated with the different dilutions of bacteria, and incubated for 6 h, were returned to the incubator after samples had been collected for testing according to the probe protocol. These tubes were incubated for a total of 18 h, after which time they were re-evaluated with the probes. Each bacterial isolate was tested four times.

The second approach was to inoculate the various selective media (recommended by the manufacturers of the probe kits) after the 6-h incubation period and to follow the guidelines in the probe-kit protocols. In the case of *E. coli*, 1 mℓ for each of the 6-h cultures was inoculated into 9-mℓ lauryl sulfate broth (LSB) (Merck) and incubated for 18 h at 35 °C. After this incubation, the procedures for the detection of *E. coli* by the probes was carried out according to the manufacturer's protocol. In the case of *Salmonella* Enteritidis, 9 mℓ of tetrathionate broth base (TT) (Merck) and 9 mℓ of selenite cystine F broth (SC) (Merck) were inoculated with 1 mℓ each, from each of the NB tubes containing the different dilutions of *Salmonella* Enteritidis, after the 6-h incubation period. The TT and SC cultures were incubated for 18 h at 37 °C after which 1 mℓ from each culture was used to inoculate 9 mℓ of GN enrichment broth (Merck). These cultures were incubated for 6 h, after which the procedures for the detection of *Salmonella* spp. by the probes were carried out according to the manufacturer's protocols.

RESULTS

Countable colonies of *Salmonella* Enteritidis were obtained on the plates of dilutions 10^{-6} and 10^{-7} in all four repetitions. The average colony count was $1{,}02 \times 10^9$ cfu/mℓ, with the highest count being $1{,}17 \times 10^9$ cfu/mℓ and the lowest count, $8{,}18 \times 10^8$ cfu/mℓ. Countable colonies of *E. coli* were also obtained in dilutions 10^{-6} and 10^{-7} for all four repetitions, with an average colony count of $4{,}5 \times 10^8$ cfu/mℓ. The highest count was $6{,}47 \times 10^8$ cfu/mℓ, while the lowest count was $2{,}68 \times 10^8$ cfu/mℓ.

After 6 h of incubation, the lowest dilution of *E. coli* which yielded positive results on the probes, was a 10^{-5} dilution in four out of four repetitions. The number

of cfu/mℓ in the tubes inoculated with 1 mℓ of the 10^{-5} dilutions of *E. coli* before incubation, can be calculated from the mean bacterial count data. If the mean bacterial count of the four 18-h cultures of *E. coli* was $4,5 \times 10^8$ cfu/mℓ, then a tube inoculated with 1 mℓ of the 10^{-5} dilution into 9 mℓ of medium would have approximately $4,5 \times 10^2$ cfu/mℓ. Thus the *E. coli* probes are capable of detecting *E. coli* in a sample after only 6 h of incubation, when there are at least approximately $4,5 \times 10^2$ cfu/mℓ in the sample before incubation (cf. Table 1).

In the case of *Salmonella* Enteritidis the lowest dilution which yielded positive probe results (after 6 h of incubation at 37 °C in NB) was 10^{-6} in three of the four repetitions, with one repetition showing a positive result at a 10^{-5} dilution. From the mean bacterial-count data for the 18-h cultures and the dilution factors used to inoculate the tubes, it can be calculated that the *Salmonella* probes can detect *Salmonella* in a sample after only 6 h of incubation, when there are at least approximately $1,02 \times 10^2$ cfu/mℓ in the sample before incubation (cf. Table 2).

When *E. coli* was incubated for longer than 6 h, in both NB and LSB, positive probe results were obtained in the 10^{-8} dilution in all cases. The estimated number of bacteria in the 10^{-8} dilutions which were used to inoculate the cultures, could also be calculated from the mean plate-count data of the 18-h-old cultures. In the case of *E. coli*, the mean plate count before dilution was $4,5 \times 10^8$ cfu/mℓ. If 1 mℓ of a 10^{-8} dilution of these cultures was used to inoculate 9 mℓ of medium, the initial bacterial counts in the culture, before incubation, would be approximately $4,5 \times 10^{-1}$ cfu/ mℓ in both the NB and the LSB cultures (cf. Table 2).

In most of the tubes—both the NB and the GN tubes—that were inoculated with various dilutions of *Salmonella* Enteritidis, the lowest dilution which showed positive results was the 10^{-9} dilution. In one of the repetitions, the 10^{-8} dilution was found to be the lowest. The lowest mean bacterial count, resulting in a positive probe result in all four repetitions, was calculated according to methods used for *E. coli*, and was $2,68 \times 10^{-1}$ cfu/mℓ (cf. Table 2).

DISCUSSION

Pienaar *et al.* (1994; 1995) established that taking the OD readings of a culture of nutrient broth in which hatching eggs had been washed and which had been incubated for 6 h at 37 °C, is a suitable method for determining the level of bacterial contamination on these eggs. With the isolation of *Salmonella* Enteritidis in South Africa, it has become very important to detect the presence of this bacterium on the shells of hatching eggs. There are numerous selective procedures to enhance the detection of *Salmonella* spp. and this bacterium can be detected on the shells of hatching eggs by conventional bacteriology.

