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CULTURE MEDIA FOR OPTIMAL ISOLATION OF *MORITELLA VISCOSA* FROM ATLANTIC SALMON (*SALMO SALAR*) WITH WINTER ULCER

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Contents

Introduction

Skin ulceration is one of the most frequently recorded disease conditions in farmed fish and can be produced by microbiological as well as by mechanical means. A disease called winter ulcers, which affects salmonid fish reared at cold temperatures, is characterized by shallow skin lesions and often by diffuse or petechial hemorrhaging in internal organs. 'Winter ulcers' have been observed in Norway, Iceland, Scotland and Canada (Lunder, 1992; Lunder *et al.*, 1995; Benediktsdóttir *et al.*, 1991; Benediktsdóttir *et al.*, 1998; Bruno *et al.*, 1998; Laidler *et al.*,1999; Whitman *et al.*, 2000).

Two species of Gram-negative bacteria, *Moritella viscosa* and *Vibrio wodanis,* are most often isolated from the ulcers or the internal organs, either one of them or a mixed culture of both species (Benediktsdóttir *et al*, 2000; Lunder et.al, 2000). Two strains of *M.viscosa* have been tested for pathogenicity in Norway and Iceland respectively and both were shown to be pathogenic for Atlantic salmon parr, whereas strains of *V. wodanis* that have been tested for pathogenicity have not been shown to be virulent to Atlantic salmon (Lunder *et al.*, 1995; Benediktsdóttir *et al.*, 1998).

Blood agar plates are most often used for isolation of *M. viscosa* and samples from kidneys and ulcers from diseased fish are streaked out on blood agar plates supplemented with 2% NaCl and incubated at 15°C for 2-3 days.

V. wodanis has been isolated from outbreaks where no other suspect or known pathogenic or opportunistic microbe was found. *M. viscosa*, which is easily overgrown and sensitive in transport, might have been present but not detected (Benediktsdóttir *et al.*, 1998). No research has been done on the effect of blood in the media or in developing selective media for *M. viscosa.*

Material and Methods

Bacterial strains

M. viscosa: NVI 88/478^T was kindly supplied by E. Myhr from the National Veterinary Institute in Oslo Norway, AL266 and AL 267 kindly supplied by E. Greger ALPharma, Bellevue, WA, USA, all isolated from diseased fish in Norway. MT 1887 was kindly supplied by D.W. Bruno, FRS Marine Laboratory, Aberdeen, UK, isolated from diseased salmon. K58 and K56 were isolated from diseased salmon in Iceland, and F57 isolated from a healthy lumpsucker.

V. wodanis NVI 88/441^T was kindly supplied by E. Myhr at the National Veterinary Institute in Oslo Norway , isolated from diseased salmon in Norway and K31 isolated from diseased salmon in Iceland.

Shewanella sp. strain PA-43 was kindly supplied by Haflidi M. Gudmundsson at the Institute of Biology, University of Iceland.

The reference strains *Photobacterium phosphoreum* NCIMB 1282^T and *Vibrio logei* NCIMB 2252^T were obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, UK. *Shewanella hanedai* DSM 6066^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Media

Antibacterial agents

Resistance against 24 different antibacterial agents was tested with the disc diffusion method (Table 1). Young cultures of type strains of *M. viscosa* and *V. wodanis* were cultured in Tryptic Soy Broth (Difco) supplemented with 1.5% NaCl for 4 hours. Tryptic Soy Agar (Difco) plates, supplemented with 1.5% NaCl , were floated with 1.0 ml of the culture and the rest pipetted off. The plates were dried in a Laminar flow Hood before the discs were put on them. All the discs were from Oxoid. The plates were incubated at 15 °C for 24 hours and the sensitivity estimated by measuring the inhibition zone.

Table 1. Antibacterial agents tested with disc diffusion method.

Penicillins:	**Quinolones:**
• Methicillin	• Ciprofloxacin
• Carbenicillin	• Nalidixic Acid
• Imibenem	**Other agents:**
• Ampicillin	• Vancomycin
• Mecillinam	• Clindamycin
Cephalosporins:	• Novobiocin
• Cefuroxime	• Bacitracin
• Cefotaxime	• Polymyxin
• Ceftazidime	• Sulfamethoxazole
Aminoglycoside	• Nitrofurantoin
• Gentamicin	• Chloramphenicol
• Tobramycin	• Tetracycline
Macrolides	• O129
• Erythromycin	
• Azithromycin	

Minimal inhibitory concentration (MIC), in Marine Broth (Difco), was established for 4 compounds. MIC for Chloramphenicol (Sigma), Tobramycin (BUFA) and Erythromycin (BUFA) were established for *M. viscosa* NVI 88/478^T and *V. wodanis* NVI 88/441^T and for the Vibriostat O129 (2,4-Diamino-6,7-diisopropylpteridine Phosphate Salt, Sigma product No. D-0781) for NVI 88/478^T, K58, MT 1887, AL 266, AL 267 and F57 of *M. viscosa*, *V. wodanis* NVI 88/441^T, *P. phosphoreum* NCIMB 1282^T, *S. hanedai* DSM 6066^T, *Vibrio logei* NCIMB 2252^{T}and *Shewanella* sp. PA-43.

Different pH

Tryptic Soy Agar (TSA), supplemented with 1.5% NaCl and 0.05 M Tris-HCl was used and pH was adjusted by using 1.0 M HCl and 1.0 M NaOH. The pH values tested were 6.0, 6.5, 7.3, 8.0, 8.5, 9.0 and 9.5. The pH value 7.3 was used as control for the effect of Tris. Young cultures were streaked out on the media and incubated at 15 °C for 4 days. Growth was estimated by measuring the diameter of single colonies each day. The growth of NVI 88/478^T, K58, MT 1887, AL 266, AL 267 and F57 of *M. viscosa*, *V. wodanis* NVI 88/441^T, *P. phosphoreum* NCIMB 1282^T, *S. hanedai*

DSM 6066^T, *Vibrio logei* NCIMB 2252^{T}and *Shewanella* sp. PA-43 were tested at pH 6.5, 7.3 and 8.0.

NVI 88/478^{T}and K58 of *M. viscosa*, NVI 88/441^{T}and K31 of *V. wodanis* were tested at pH 8.5, 9.0 and 9.5, and NVI 88/478^{T}and K58 of *M. viscosa*, *V. wodanis* NVI 88/441^T, *P. phosphoreum* NCIMB 1282^T, *S. hanedai* DSM 6066^T, *Vibrio logei* NCIMB 2252^T and *Shewanella* sp. PA-43 at pH 6.0.

Enhancers

Tryptic Soy Broth supplemented with 1.2% Agar and 1.5% NaCl was used as a base for testing media supplemented with 5% horse blood or media with 0.1% charcoal. TSA + 1.5% NaCl was used as a base for testing growth on media supplemented with the following agents: 0.5% NAG, 0.5% Ribose, 0.5% glycerol, 1.0% Tween 80. TSA + minerals (1.9% NaCl, 0.7% $MgSO_4$ x 7 H_2O and 0.075% KCl) was used as a base for testing media containing 0.017% $FeSO_4$ x 7 H_2O, 0.001% $CaCl_2$, 1.0% casein acidic hydrolysate or 1.0% casein enzymatic hydrolysate. Young cultures were streaked out on the media and incubated at 15 °C for 4 days. Growth was estimated by measuring the diameter of single colonies each day. The species and strains used in this part of the study were NVI 88/478^T, K58, MT 1887, Al 266, AL 267 and F57 of *M. viscosa*, *V. wodanis* NVI 88/441^T, *P. phosphoreum* NCIMB 1282^T, *S. hanedai* DSM 6066^T, *Vibrio logei* NCIMB 2252^T and *Shewanella* sp. PA-43.

Co-cultivation

In order to see if *V. wodanis* inhibits the growth of *M. viscosa* 24 hour liquid cultures of *M.* viscosa NVI 88/478^T and *V. wodanis* NVI 88/441^T were mixed in various proportions and inoculated on blood agar media. As a control the cultures were also diluted in sterile broth. The results gave reason for further testing and the culture of *V.wodanis* was centrifuged and the cells dissolved in sterile broth before mixing with *M. viscosa* culture. *M. viscosa* culture was also diluted with spent media from *V. wodanis*, with the control as in the former test. A viable count was carried out for both cultures.

Finally different mixing of *M. viscosa* and *V. wodanis* cultures were streaked on media containing different concentration of O129 that was added to the media

prior to autoclaving. In all of these testing a standard 10 µl loop was used to streak with.

Comparison of media

Four different media were tested on samples taken from diseased fish. TSA, supplemented with minerals (1.9% NaCl, 0.7% $MgSO_4$ x 7 H_2O, 0.075% KCl, 0.017% $FeSO_4$ x 7 H_2O and 0.001% $CaCl_2$) was used as a base for all the media. Media I consisted of the base medium (TSA*), media II was the base medium supplemented with 0.5µg/ml O129 (TSA*+O129), media III was the base medium supplemented with 5% bovine or horse blood and 0.5µg/ml O129(TSA*+B+O129), and finally media IV was the base medium supplemented with 5% blood (TSA*+B).
The samples were randomly streaked on these four media and the plates were then incubated at 15 °C and examined every other day for one week. For identification 120 representative strains were isolated and biochemical tests and whole cell SDS-PAGE electrophoresis followed by Comassie staining were performed.

Fish

Samples were taken at four different fish farms off the west coast of Norway during outbreaks of winter ulcer. All together the kidney and ulcers of 107 Atlantic salmon (*Salmo salar*) with skin ulcers were sampled . The kidney samples were taken under aseptic conditions with a sterile loop and streaked on the media. Ulcer samples were taken at the edge of the ulcer with a sterile loop and streaked on the media. A new sample was taken for each medium. The fish was caught, blooded and sampled. All fish had been vaccinated prior to smoltification, with different vaccines.
Location A: Samples were taken from 23 Atlantic salmon. The fish smolted in May 2000 and weighed about 1 kg.
Location B: Samples were taken from 28 Atlantic salmon. The fish smolted in December 2000 and weighed about 100 g. The salmon were heavily infected and the death rate was about 40 fish/day. Antibiotic treatment, with oxolinic acid, had been going on for 3 days.
Location C: Samples were taken from 27 Atlantic salmon that smolted in October 2000 and weighed about 100 – 150g.

Location D: Samples were taken from 30 Atlantic salmon that smolted in October 2000 and weighed about 200- 250g.

Biochemical tests

All tests performed were according to Benediktsdóttir *et al* (1998). Incubation was at 15 °C except when testing for growth at different temperatures. Oxidative and fermentative degradation of glucose, gas production and motility were tested and read every day for one week. Sensitivity to the vibriostatic agent O129 was tested with the disc diffusion method, using 10 and 150 µg discs (Oxoid) on MB agar (Marine Broth 2216 from Difco supplemented with 1.2% Agar No.1 from Oxoid). The salt requirement was tested using Nutrient broth (Difco), with 0%, and 3% NaCl. Luminescence and growth at 4 °C and 26 °C were tested on MB agar. As soon as growth was visible on the plates, luminescence was observed after five minutes in the dark, but growth at different temperatures and with different salt concentrations was examined after one week's incubation time.

Tests for the production of amino acid decarboxylase and dihydrolase tests and production of indole from tryptophane were performed and examined after one week's incubation time at 15 °C.

SDS-PAGE

For further identification SDS-PAGE electrophoresis according to Laemmli (1970) was performed on 21 strains assumed to be *M. viscosa* and 26 strains assumed to be *V. wodanis* according to biochemical tests. In addition 15 strains of unknown species were tested to see if they were *M. viscosa* or *V. wodanis*. The samples were run in 1.5mm 4% acrylamide stacking gel, pH 6.8, and a 12% acrylamide separating gel, pH 8.8, at 35 mA/gel using Biorad Protean II xi electrophoresis equipment. After fixation in 40% methanol and 10% acetic acid the gels were stained with Comassie blue, destained and evaluated. Samples of *M. viscosa* NVI 88/478^{T} and *V. wodanis* NVI 88/441^{T} were run on each gel as a standard.

Statistics

When examining the plates the growth was rated in 5 groups: 0 = no growth, 1 = 1-10 colony forming units (CFU), 2 = 10-50 CFU, 3 = 50-100 CFU and 4 = more than 100 CFU, for *M. viscosa*, *V. wodanis*, and other bacteria and fungus.

The results were tested with the chi-square test, both for negative-positive and also for grouped results. All the media were compared in pairs and also all together.

Results

Development of the media

Antibacterial agents

M. viscosa and *V. wodanis* showed similar sensitivity for all agents tested (Table. 1) except Tobramycin, Erythromycin, Chloramphenicol and O129 when the disc diffusion method was used.

MIC was the same for *M. viscosa* and *V. wodanis* for Chloramphenicol (1.17 µg/ml) and Erythromycin (2.34 µg/ml) but slightly higher for Tobramycin for *M. viscosa* (9.38 µg/ml) than for *V. wodanis* (4.69 µg/ml). The MIC for O129 was 2.34 µg/ml for all the strains of *M. viscosa* tested except F57 where it was slightly higher or 9.38 µg/ml. MIC for O129 for *V. wodanis* NVI 88/441^T, *P. phosphoreum* NCIMB 1282^T was 0.29 µg/ml, for *Vibrio logei* NCIMB 2252^T it was 0.15 µg/ml, and 4.69 µg/ml for *S. hanedai* DSM 6066^T and *Shewanella* sp. PA-43.

Different pH

M. viscosa grew at all the pH tested but at a pH below 6.5 or above 8.0 growth was suppressed. No difference was seen between different strains of *M. viscosa*. The same applied to *V. wodanis*. Slight suppression could be seen on the growth of *S. hanedai* DSM 6066^T and *Shewanella* sp. PA-43 at pH 6.5.

Enhancers

No significant difference was seen in the growth of *M. viscosa* or other species when testing several enhancers. The growth of *M. viscosa* was slightly better when the

media contained minerals (1.9% NaCl, 0.7% $MgSO_4$ x 7 H_2O, 0.075% KCl, 0.017% $FeSO_4$ x 7 H_2O, and 0.001% $CaCl_2$), and also when it contained blood.

Co-cultivation of *M. viscosa* and *V. wodanis*

When cultures of *M. viscosa* and *V. wodanis* were mixed in even proportions and inoculated on blood agar plates only *V. wodanis* was observed (**Fig. 1,** plate 1). The spent media from the *V. wodanis* culture did not affect the growth of *M. viscosa* (**Fig. 1,** plate 2).

Adding O129 to the media inhibited the growth of *V. wodanis* even though there was 10 times more *V. wodanis* than *M. viscosa*. It was found that 0.25µg/ml of O129 was enough to inhibit the growth of *V. wodanis* if the media also contained blood (**Fig. 2,** plates 2, 3 and 4). When TSA* without blood was used mixed culture of *M. viscosa* and *V. wodanis* was seen on plates containing 0.25 and 0.5 µg/ml of O129 but only *M. viscosa* was seen on a plate containing 1.0µg/ml O129 (**Fig. 3,** Plate 2) .

Plate 1. Inoculated with *M. viscosa* $2x10^3$ and *V. wodanis* $2x10^3$

Plate 2. Inoculated with *M. viscosa* $2x10^3$ in 10% spent media from *V. wodanis*

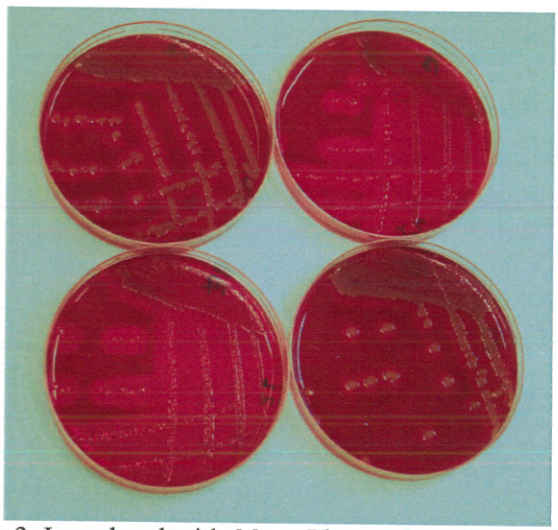

Plate 3. Inoculated with *M. viscosa* $2x10^3$

Plate 4. Inoculated with *V. wodanis* $2x10^3$

Fig.1
Growth of *M. viscosa* and *V. wodanis* on blood agar plates. On plate 1 only *V. wodanis* was observed, whereas on plate 2 only *M. viscosa* was observed.

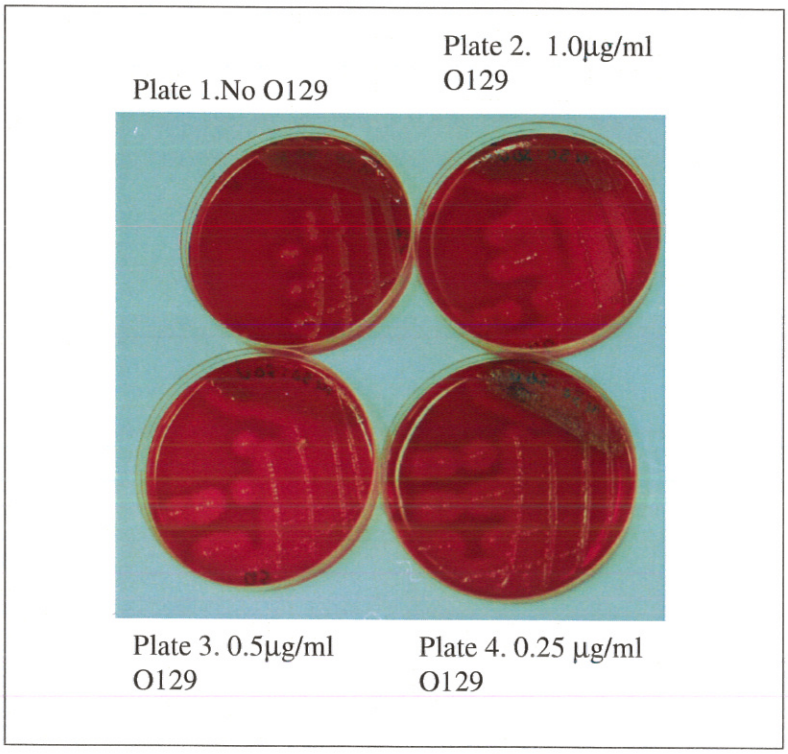

Fig. 2

Plates were inoculated with *M. viscosa* 1 x 10^3 and *V. wodanis* 1 x 10^4 on TSA*+B containing various concentrations of O129. On plate 1 only *V. wodanis* was observed, whereas on plates 2, 3 and 4 only *M. viscosa* was observed.

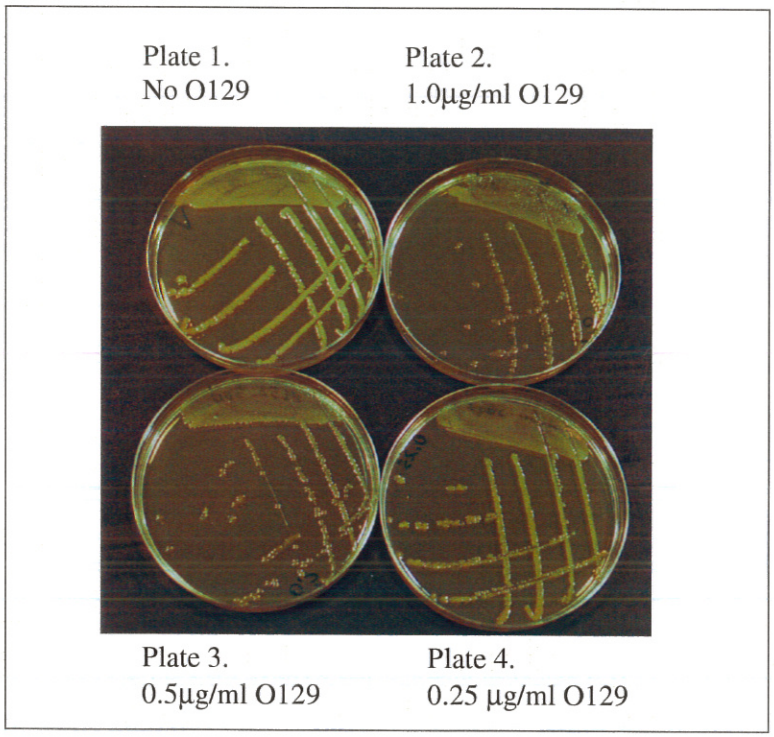

Fig.3

Plates were inoculated with *M. viscosa* 1 x 10^3 and *V. wodanis* 1 x 10^4 on TSA* containing various concentrations of O129. On plate 1 only *V. wodanis* was observed, whereas on plate 2 only *M. viscosa* was observed.

Both species grew on plate 3 and 4 but better suppression of *V. wodanis* was observed on plate 3.

Comparison of media

Grouping of the growth did not change the results as to whether they were positive or negative; therefore only results from the latter are presented. Further results from pair comparisons between the media, as well as for each location, are presented in the Appendix.

Isolation of M. viscosa

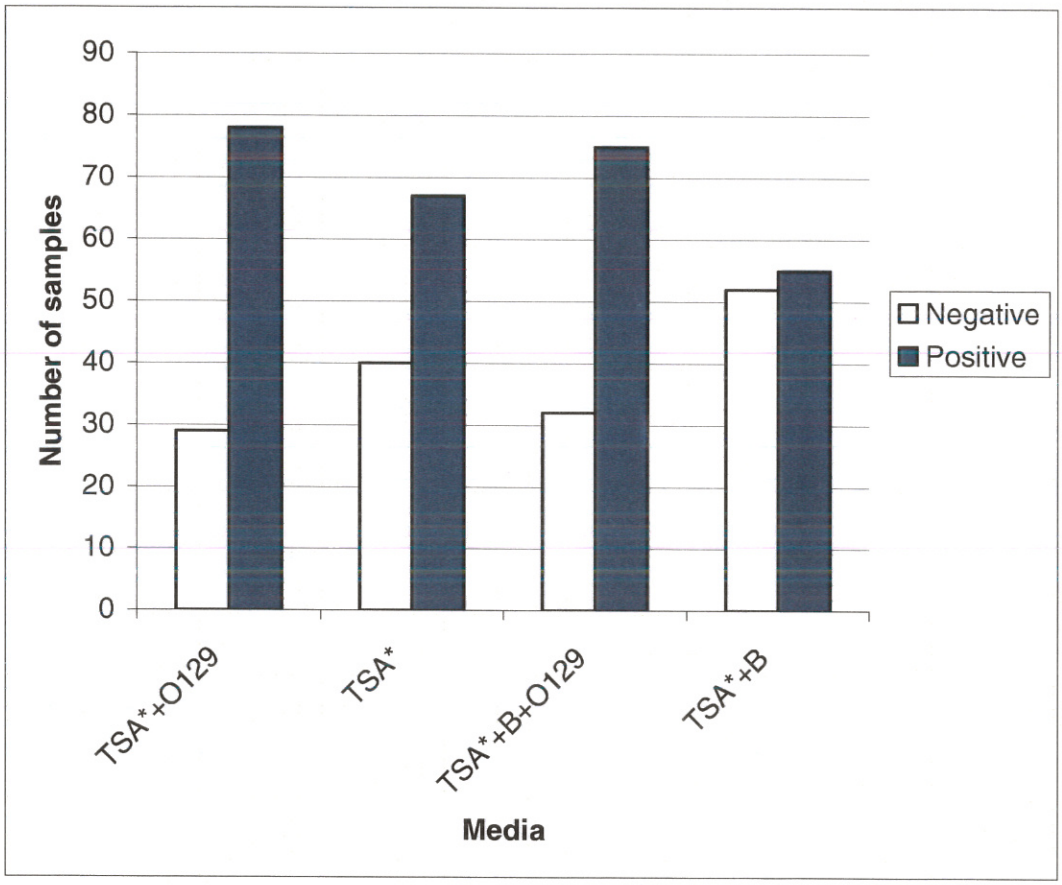

Fig. 4. Growth of *M.viscosa*. All locations. Kidney samples. See also table 1 in Appendix.