However, with the advent of commercially available, non-radioactive probes, the detection of *Salmonella* spp. and, indeed, other pathogens has become more efficient. One of the main advantages of using probes to detect specific pathogens is that the probe will detect the specific genetic material (in this case ribosomal RNA) of the target organism amongst a host of other contaminating genetic materials.

The suppliers of the probe kits have included various selective media and procedures in their protocols to enhance the detection of the particular pathogen.

In this experiment, we determined that the probes were sufficiently sensitive to detect specific pathogens if present in numbers as low as $4,5 \times 10^2$ cfu/mℓ for *E. coli*, and $2,86 \times 10^2$ cfu/mℓ for *Salmonella* Enteritidis, after only 6 h of incubation at 37 °C. The main objective of this work was to investigate the feasibility of using commercially available probes to detect specific pathogens within the incubation parameters set by Pienaar *et al.* (1994; 1995) for the determination of bacterial contamination on hatching eggs by the reading of OD values.

From a practical point of view, the sensitivity of the probes could be further increased by using the NB culture, after 6 h of incubation, and after a sample had been taken to determine the OD readings, to inoculate specific selective media, or for further incubation. The selective media could then be incubated overnight and the detection procedure, with the use of the probes, could be carried out the following morning. When this was done, the lowest mean number of *E. coli* that could be detected was $4,5 \times 10^{-1}$ cfu/mℓ, while the lowest mean number of *Salmonella* Enteritidis was $2,86 \times 10^{-1}$ cfu/mℓ.

When pure cultures of *E. coli* or *Salmonella* Enteritidis were used, no difference in sensitivity could be detected between the NB cultures that had been incubated for a further 18 h at 37 °C, and the samples that had been removed after 6 h for inoculation into specific selective media. In fact, when the OD readings were examined, higher OD readings had been obtained from the NB tubes after 18 h than from the various selective media. In mixed cultures, however, such as would be found on hatching eggs, the use of selective media may enhance the detection of low levels of the specific pathogens.

From this data, it can be concluded that the commercially available probes are sufficiently sensitive to detect low levels of specific pathogens which may occur on the surface of hatching eggs in NB samples which have been incubated for 6 h in order to determine the levels of bacterial contamination on the surface of the eggs (Pienaar *et al.* 1995). It might be more practical, however— particularly if large numbers of eggs are to be tested according to the methods of Pienaar *et al.* (1995)—to use the NB culture, after having taken the

TABLE 1 Results of probes performed after 6 h of incubation at 37 °C and after 18 h of incubation at 37 °C, of cultures made from the different dilutions of *Escherichia coli*

No.	Media	Time (h)	Dilution	OD (450 nm) (probes)	Probe result	Calculated bacterial counts (cfu/mℓ)
1	NB*	6	−4 −5 −6	1,800 1,436 0,044	Pos. Pos. Neg.	$6,47 \times 10^{3}$ $6,47 \times 10^{2}$ $6,47 \times 10^{1}$
2	NB	6	−4 −5 −6	1,577 1,095 0,072	Pos. Pos. Neg.	$3,7 \times 10^{3}$ $3,7 \times 10^{2}$ $6,47 \times 10^{1}$
3	NB	6	−4 −5 −6	1,218 1,457 0,020	Pos. Pos. Neg.	$5,17 \times 10^{3}$ $5,17 \times 10^{2}$ $5,17 \times 10^{1}$
4	NB	6	−4 −5 −6	1,266 0,646 0,066	Pos. Pos. Neg.	$2,68 \times 10^{3}$ $2,68 \times 10^{2}$ $6,68 \times 10^{1}$
Mean count of lowest dilutions showing positive probe results = $4,5 \times 10^{2}$ cfu/mℓ						
1	NB	18	−8 −9 −10	2,256 0,076 0,034	Pos. Neg. Neg.	$6,47 \times 10^{-1}$ $6,47 \times 10^{-2}$ $6,47 \times 10^{-3}$
2	NB	18	−8 −9 −10	2,034 0,088 0,042	Pos. Neg. Neg.	$3,7 \times 10^{-1}$ $3,7 \times 10^{-2}$ $3,7 \times 10^{-3}$
3	NB	18	−8 −9 −10	2,275 0,045 0,017	Pos. Neg. Neg.	$5,17 \times 10^{-1}$ $5,17 \times 10^{-2}$ $5,17 \times 10^{-3}$
4	NB	18	−8 −9 −10	2,415 0,075 0,021	Pos. Neg. Neg.	$2,68 \times 10^{-1}$ $2,68 \times 10^{-2}$ $2,68 \times 10^{-3}$
Mean count of lowest dilutions showing positive probe results = $4,5 \times 10^{-01}$ cfu/mℓ						
1	LSB**	18	−8 −9 −10	1,124 0,076 0,044	Pos. Neg. Neg.	$6,47 \times 10^{-1}$ $6,47 \times 10^{-2}$ $6,47 \times 10^{-3}$
2	LSB	18	−8 −9 −10	0,762 0,077 0,055	Pos. Neg. Neg.	$3,7 \times 10^{-1}$ $3,7 \times 10^{-2}$ $3,7 \times 10^{-3}$
3	LSB	18	−8 −9 −10	0,737 0,075 0,021	Pos. Neg. Neg.	$5,17 \times 10^{-1}$ $5,17 \times 10^{-2}$ $5,17 \times 10^{-3}$
4	NB	18	−8 −9 −10	0,972 0,061 0,038	Pos. Neg. Neg.	$2,68 \times 10^{-1}$ $2,68 \times 10^{-2}$ $2,68 \times 10^{-3}$
Mean count of lowest dilutions showing positive probe results = $4,5 \times 10^{-01}$ cfu/mℓ						