In isolating *M. viscosa* from kidney samples, media TSA*+O129 and TSA*+B+O129 were significantly better than TSA*+B (**P=0.0049).

M. viscosa was isolated on 73% of the plates of TSA*+O129 and 70% for TSA*+B+O129 from kidney samples. *M. viscosa* was 42% more often isolated on TSA*+O129 than on TSA*+B and 36% more often on TSA*+B+O129 than on TSA*+B from kidney samples.

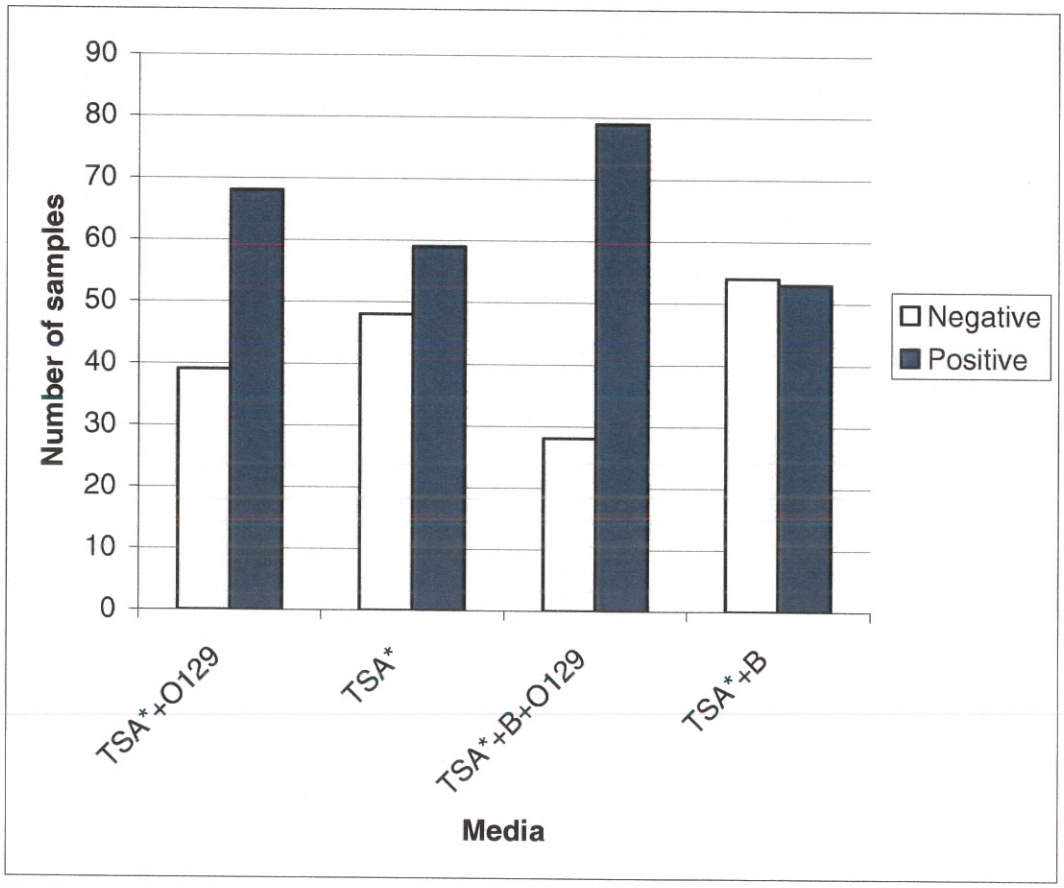

Fig. 5. Growth of *M.viscosa*. All locations. Ulcer samples. See also table 2 in Appendix.

In isolating *M. viscosa* from ulcer samples, media TSA*+O129 and TSA*+B+O129 were significantly better than TSA*+B and TSA* (**P=0.0018). *M. viscosa* was isolated on 64% of the plates of TSA*+O129 and 74% for TSA*+B+O129 from ulcer samples. *M. viscosa* was 49% more often isolated on TSA*+B+O129 than on TSA*+B from ulcer samples.

Inhibition of V. wodanis

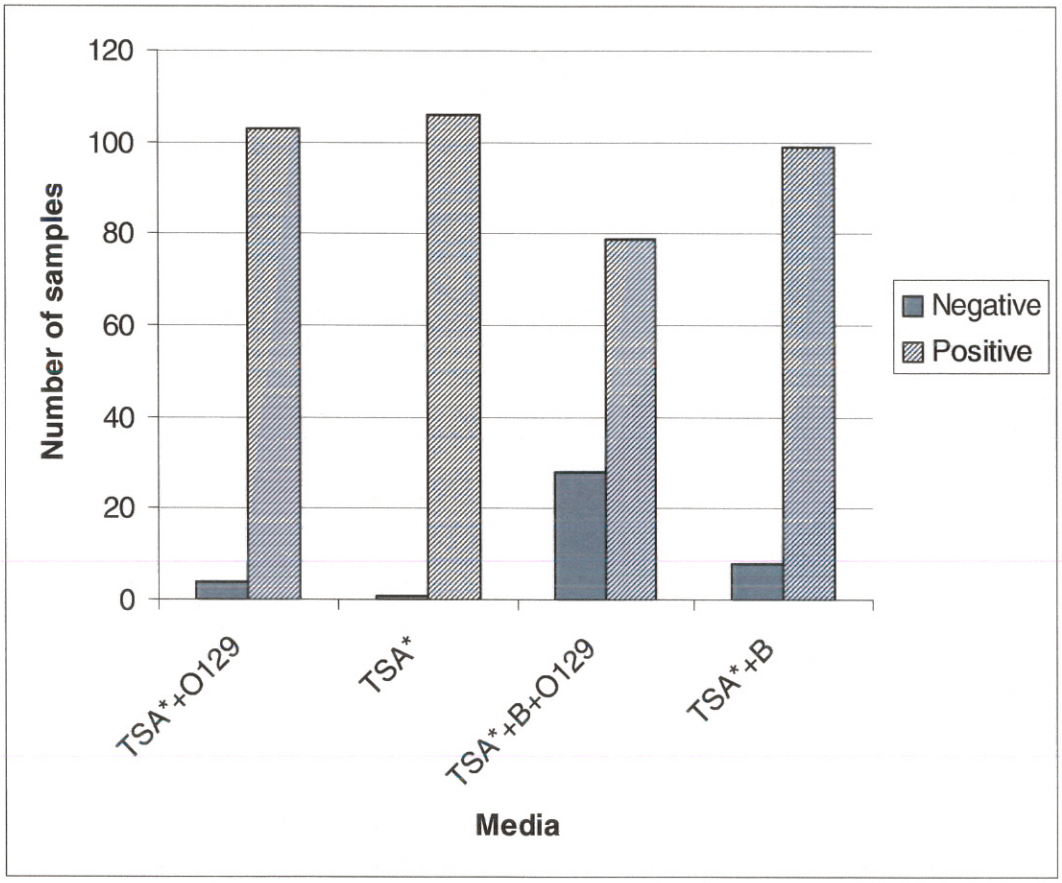

Fig. 6. Growth of *V. wodanis*. All locations. Kidney samples. See also table 11 in Appendix.

In inhibiting *V. wodanis* there was a significant difference between the media for kidney samples, in favor of the media TSA*+B+O129 (***** $P = 8{,}9*10^{-10}$).
V. wodanis was isolated on 74% of the TSA*+B+O129 plates but on over 90% of the plates for all the other media from kidney samples.

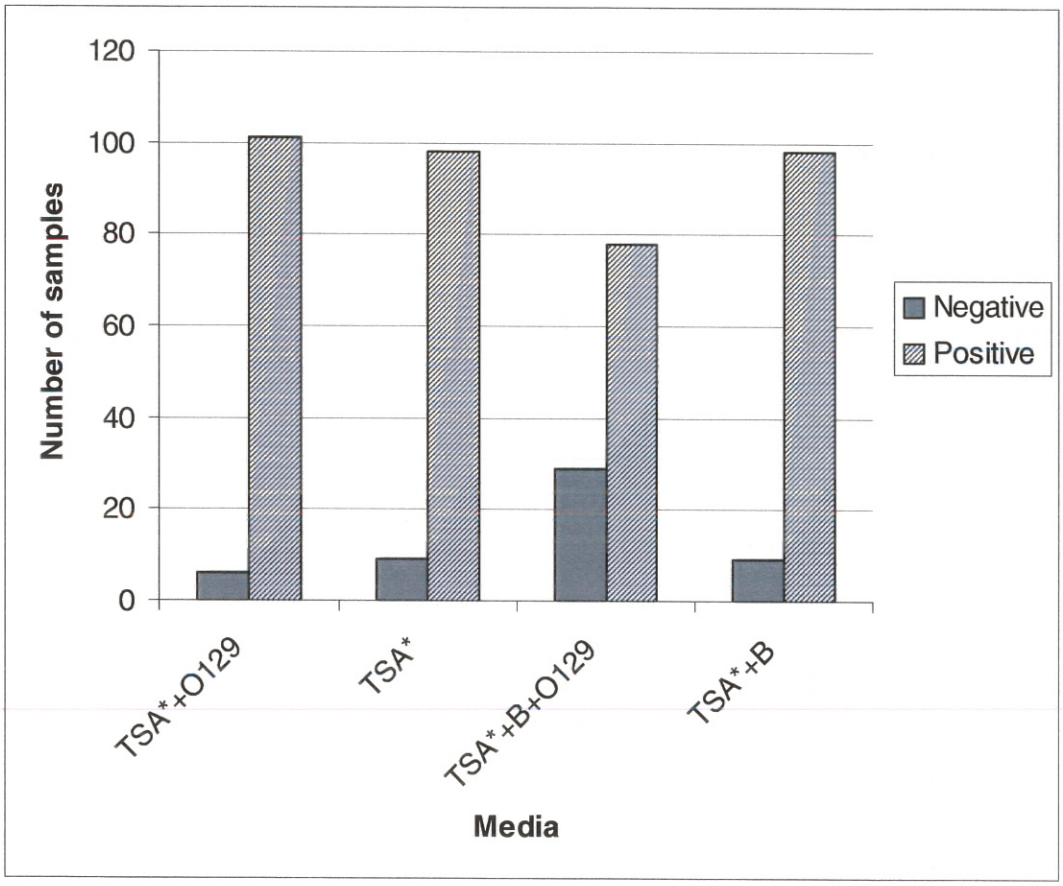

Fig. 7. Growth of *V. wodanis*. All locations, ulcer samples. See also table 12 in Appendix.

In inhibiting *V. wodanis* from ulcer samples, a significant difference was seen in comparison of all the media (***** P=2.2*10-6), in favor of TSA*+B+O129. *V. wodanis* was isolated on 74% of the TSA*+B+O129 plates but on over 90% of the plates for all the other media from ulcer samples.

Other isolates

In 89% of the incidence for kidney samples and 77% of the incidence for ulcer samples, other isolates were fungi or 1-10 colonies of bacteria (Tables 21 and 22 in Appendix).

Discussion

In this report we have shown that *V. wodanis* inhibits the growth of *M. viscosa* on blood agar when the inoculate is composed of an even mixture of both species. The effect is caused by the growing cells and not by extracellular products present in spent media. This indicates that the absence of *M. viscosa* on culture plates when fish with winter ulcers are sampled and *V. wodanis* is present is probably caused by this inhibition and gives further support to the suppotition that *M. viscosa* is the primary pathogen in winter ulcers and *V. wodanis* is an opportunistic pathogen or saphrophytic.
The results also show that for effective detection of *M. viscosa* in fish samples, it is important to inhibit growth of *V. wodanis*. However, as growth of *V. wodanis* can be considered a strong indicatior of winter ulcers, use of agar media both with and without O129 would be recommended.

From the experiments with laboratory strains the conclusion was drawn, that 0.5 µg/ml of O129 in the media was enough to inhibit the growth of *V. wodanis* if blood is also included. The results of sampling experiments from fish farms showed that *V.wodanis* was only partly suppressed by this concentration, as it did grow on 74% of the plates of that media but on over 90% of the plates with other media tested. However O129 enhanced the isolation efficiency of *M. viscosa* on TSA*+B+O129 by 36% from kidney samples and 49% from ulcer samples, compared with TSA*+B. Probably better results would be achieved if media with higher concentration, 1 µg/ml, were used.
Including blood in the media enhanced the inhibition effect of O129 on *V. wodanis*, but it is not nesseccary for the growth of *M. viscosa* on first isolation from fish, as no difference in isolation efficiency of *M. viscosa* was seen between TSA* with and without blood. However, because most strains of *M. viscosa* are hemolytic, the use of blood would be recommended for easier detection of colonies.

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Appendix

Contents

Comparison of Media
Isolation of *M. viscosa*
All locations, kidney samples

Table 1. Growth of *M. viscosa*. All locations. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	29	78	107
TSA*	40	67	107
TSA*+B+O129	32	75	107
TSA*+B	52	55	107
Total	153	275	428

There was a significant difference between media TSA*+B+O129 and TSA*+B (**P=0.0051) and also between TSA*+O129 and TSA*+B (***P= 0.001) in favor of the media containing O129.

All locations, ulcer samples

Table 2. Growth of *M. viscosa*. All locations. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	39	68	107
TSA*	48	59	107
TSA*+B+O129	28	79	107
TSA*+B	54	53	107
Total	169	259	428

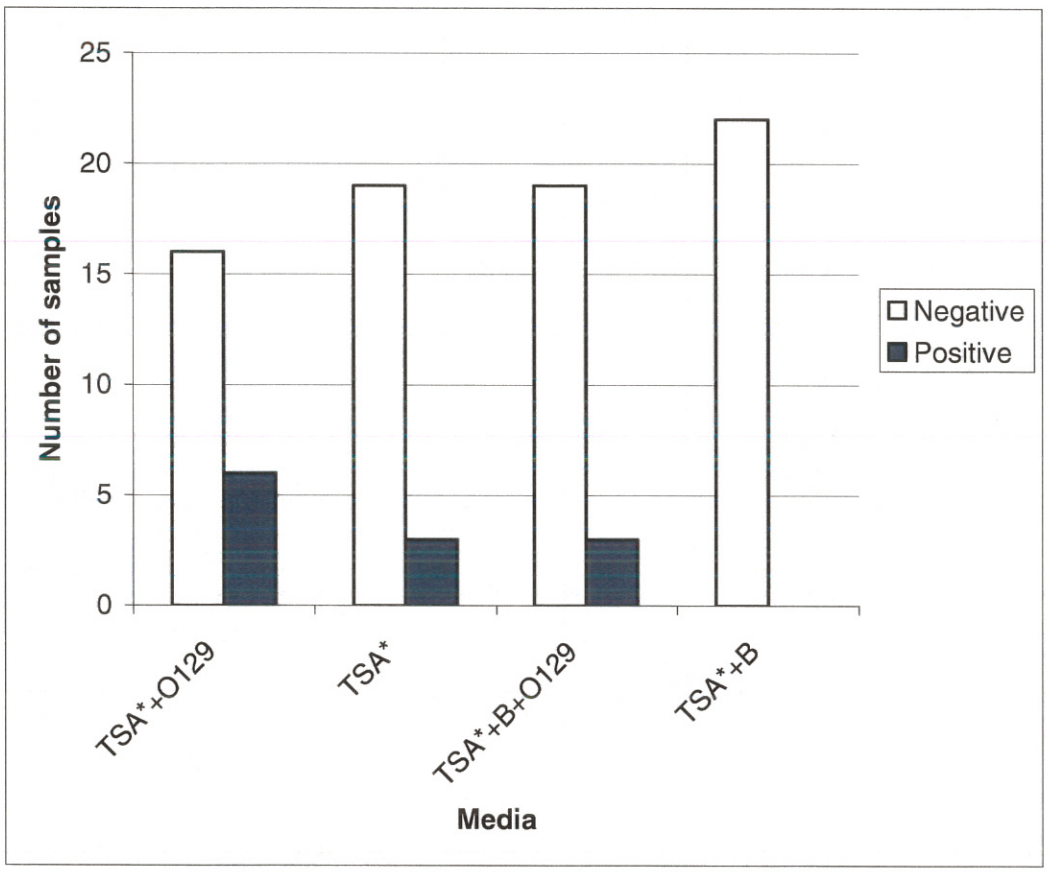
There was a significant difference between TSA*+B+O129 and TSA*+B (***P=$3.0*10^{-4}$) and between TSA* and TSA*+B+O129 (**P=0.004), both in favor of TSA*+B+O129 isolating *M. viscosa*. There was also a significant difference between the media TSA*+O129 and TSA*+B (*P=0.039) in favor of TSA*+O129.

Location A. Kidney samples

Table 3. Isolation of *M. viscosa*. Location A. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	16	6	22
TSA*	19	3	22
TSA*+B+O129	19	3	22
TSA*+B	22	0	22
Total	76	12	88

Fig. 1. Isolation of *M.viscosa*. Location A. Kidney samples.



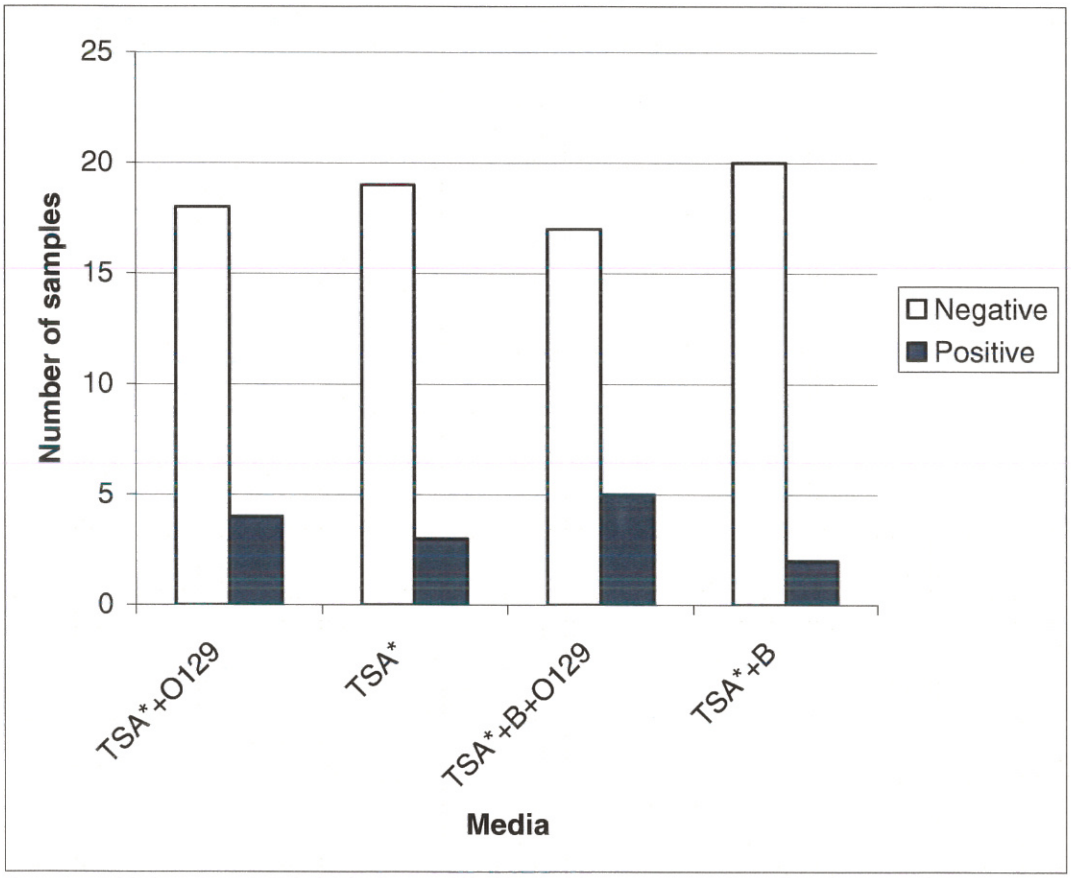
A significant difference was seen between media TSA*+O129 and TSA*+B (**P=0.008) in favor of the former in isolating *M. viscosa*.

Location A. Ulcer samples

Table 4. Isolation of *M. viscosa*. Location A. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	18	4	22
TSA*	19	3	22
TSA*+B+O129	17	5	22
TSA*+B	20	2	22
Total	74	14	88

Fig. 2. Isolation of *M.viscosa*. Location A. Ulcer samples.



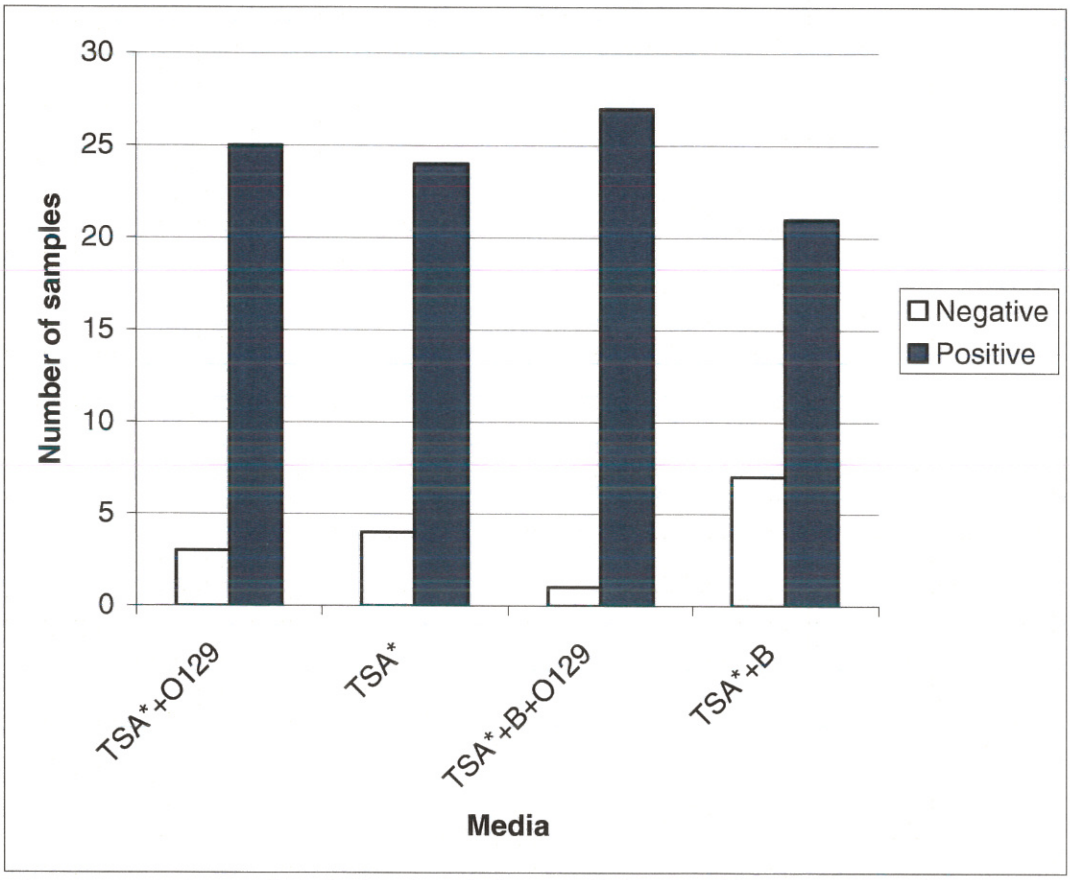
There was no significant difference between the media in isolating *M. viscosa*.

Location B. Kidney samples

Table 5. Isolation of *M. viscosa*. Location B. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	3	25	28
TSA*	4	24	28
TSA*+B+O129	1	27	28
TSA*+B	7	21	28
Total	15	97	112

Fig. 3. Isolation of *M.viscosa*. Location B. Kidney samples.