* Nutrient broth
** Lauryl sulfate broth

TABLE 2 Results of probes performed after 6 h of incubation at 37 °C and after 18 h incubation at 37 °C, of cultures made from the different dilutions of *Salmonella* Enteritidis

No.	Media	Time (h)	Dilution	OD (450 nm) (probes)	Probe result	Calculated bacterial counts (cfu/mℓ)
1	NB*	6	−4 −5 −6	2,507 1,436 0,044	Pos. Pos. Neg.	$8{,}18 \times 10^{3}$ $8{,}18 \times 10^{2}$ $8{,}18 \times 10^{1}$
2	NB	6	−4 −5 −6 −7	2,577 1,095 0,672 0,065	Pos. Pos. Pos. Neg.	$1{,}08 \times 10^{4}$ $1{,}08 \times 10^{3}$ $1{,}08 \times 10^{2}$ $1{,}08 \times 10^{1}$
3	NB	6	−4 −5 −6 −7	2,853 1,940 0,160 0,034	Pos. Pos. Pos. Neg.	$1{,}02 \times 10^{4}$ $1{,}02 \times 10^{3}$ $1{,}02 \times 10^{2}$ $1{,}02 \times 10^{1}$
4	NB	6	−4 −5 −6 −7	2,863 2,073 0,195 0,088	Pos. Pos. Pos. Neg.	$1{,}17 \times 10^{4}$ $1{,}17 \times 10^{3}$ $1{,}17 \times 10^{2}$ $1{,}17 \times 10^{1}$
Mean count of lowest dilutions showing positive probe results = $2{,}68 \times 10^{2}$ cfu/mℓ						
1	NB	18	−8 −9 −10	2,746 0,613 0,026	Pos. Pos. Neg.	$8{,}18 \times 10^{-1}$ $8{,}18 \times 10^{-2}$ $8{,}18 \times 10^{-3}$
2	NB	18	−8 −9 −10	2,034 0,088 0,042	Pos. Neg. Neg.	$1{,}08 \times 10^{0}$ $1{,}08 \times 10^{-1}$ $1{,}08 \times 10^{-2}$
3	NB	18	−8 −9 −10	2,822 0,213 0,017	Pos. Pos. Neg.	$1{,}02 \times 10^{0}$ $1{,}02 \times 10^{-1}$ $1{,}02 \times 10^{-2}$
4	NB	18	−8 −9 −10	2,840 2,837 0,095	Pos. Pos. Neg.	$1{,}17 \times 10^{0}$ $1{,}17 \times 10^{-1}$ $1{,}17 \times 10^{-2}$
Mean count of lowest dilutions showing positive probe results = $2{,}68 \times 10^{-1}$ cfu/mℓ						
1	GN**	18	−8 −9 −10	1,356 0,135 0,007	Pos. Pos. Neg.	$8{,}18 \times 10^{-1}$ $8{,}18 \times 10^{-2}$ $8{,}18 \times 10^{-3}$
2	GN	18	−8 −9 −10	1,939 1,230 0,043	Pos. Pos. Neg.	$1{,}08 \times 10^{0}$ $1{,}08 \times 10^{-1}$ $1{,}08 \times 10^{-2}$
3	GN	18	−8 −9 −10	2,818 1,085 0,005	Pos. Pos. Neg.	$1{,}02 \times 10^{0}$ $1{,}02 \times 10^{-1}$ $1{,}02 \times 10^{-2}$
4	GN	18	−8 −9 −10	2,690 0,221 0,015	Pos. Pos. Neg.	$1{,}17 \times 10^{0}$ $1{,}17 \times 10^{-1}$ $1{,}17 \times 10^{-2}$
Mean count of lowest dilutions showing positive probe results = $2{,}68 \times 10^{-1}$ cfu/mℓ						

* Nutrient broth
** GN enrichment broth

OD reading, for further overnight incubation. This would also increase the sensitivity of the probes for the detection of specific pathogens. It must be noted, though, that the commercially available probe kits for the detection of *Salmonella* are not specific for *Salmonella* Enteritidis, but will detect any *Salmonella* spp. which are present on the egg shell.

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