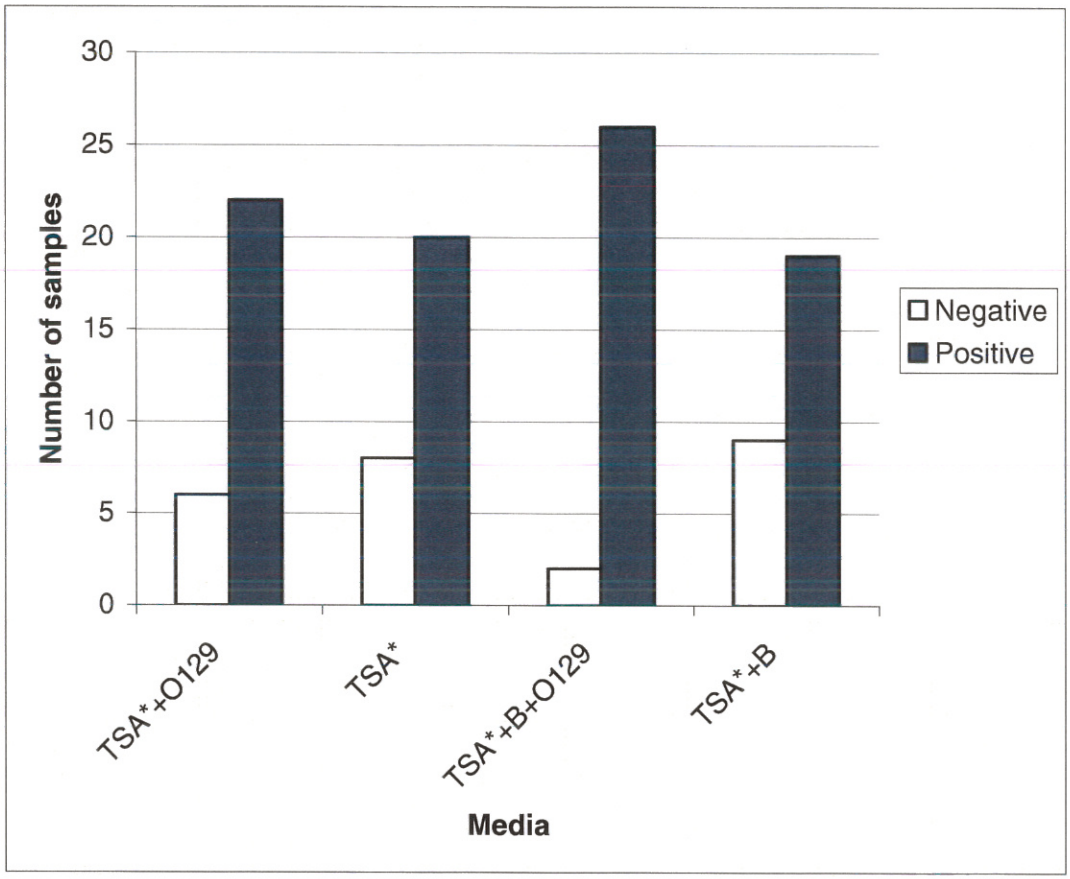
There was a significant difference between media TSA*+B+O129 and TSA*+B (*P=0.022) in favor of the former in isolating *M. viscosa*.

Location B. Ulcer samples

Table 6. Isolation of *M. viscosa*. Location B. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	6	22	28
TSA*	8	20	28
TSA*+B+O129	2	26	28
TSA*+B	9	19	28
Total	25	87	112

Fig. 4. Isolation of *M.viscosa*. Location B. Ulcer samples.



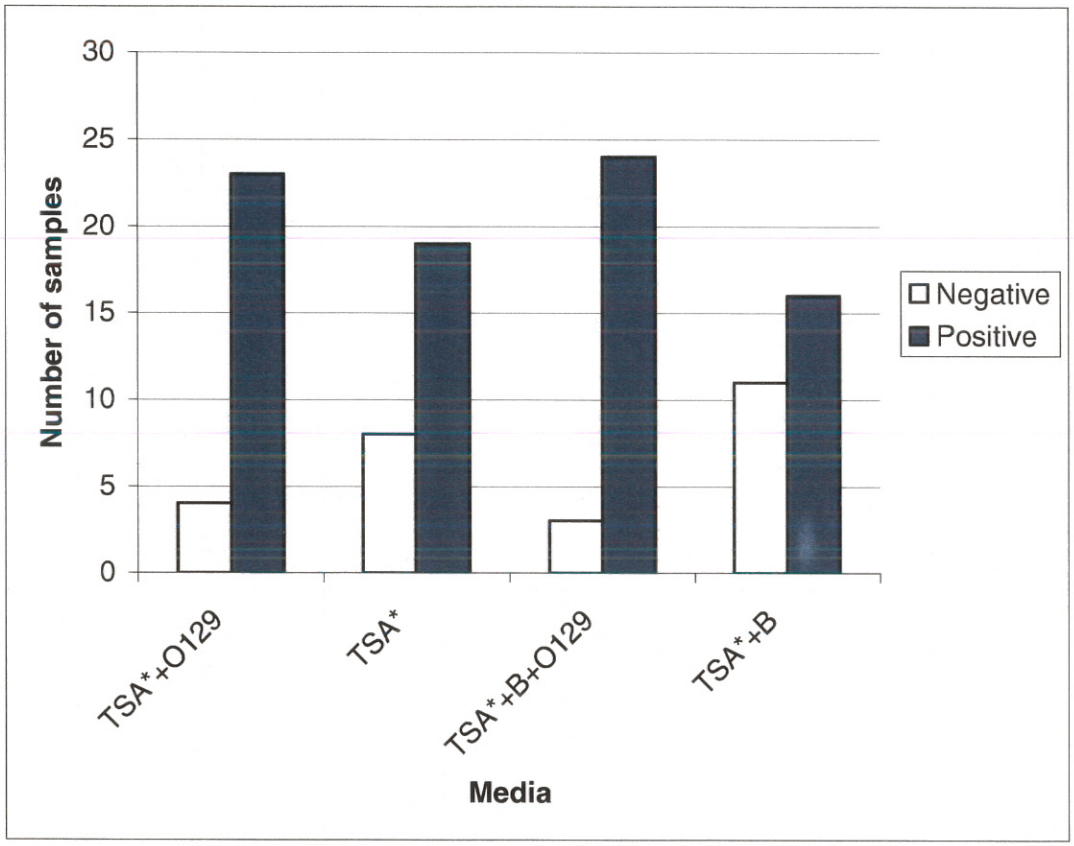
There was significant difference between media TSA*+B+O129 and TSA* (*P=0.036) in isolating *M. viscosa*, and also between TSA*+B+O129 and TSA*+B (*P=0.02) both in favor for TSA*+B+O129.

Location C. Kidney samples

Table 7. Isolation of *M. viscosa*. Location C. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	4	23	27
TSA*	8	19	27
TSA*+B+O129	3	24	27
TSA*+B	11	16	27
Total	26	82	108

Fig. 5. Isolation of *M.viscosa*. Location C. Kidney samples.



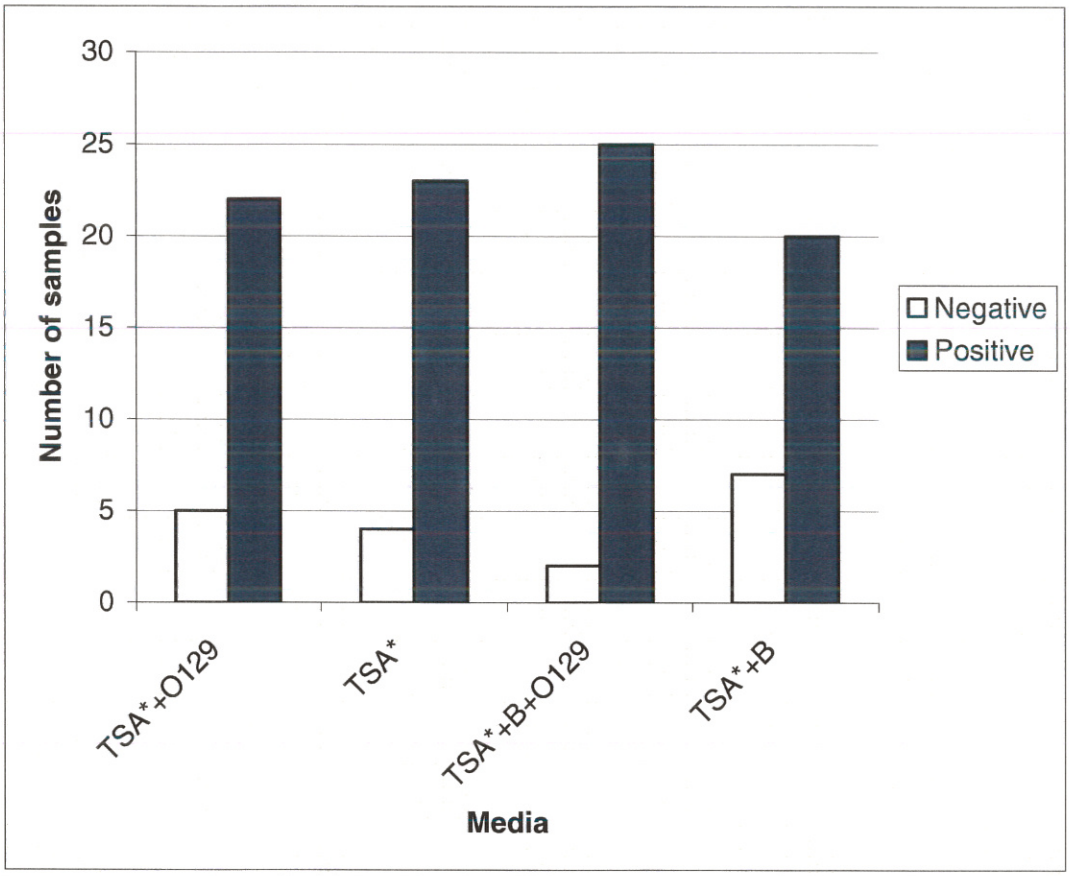
Media TSA*+B was significantly poorer in isolating *M. viscosa* in comparison to the other media (*P=0.04). There was a significant difference between TSA*+O129 and TSA*+B in isolating *M. viscosa*, in favor of the former (*P=0.03) and also between TSA*+B+O129 and TSA*+B in isolating *M. viscosa*, in favor of the former (*P=0.01).

Location C. Ulcer samples

Table 8. Isolation of *M. viscosa*. Location C. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	5	22	27
TSA*	4	23	27
TSA*+B+O129	2	25	27
TSA*+B	7	20	27
Total	18	90	108

Fig. 6. Isolation of *M.viscosa*. Location C. Ulcer samples.



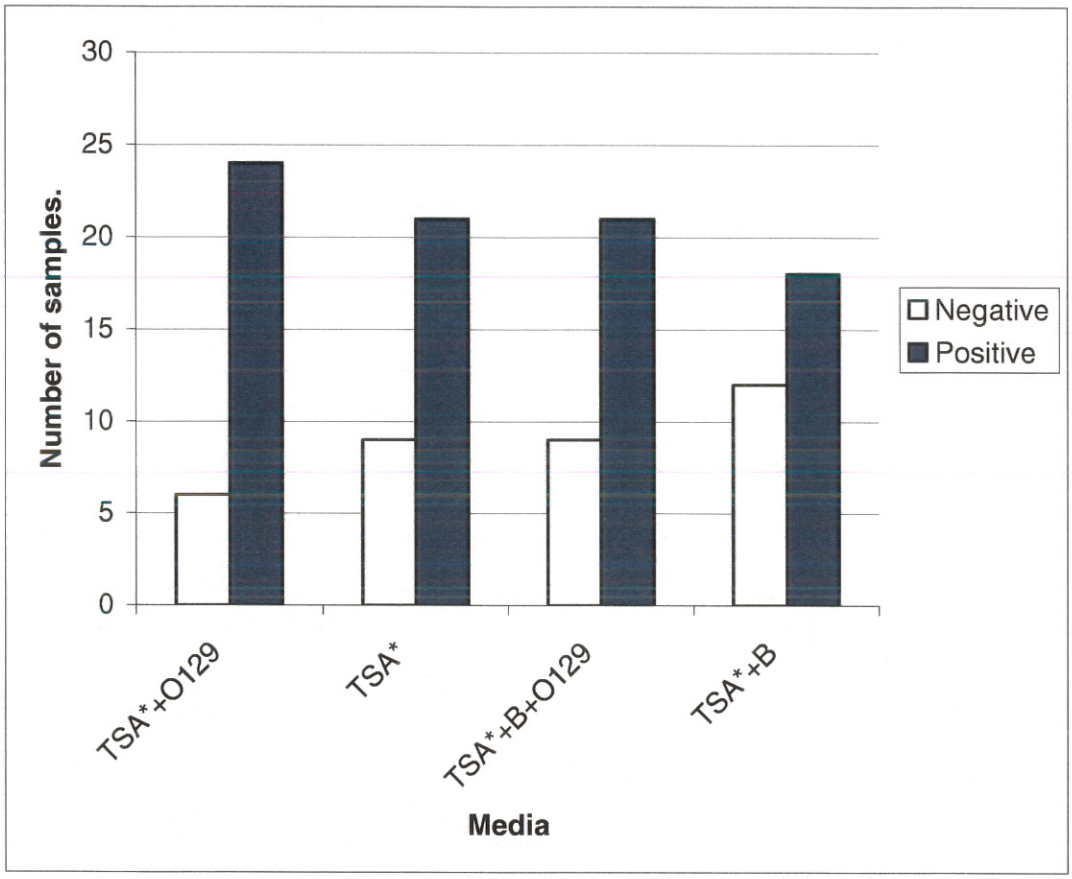
There was no significant difference between the media in isolating *M. viscosa*.

Location D. Kidney samples

Table 9. Isolation of *M. viscosa*. Location D. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	6	24	30
TSA*	9	21	30
TSA*+B+O129	9	21	30
TSA*+B	12	18	30
Total	36	84	120

Fig. 7. Isolation of *M.viscosa*. Location D. Kidney samples.



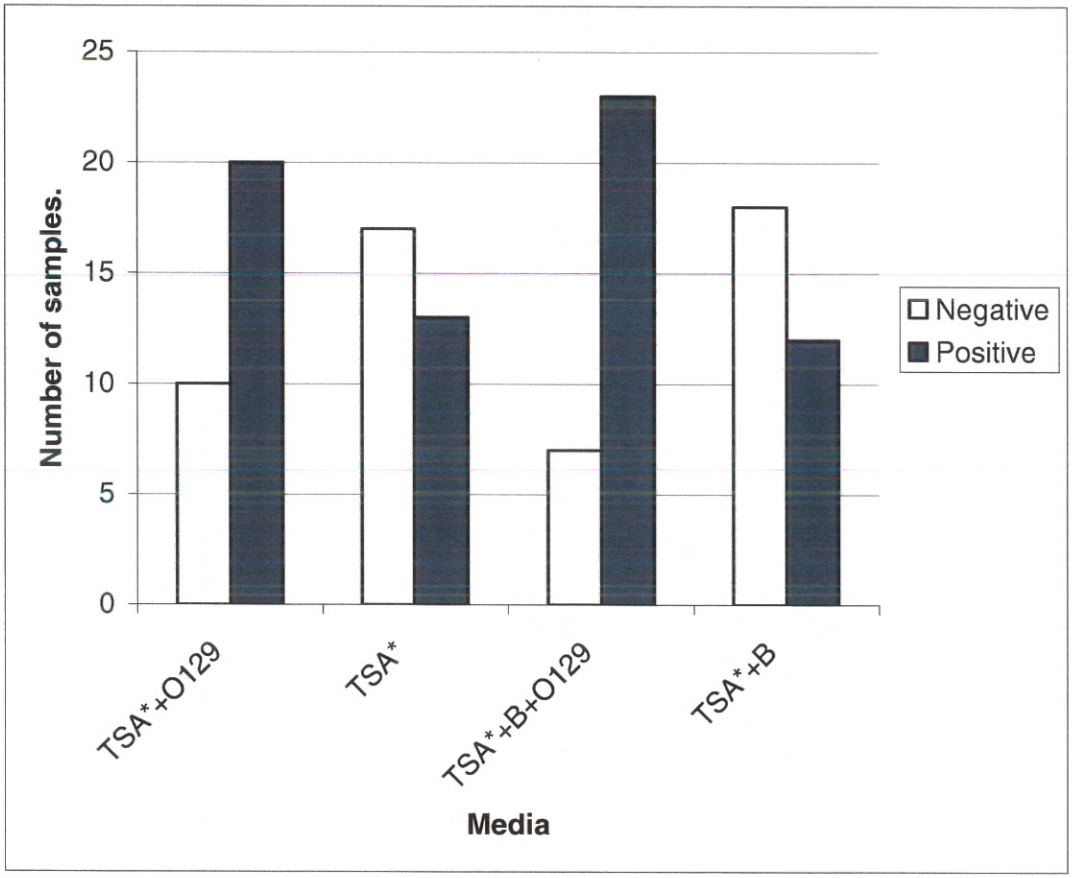
There was no significant difference between the media in isolating *M. viscosa*.

Location D. Ulcer samples

Table 10. Isolation of *M. viscosa*. Location D. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	10	20	30
TSA*	17	13	30
TSA*+B+O129	7	23	30
TSA*+B	18	12	30
Total	52	68	120

Fig. 8. Isolation of *M.viscosa*. Location D. Ulcer samples.



Media TSA*+O129 and TSA*+B+O129 were significantly better in isolating *M. viscosa* than the other two (**P=0.009). There was a significant difference between TSA*+O129 and TSA*+B (*P=0.038) in favor of the former in isolating *M. viscosa*. There was also a significant difference between TSA*+B+O129 and TSA* (** P=0.008) and between TSA*+B+O129 and TSA*+B (** P=0.004) in favor of TSA*+B+O129 in isolating *M. viscosa*.

Inhibition of *V. wodanis*
All locations. Kidney samples

Table 11. Growth of *V. wodanis*. All locations. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	4	103	107
TSA*	1	106	107
TSA*+B+O129	28	79	107
TSA*+B	8	99	107
Total	41	387	428

A significant difference was seen between TSA*+O129 and TSA*+B+O129 (*****P= $4.2*10^{-6}$), TSA* and TSA*+B+O129 (*****P= $3.1*10^{-7}$), and between TSA*+B+O129 and TSA*+B (*** P=$3.0*10^{-4}$), all in favor of TSA*+B+O129 in inhibiting *V. wodanis*. Also between TSA* and TSA*+B (*P=0.05) in favor for the latter.

All locations. Ulcer samples.

Table 12. Growth of *V. wodanis*. All locations. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	6	101	107
TSA*	9	98	107
TSA*+B+O129	29	78	107
TSA*+B	9	98	107
Total	53	375	428

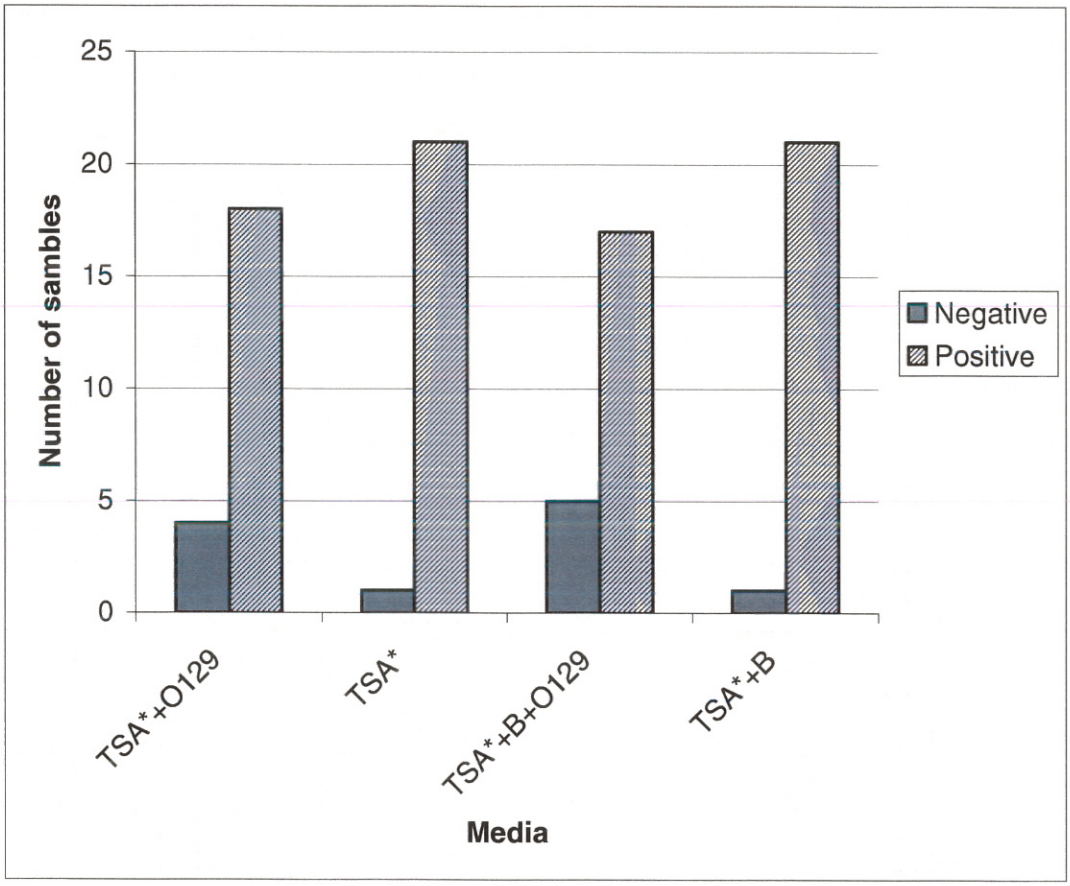
A significant difference was seen between TSA*+O129 and TSA*+B+O129 (**** P=$2.1*10^{-5}$), TSA* and TSA*+B+O129 (*** P=$3.5*10^{-4}$) and between TSA*+B+O129 and TSA*+B (*** P=$3.0*10^{-4}$) all in favor of TSA*+B+O129 in inhibiting *V. wodanis*.

Location A. Kidney samples

Table 13. Inhibition of *V. wodanis*. Location A.Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	4	18	22
TSA*	1	21	22
TSA*+B+O129	5	17	22
TSA*+B	1	21	22
Total	11	77	88

Fig. 9. Inhibition of *V. wodanis*. Location A. Kidney samples.



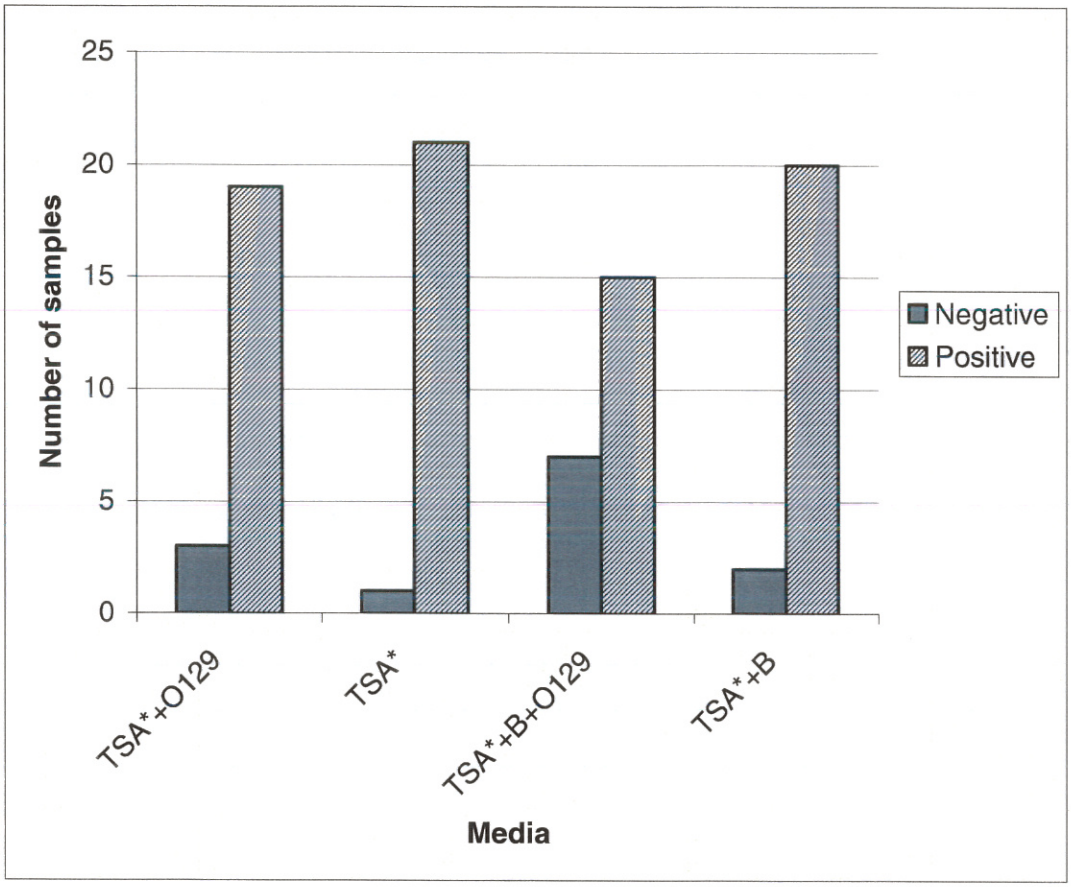
There was no significant difference between the media in inhibiting *V. wodanis*.

Location A. Ulcer samples

Table 14. Inhibition of *V. wodanis*. Location A.Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	3	19	22
TSA*	1	21	22
TSA*+B+O129	7	15	22
TSA*+B	2	20	22
Total	13	75	88

Fig. 10. Inhibition of *V. wodanis*. Location A. Ulcer samples



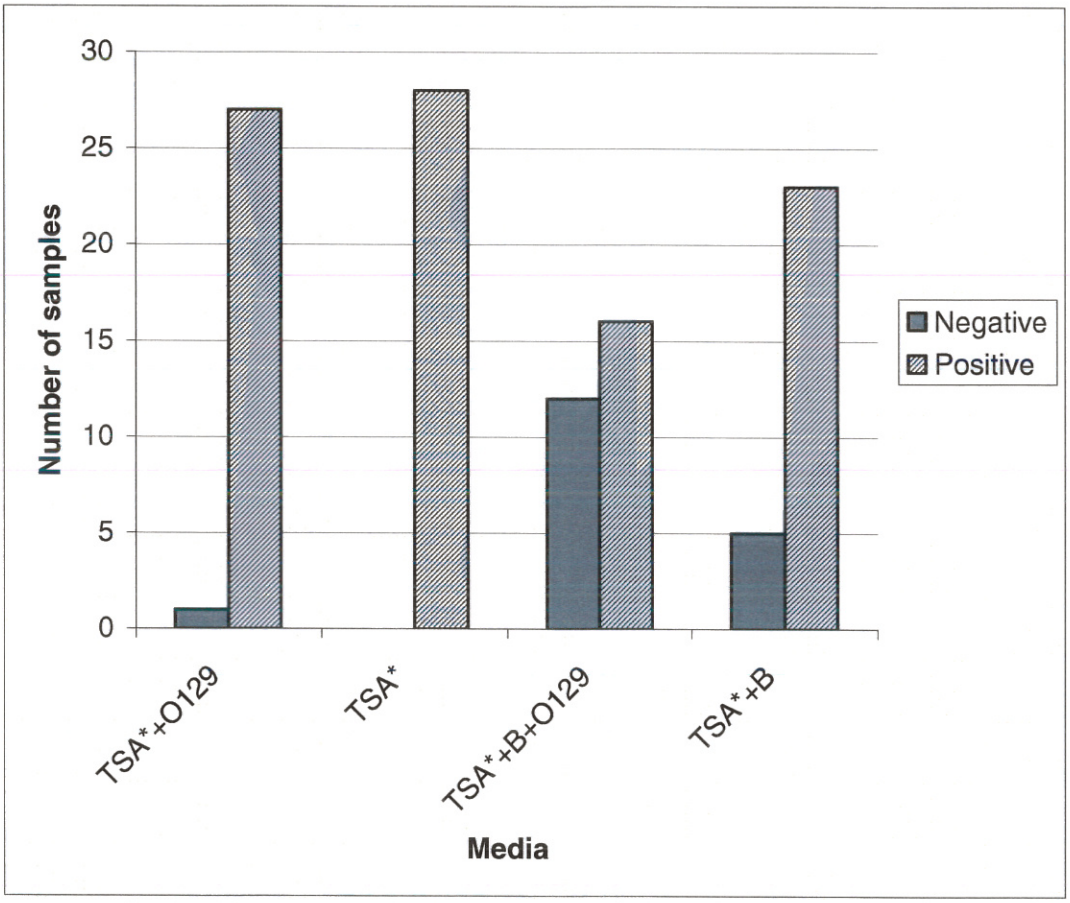
A significant difference was only seen between TSA* and TSA*+B+O129 (* P=0.019) in inhibiting *V. wodanis* in favor for the latter.

Location B. Kidney samples

Table 15. Inhibition of *V. wodanis*. Location B. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	1	27	28
TSA*	0	28	28
TSA*+B+O129	12	16	28
TSA*+B	5	23	28
Total	18	94	112

Fig. 11. Inhibition of *V. wodanis*. Location B. Kidney samples.



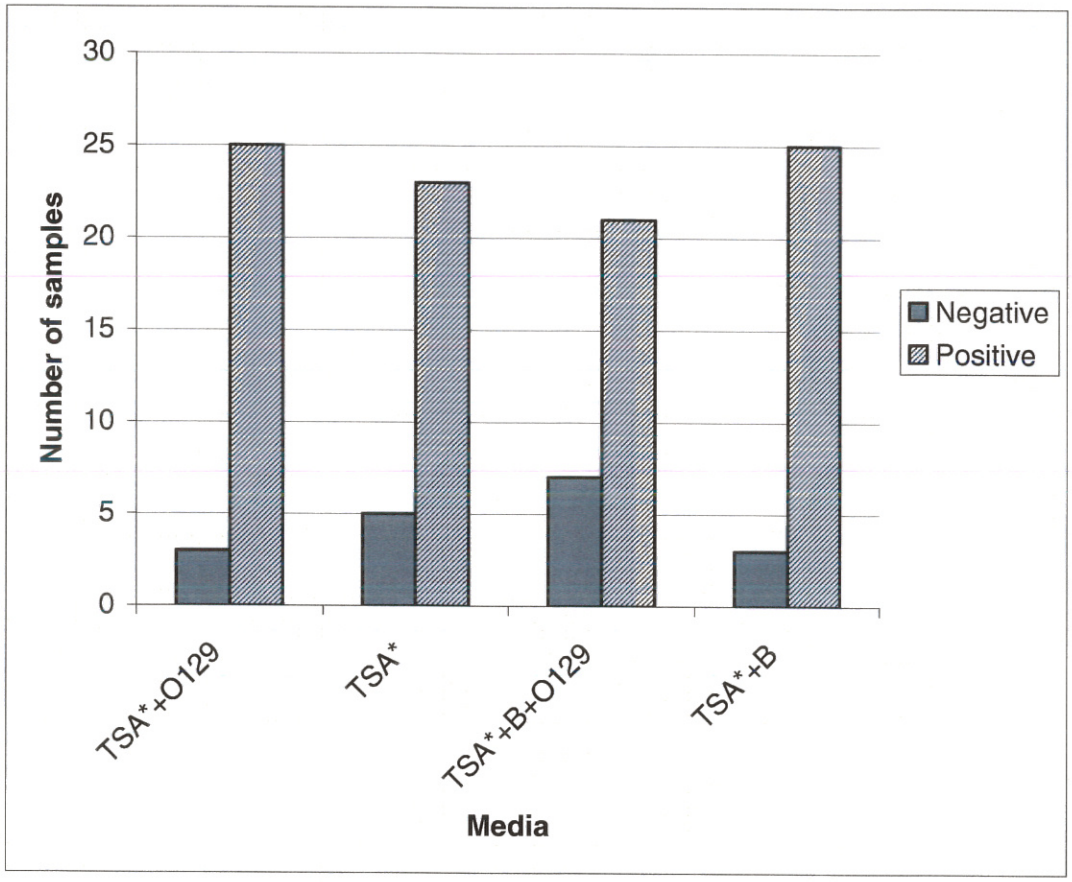
A significant difference was seen in inhibiting *V. wodanis* in favor of TSA*+B+O129 in comparison to all the media (**** $P=3.1*10^{-5}$), between TSA*+O129 and TSA*+B+O129 (***$P=5.0*10^{-4}$), TSA* and TSA*+B+O129 (**** $P=9.3*10^{-5}$); TSA* and TSA*+B in favor of TSA*+B (*P=0.02); and between TSA*+B+O129 and TSA*+B, in favor of the former (*P=0.04)

Location B. Ulcer samples

Table 16. Inhibition of *V. wodanis*. Location B. Ulcer samples

Media	Negative	Positive	Total
TSA*+O129	3	25	28
TSA*	5	23	28
TSA*+B+O129	7	21	28
TSA*+B	3	25	28
Total	18	94	112

Fig. 12. Inhibition of *V. wodanis*. Location B. Ulcer samples.



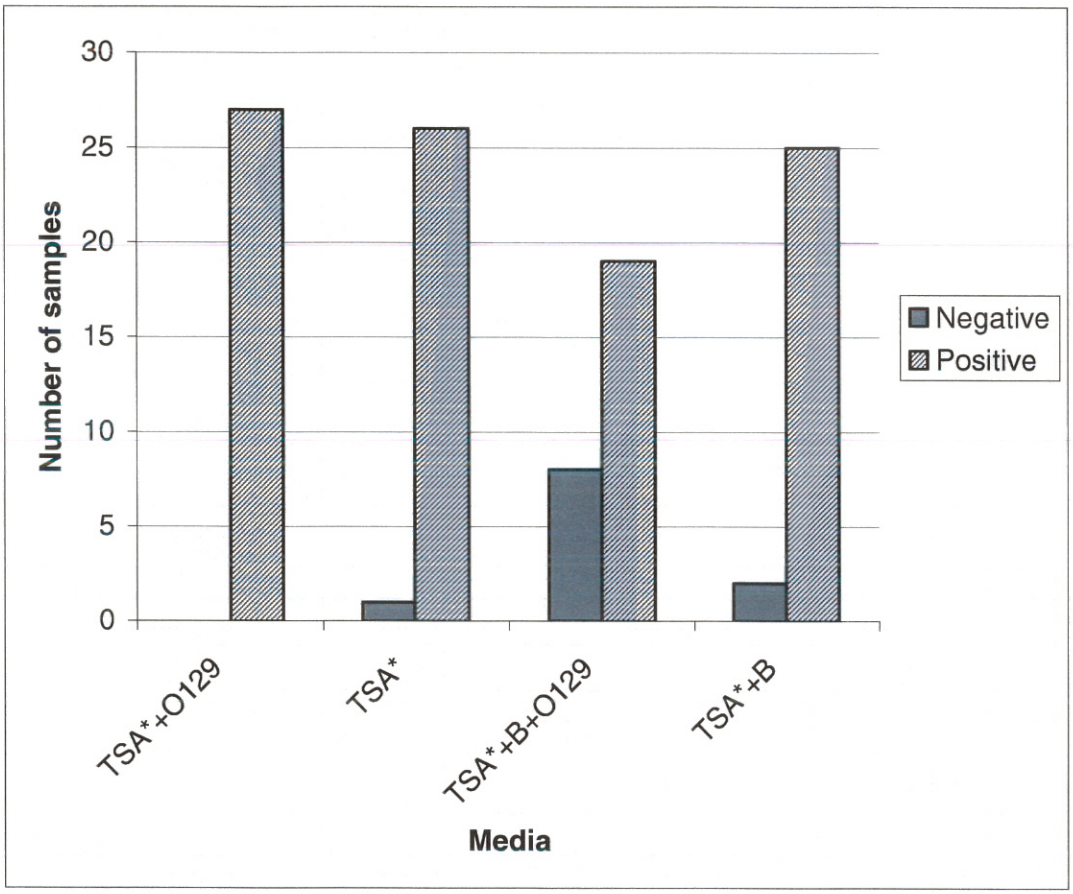
No significant difference was found between the media in inhibiting *V. wodanis*.

Location C. Kidney samples

Table 17. Inhibition of *V. wodanis*. Location C. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	0	27	27
TSA*	1	26	27
TSA*+B+O129	8	19	27
TSA*+B	2	25	27
Total	11	97	108

Fig. 13. Inhibition of *V. wodanis*. Location C. Kidney samples.



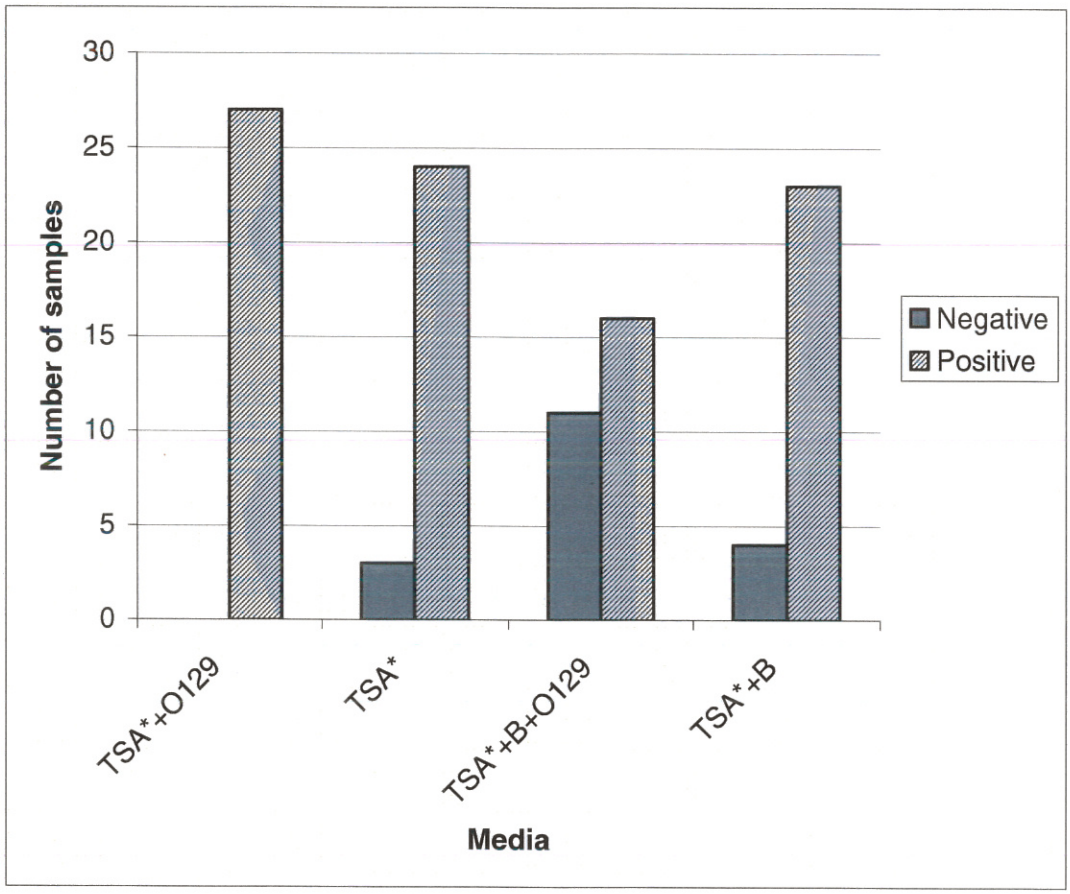
A significant difference was seen in inhibiting *V. wodanis* in favor of TSA*+B+O129 in comparison to all the media (** P= 0.001); between TSA*+O129 and TSA*+B+O129 (** 0.002), TSA* and TSA*+B+O129 (* P=0.01); and between TSA*+B+O129 and TSA*+B (*P=0.036)

Location C. Ulcer samples

Table 18. Inhibition of *V. wodanis*. Location C. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	0	27	27
TSA*	3	24	27
TSA*+B+O129	11	16	27
TSA*+B	4	23	27
Total	18	90	108

Fig. 14. Inhibition of *V. wodanis*. Location C. Ulcer samples.



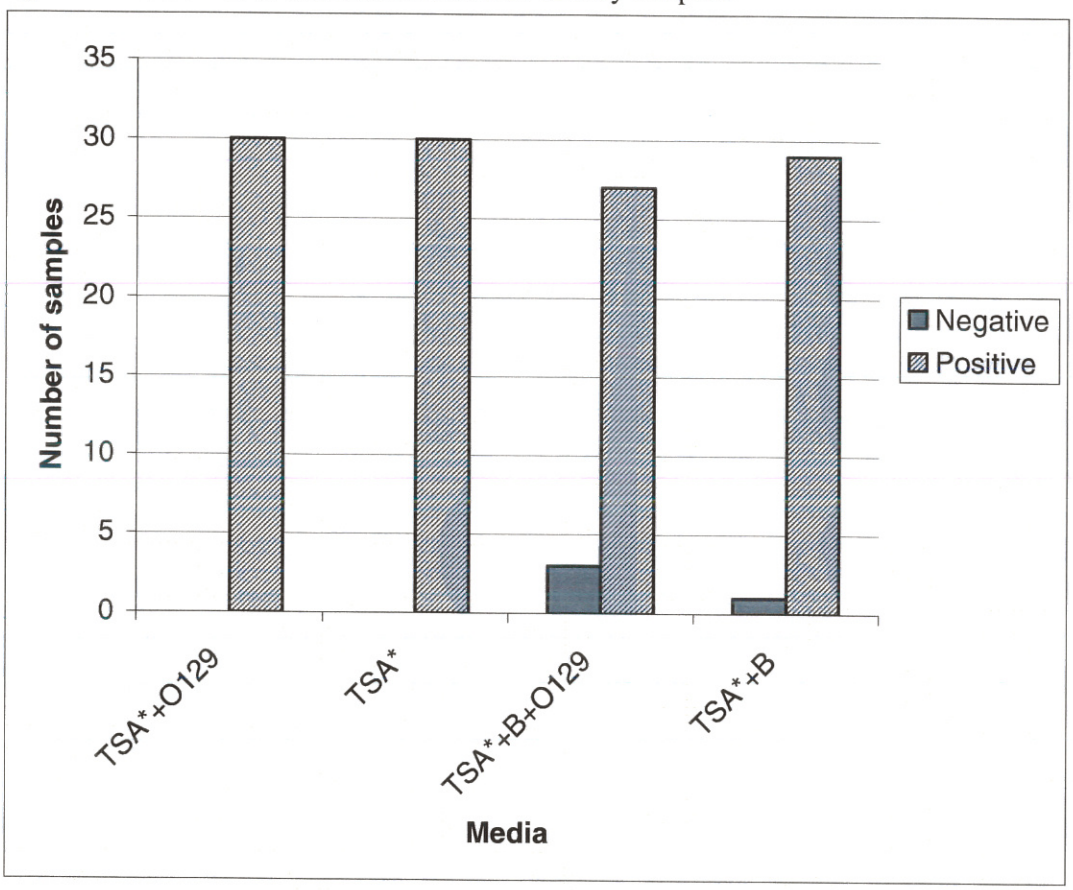
A significant difference was seen in comparison to all the media, in favor of TSA*+B+O129 inhibiting *V. wodanis* (*** $P=6.0*10^{-4}$), between TSA*+O129 and TSA*+B+O129 (***$P=2.0*10^{-4}$); and between TSA* and TSA*+B+O129 (*P= 0.013); and also between TSA*+O129 and TSA*+B, in favor of the latter (*P=0.038)

Location D. Kidney samples

Table 19. Inhibition of *V. wodanis*. Location D. Kidney samples.

Media	Negative	Positive	Total
TSA*+O129	0	30	30
TSA*	0	30	30
TSA*+B+O129	4	26	30
TSA*+B	0	30	30
Total	4	116	120

Fig. 15. Inhibition of *V. wodanis*. Location D. Kidney samples.



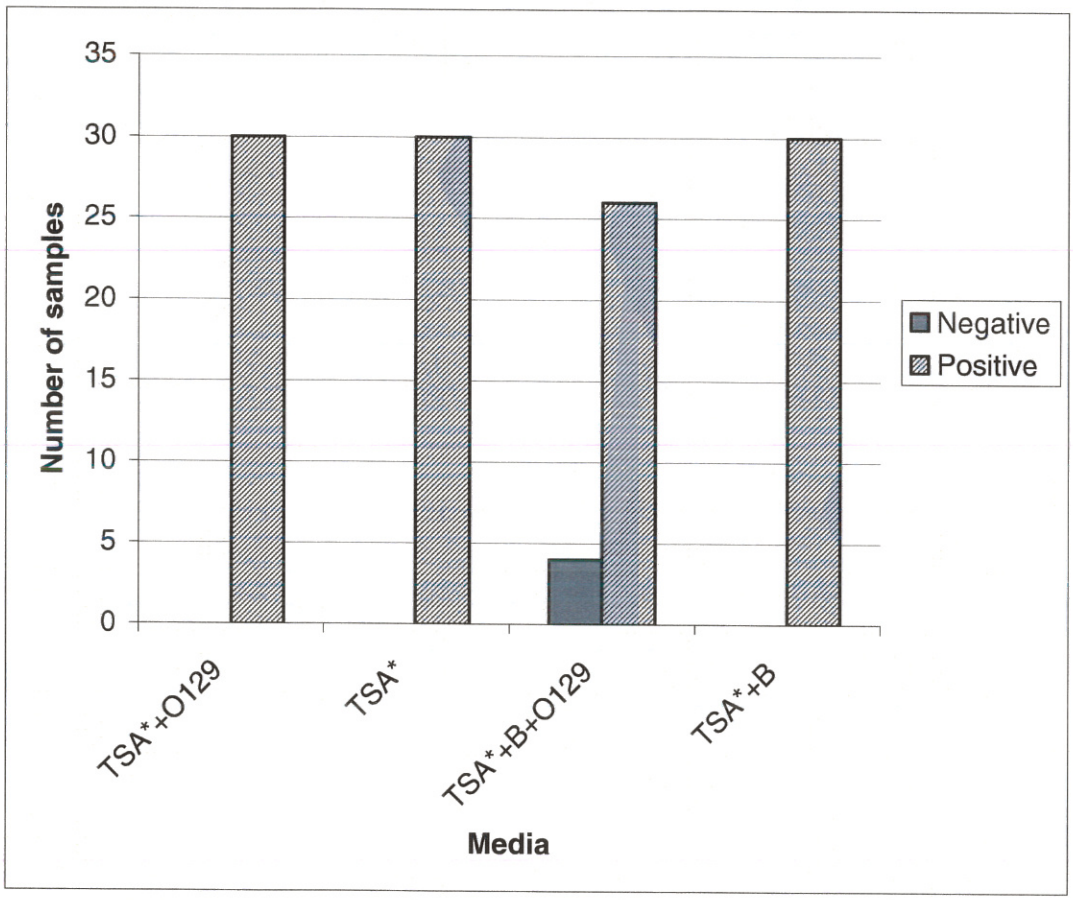
There was no significant difference between the media in inhibiting *V. wodanis*.

Location D. Ulcer samples

Table 20. Inhibition of *V. wodanis*. Location D. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	0	30	30
TSA*	0	30	30
TSA*+B+O129	4	26	30
TSA*+B	0	30	30
Total	4	116	120

Fig. 16. Inhibition of *V. wodanis*. Location D. Ulcer samples



A significant difference was seen in comparison to all the media in favor of TSA*+B+O129 inhibiting *V. wodanis* (** P=0.0061); between TSA*+O129 and TSA*+B+O129 (*P=0.042); TSA*+B+O129 and TSA* (*P=0.038); and also between TSA*+B+O129 and TSA*+B (*P=0.038) all in favor for TSA*+B+O129.

Other isolates
All locations

Table 21. Other isolates. All locations Kidney samples

Media	Negative	Positive	Total
TSA*+O129	66	41	107
TSA*	67	40	107
TSA*+B+O129	63	44	107
TSA*+B	73	34	107
Total	269	159	428

Table 22. Other isolates. All locations. Ulcer samples.

Media	Negative	Positive	Total
TSA*+O129	78	29	107
TSA*	79	28	107
TSA*+B+O129	47	60	107
TSA*+B	61	46	107
Total	265	163	428